BIOTECHNOLOGY

Kareen Martin & Patricia Zuk
West Los Angeles College

LibreTexts™

Biotechnology

(K. Martin and P. Zuk)

West Los Angeles College

This text is disseminated via the Open Education Resource (OER) LibreTexts Project (https://LibreTexts.org) and like the thousands of other texts available within this powerful platform, it is freely available for reading, printing, and "consuming."

The LibreTexts mission is to bring together students, faculty, and scholars in a collaborative effort to provide an accessible, and comprehensive platform that empowers our community to develop, curate, adapt, and adopt openly licensed resources and technologies; through these efforts we can reduce the financial burden born from traditional educational resource costs, ensuring education is more accessible for students and communities worldwide.

Most, but not all, pages in the library have licenses that may allow individuals to make changes, save, and print this book. Carefully consult the applicable license(s) before pursuing such effects. Instructors can adopt existing LibreTexts texts or Remix them to quickly build course-specific resources to meet the needs of their students. Unlike traditional textbooks, LibreTexts' web based origins allow powerful integration of advanced features and new technologies to support learning.

LibreTexts is the adaptable, user-friendly non-profit open education resource platform that educators trust for creating, customizing, and sharing accessible, interactive textbooks, adaptive homework, and ancillary materials. We collaborate with individuals and organizations to champion open education initiatives, support institutional publishing programs, drive curriculum development projects, and more.

The LibreTexts libraries are Powered by NICE CXone Expert and was supported by the Department of Education Open Textbook Pilot Project, the California Education Learning Lab, the UC Davis Office of the Provost, the UC Davis Library, the California State University Affordable Learning Solutions Program, and Merlot. This material is based upon work supported by the National Science Foundation under Grant No. 1246120, 1525057, and 1413739.

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation nor the US Department of Education.

Have questions or comments? For information about adoptions or adaptions contact info@LibreTexts.org or visit our main website at https://LibreTexts.org.

This text was compiled on 03/04/2026

TABLE OF CONTENTS

Licensing

1: Introduction to Biotechnology

- 1.1: Definition and a Short History of Biotechnology
- 1.2: Applications of Biotechnology
- 1.3: Common Biotechnology Equipment
- 1.4: Advanced Biotechnology Equipment

2: The Molecules of Life

- 2.1: Water
- 2.2: Carbon
- 2.3: Macromolecules
- 2.4: Carbohydrates
- 2.5: Lipids
- 2.6: Proteins
- 2.7: Nucleic Acids

3: Molecular Biology Fundamentals

- 3.1: DNA Structure
- 3.2: Replication of DNA
- 3.3: Transcription of RNA
- 3.4: Translation of the polypeptide
- 3.5: Mutations and DNA Repair

4: Genetic Engineering and Recombinant DNA Technology

- 4.1: Principles of Genetic Engineering
- 4.2: Creating Recombinant DNA
- 4.3: Applications of Recombinant DNA Technology

5: Bioinformatics- Genomics and Proteomics

- 5.1: Introduction to Bioinformatics
- 5.2: Genomics
- 5.3: Genome Sequencing
- 5.4: Comparative Genomics
- 5.5: Functional Genomics
- 5.6: Proteomics

6: Mammalian Cell Culture Fundamentals

- 6.1: Principles of Mammalian Cell Culture
- 6.2: Principles of Aseptic Technique
- 6.3: Cell Culture Equipment

7: Microbial Biotechnology

- 7.1: Microbe classification
- 7.2: Microbial Identification
- 7.3: Microbial Cultivation and Manipulation
- 7.4: Industrial Applications of Microbial Biotechnology
- 7.5: Medical Applications of Microbial Biotechnology

8: Animal Biotechnology

- 8.1: Applications of Animal Biotechnology
- 8.2: Genetic Engineering in Animals

9: Agricultural and Environmental Biotechnology – “Green” and "Gray" Biotechnology

- 9.1: "Green" vs. "Gray" Biotechnology
- 9.2: Applications of "Green" Biotechnology
- 9.3: Applications of "Gray" Biotechnology
- 9.4: Benefits of "Green" Biotechnology

10: Medical Biotechnology – “Red” Biotechnology

- 10.1: Principles of Medical Biotechnology
- 10.2: Diagnostic Biotechnology
- 10.3: Therapeutic Biotechnology
- 10.4: Regenerative Medicine

11: Industrial Biotechnology – “White” Biotechnology

- 11.1: Applications of Industrial Biotechnology
- 11.2: Bioprocess Engineering
- 11.3: Synthetic Biology

12: Quality Assurance and Quality Control in Biotechnology

- 12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
- 12.2: QA/QC Documentation- SOPs, Batch Records, and COAs

13: Biotechnology Lab Protocols

- 13.1: Lab Technique - Pipetting
- 13.2: Lab Technique - Polymerase Chain Reaction (PCR)
- 13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
- 13.4: Lab Technique - Restriction Digest of DNA
- 13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
- 13.6: Lab Technique - Bacterial Transformation
- 13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
- 13.8: Lab Technique - Quantitation of DNA
- 13.9: Lab Technique - DNA Sequencing
- 13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
- 13.11: Lab Technique - SDS-PAGE
- 13.12: Lab Technique - Western Blotting

- 13.13: Lab Technique - Cell Culture
- 13.14: Lab Technique - Light Microscopy
- 13.15: Bacterial Identification Methods
- 13.16: Basics of Bacterial Culture

14: Metric System and Measurements

- 14.1: Metric System Fundamentals

Index

Glossary

Detailed Licensing

Detailed Licensing

Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

CHAPTER OVERVIEW

1: Introduction to Biotechnology

1.1: Definition and a Short History of Biotechnology
1.2: Applications of Biotechnology
1.3: Common Biotechnology Equipment
1.4: Advanced Biotechnology Equipment

1: Introduction to Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

1.1: Definition and a Short History of Biotechnology

Biotech Focus

The rise of modern textile production comes with an increased release of harmful chemicals dyes into the environment and significant water consumption. Biotechnology in textile dyeing offers significant environmental and economic benefit. It can be used not only to to create eco-friendly dyes but also to clean up wastewater at the end of textile processing. Today, the textile industry has begun developing an alternative dyeing method through fermentation, using bacteria that have been genetically modified to produce pigments, thus reducing the use of toxic dyes and water.

Watch this TED talk from Natasi Audrey Chiexa, the founder of Faber Futures, who designed this technology.

Fashion has a pollution problem - can biology fix it?

Overview of biotechnology

Biotechnology is a multidisciplinary field that combines biology and technology in order to develop products and processes that improve the quality of life and the environment. It involves the manipulation of living organisms or their components, using them to solve problems and produce useful products. Biotechnology plays an essential role in our everyday life, from the production of medicine, to the food we eat, to the clothes we wear. It applications range of from agriculture to medicine, energy production, chemical processing, industrial manufacturing, and bioremediation.

Learning Objectives

By the end of this section, you will be able to:

- list some of the areas of biotechnology
- outline some of the major events in the history of biotechnology
- describe the interdisciplinary nature of biotechnology

Areas of Biotechnology

Biotechnology profoundly impacts our everyday lives. Its broad scope encompasses several key areas that contribute to the quality of human life worldwide. Some of the areas of biotechnology in use today are:

1. **Medical and Health Biotechnology ("Red Biotechnology)**
2. **Agricultural Biotechnology ("Green Biotechnology)**
3. **Food Biotechnology ("Yellow" Biotechnology**
4. **Industrial and Manufacturing Biotechnology ("White Biotechnology)**
5. **Marine Biotechnology ("Blue" Biotechnology)**
6. **Environmental Biotechnology ("Gray" Biotechnology)**
7. **Land Management Biotechnology ("Brown" Biotechnology)**

8. **Bio-defense Biotechnology ("Black Biotechnology)**

Many of these areas will be discussed in subsequent chapters in this textbook.

Brief History of Biotechnology

Throughout history, humans have used biology to manipulate living organisms, from altering bacteria to enhance fermentation, to the selective breeding of plants or animals to improve desired traits. Such careful manipulation of their biology has created the modern organisms we see and use today. If one were to compare the modern apple with its ancestral counterpart, one would be shocked at the difference. Ancestral apples were small and tart (Figure **1.1.1**). Through selective breeding principles, the modern apple is significantly larger and sweeter. These breeding principles are an example of how biotechnology can be used to change an organism.

Figure **1.1.1**: Wild Siberian crabapples are very similar to the early apples that were eaten by humans. Biotechnology, using selective breeding, has increased the size of apples and their sweetness and produced the modern varieties enjoyed today. (Siberian crabapple by Jackie Diectrich; CC-BY-NC 3.0).

The identification of bacteria and their role in fermentation and disease, together with technological advancements in biology, such as the development of pure bacterial cultures, paved the way for the development of modern biotechnology. With the discovery of DNA and its role in heredity and genetics, new means for manipulating living organisms became available. In 1973, the first **genetically-modified organism (GMO)** was created by Stanley Cohen and Herbert Boyer, who used biotechnology procedures to introduce foreign DNA into a bacterial genome. In 1977, Herbert Boyer and Keiichi Itakura succeeded in inducing genetically-modified bacteria to produce the first human protein (i.e., somatostatin). Their recombinant somatostatin was found to be identical to its natural counterpart and their studies marked the start of the biotechnology industry. One year later, the first synthetic “human” insulin was produced using genetically-engineered *E. coli* bacteria. In 1982, Humulin®, human insulin used for diabetes, was the first medication developed using biotechnology and the first recombinant pharmaceutical approved by the FDA for commercial use in the United States (Figure **1.1.2**).

Figure **1.1.2**: A vial of the first Humulin® solution developed by Genentech and licensed to Eli Lilly (Humulin R by National Museum of American History; CC0)

The importance of biotechnology and its creation of Humulin® cannot be understated. Prior to the advent of biotechnology, scientists relied either on chemically synthesized human therapeutics made in the laboratory, or on a microbe or animal that

naturally produced the desired compound. Prior to 1982, Type I diabetics used insulin extracted from cows and pigs. These animal-derived insulin therapies induced minor allergic reactions in some patients. Furthermore, fears emerged that demand for insulin would soon outstrip the supply from pigs and cows. Through biotechnology and its creation of Humulin®, diabetic patients now had the option to use genetically-engineered insulin that was cheaper, safer, and could be mass produced.

The 1970s were marked by the development of the first commercial method for sequencing DNA. Developed by Frederick Sanger in 1977, the "Sanger method", or dideoxy sequencing, revolutionized biotechnology by allowing scientists to determine the precise order of nucleotides in DNA. In the following decades, advancements in sequencing technologies, such as the development of automated sequencing machines in the 1980s and the introduction of next-generation sequencing (NGS) in the early 2000s, exponentially increased sequencing speed and reduced costs for those working in the biotechnology field. The Human Genome Project, completed in 2003, marked a monumental achievement, providing a complete map of the human genome and ushering in a new era of genomics. Today, sequencing technologies continue to evolve, with techniques enabling real-time sequencing and expanding our understanding of genetics and its applications in medicine, agriculture, and beyond.

The 1980s was a decade of significant breakthroughs in the production of more advanced and refined GMOs. The first patent for a GMO was issued in 1980 to Ananda Chakrabarty, who engineered *Pseudomonas sp.* bacteria to breakdown crude oil spills as a means of bioremediation. Their patent became the first one to be granted to a living organism and paved the way for future biotechnology patents on modified plants, animals, and other organisms. This GMO would later be used in the 1989 cleanup of the Exxon oil spill at Prince William Sound, Alaska. In 1981, the first genetically-modified animal, also known as a **transgenic animal,** was produced in mice. Two years later, genetic modifications using biotechnology were being applied to sheep, pigs, and cattle, significantly impacting the livestock industry. In 1983, the first transgenic plant, a tobacco plant resistant to antibiotics, was introduced to the agriculture market. Two years later, Agracetus received the first patent for its genetically-modified cotton. Since then, GMOs have become widespread in many research fields and commercial markets. In 2020, genetically-modified plants accounted for about 55 percent of the total harvested cropland in the United States. Most of the corn, soybeans and cotton planted in the US have been genetically-modified either to resist pest or to be resistant to herbicides (Figure **1.1.3**). Today, genetically modifying flies, fish, or mice are a common and essential tool in research labs.

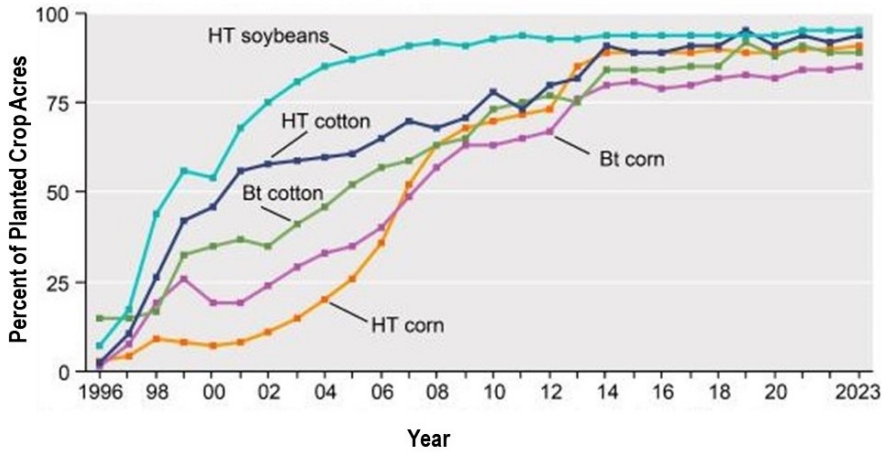


Figure **1.1.3**: Adoption of genetically-modified crops in the United States from 1996 to 2023. The percentage of acres planted with crops (i.e. percent of planted crop acres) as a function of time (in years) was plotted for Herbicide-Tolerant (HT) soybeans (teal squares), cotton (blue squares), and corn (orange squares). Insect resistant varieties containing genes from the soil bacterium *Bacillus thuringiensis* (Bt) were also plotted (Bt-corn, purple squares; Bt-cotton, green squares). The graph shows increases over time in the percent of planted crop acres for all HT- and Bt-varieties from 1996 to 2010, with the percentage of acres planted leveling off to close to 100% from 2010 to 2023. (Genetically modified crops by Patricia Zuk, CC BY 4.0; adapted from Adoption of genetically engineered crops by USDA Economic Research Service, Public Domain)

Advancements in biotechnology are currently growing at an exponential rate with improvements in techniques, such as the evolution of sequencing methods mentioned above, or creation of new methodologies constantly occurring.

The Interdisciplinary Nature of Biotechnology

Biotechnology exemplifies the interdisciplinary nature of science by its merging of diverse scientific and engineering fields to address complex biological challenges. At its core, biotechnology integrates molecular biology, cell biology, genetics, and

microbiology with other life science areas, like virology, in order to understand and manipulate living organisms at the molecular level. Chemistry and biochemistry play crucial roles in understanding the biochemical pathways and interactions within cells. Physics provides advanced techniques like X-ray crystallography and nuclear magnetic resonance, which are essential for elucidating the structures of biomolecules. Engineering principles are applied to the design of bioprocesses, bioreactors, and medical devices. Additionally, computer science and bio-informatics are indispensable for managing and analyzing the vast amounts of data generated in biotechnology research, enabling the modeling of biological systems and the development of algorithms for gene sequencing and drug design. This synergy of disciplines fosters innovation, leading to advancements in healthcare, agriculture, environmental protection, and industrial processes, ultimately improving quality of life and sustainability.

Key Concepts

Biotechnology has been used for breeding livestock and crops long before people understood the scientific basis of this field. A better understanding of cellular and molecular biology and rapid advances in new technologies has led to the growth of biotechnology. Today, biotechnology is an interdisciplinary field incorporating numerous STEM fields, including biology, biochemistry, physics, engineering, and computer science.

Some important concepts to remember are:

- there are numerous areas of biotechnology, including medical, agricultural, food, and industrial
- genetically-modified organisms (GMOs) were first created using biotechnology in 1973
- bacteria were the first GMOs to be created and used in biotechnology
- the first synthetic drug to be made through biotechnology was human insulin
- the first patented GMO was issued in 1980
- GMO animals are often called transgenic animals

Glossary

Biodiversity - the variety of life on Earth, including all living organisms, ecosystems, and genetic diversity

Bioremediation - the use of living organisms, such as bacteria, fungi, or plants, to clean up and remove environmental contaminants from soil, water, and air

Biotechnology - the use of biological systems, organisms, or derivatives to develop or create useful products and technologies

Genetic engineering - the direct modification of an organism's DNA using biotechnology; often done to introduce new traits, to improve resistance to disease, or to produce beneficial products

Genetically-modified organism (GMO) - any organism whose DNA has been altered using genetic engineering techniques

Interdisciplinary - the integration and combination of knowledge, methods, and perspectives from two or more different disciplines or fields of study

Transgenic - an alternate term used for a GMO; often used to refer to genetically-modified animals

1.1: Definition and a Short History of Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

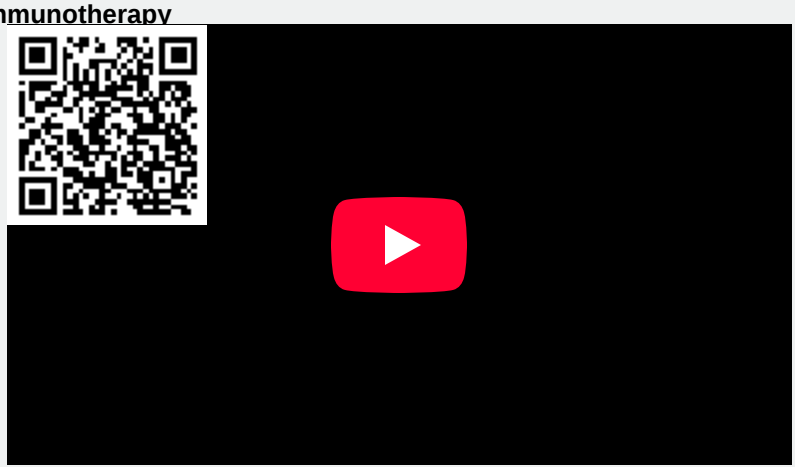
1.2: Applications of Biotechnology

Biotech Focus

Cell therapy is an exciting and emerging field in biotechnology. **CAR-T (Chimeric antigen receptor-T) cell therapy** is a type of immunotherapy treatment that uses a patient's T cells to attack cancer cells. Cells from the patients are collected, genetically modified to recognize cancer cells and reintroduced into the patient. Emily Whitehead was the first patient to receive CAR-T cell therapy as a clinical trial in 2012 in an attempt to cure her leukemia. She is now cancer free and has lived a normal life since then. To read more about CAR-T cell therapy, see Chapter 10.1: Uses of Medical Biotechnology.

The video below shows Emma's journey and how this type of therapy has since been used to save other patient's lives.

New Cancer Cure: Immunotherapy



Introduction

The rapid evolution of techniques used in biotechnology has enabled researchers to sequence segments of DNA or entire genomes in record time; to analyze specific DNA and protein sequences; to enhance microbial and eukaryotic cell culture methods; to engineer enzymes with novel or improved characteristics; to add, delete, or edit specific genes to produce desired traits in organisms. These techniques provide extensive opportunities for various applications in biotechnology, including designing new drugs, the improvement of animal and plant specific, the production of renewable energy, and protecting the environment.

Learning Objectives

By the end of this section, you will be able to:

- describe how biotechnology is used in different areas

Biotechnology in Medicine

Biotechnology offers new ways to diagnose, treat, and prevent diseases. It enables the creation of advanced tools for disease detection and monitoring, such as PCR tests and biosensors like the glucose monitor (Figure **1.2.1**). Innovations in genetic engineering and bio-processing have led to the development of new pharmaceutical products that target diseases at their molecular roots, and improvements of existing ones, such as monoclonal antibodies, vaccines, and gene therapy. Progress in regenerative medicine allows for the regeneration of damaged tissues and organs through the use of stem cells and tissue engineering. Biotechnology also plays a critical role in vaccine development, as seen with the rapid creation of COVID-19 vaccines using mRNA technology. For more about biotechnology in medicine, go to Chapter 10: Medical Biotechnology.

Figure **1.2.1**: Biotechnology and Diseases. Biotechnology can be applied to the treatment of diseases such as diabetes. Biosensors, like the disc applied to the arm, can continuously monitor and then display blood glucose levels in diabetic patients (BGM twopart by Sjö; CC BY-SA 4.0)

Biotechnology in Animals

Genetic engineering allows for the creation of genetically-modified (i.e., **transgenic**) animals with enhanced growth rates, improved disease resistance, increased milk or meat production. The development of such genetically-modified animals reduces the need for antibiotics and enhances their welfare. Cloning and reproductive technologies, such as ***in vitro* fertilization** and **somatic cell nuclear transfer (SCNT)** has helped preserve endangered species and improve breeding programs for livestock (Figure **1.2.2**). The Frozen Zoo at the San Diego Zoo has been collecting and preserving living cell lines, gametes, and embryos since 1975 making it the largest repository of its kind. Such collections provide invaluable resources for conservation, research, and potential future restoration of endangered or extinct species. For more about the application of biotechnology to animals, go to Chapter 8: Animal Biotechnology.

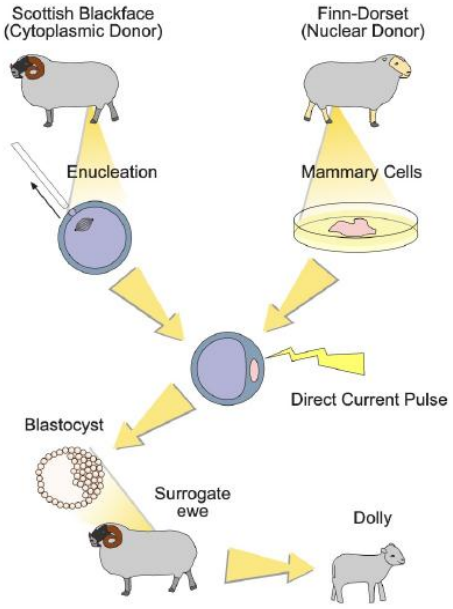


Figure **1.2.2**: Somatic cell nuclear transfer (SCNT). Dolly the sheep was the first mammal to be cloned using SCNT. To clone Dolly, "cytoplasmic donor" eggs from a Scottish Blackface sheep had their nuclei extracted in a process called enucleation. Somatic mammary cells were extracted from a Finn Dorset sheep and their nuclei removed. A direct electric current was used to fuse the nucleus from the Finn Dorset sheep to the enucleate egg of the Blackface sheep. The egg was then allowed to divide to the blastocyst stage, a sphere of cells containing a cluster of stem cells on one side. The blastocyst was implanted in a surrogate mother, who later gave birth to Dolly the sheep. (Dolly clone by Squidonius; Public Domain)

Biotechnology in Agriculture

Agriculture has greatly benefited from advances in biotechnology, such as the creation of genetically-modified organisms (GMOs). GMOs like **genetically-modified crops** has led to the development of crops with desired traits. For example, crops engineered to be resistant to pests or herbicides reduce the need for chemical pesticides and herbicides, lowering environmental and health risks. Additionally, the production of bio-fertilizers and bio-pesticides, which use natural organisms to enhance soil fertility and control pests, can increase crop yields and prevent runoff into water bodies. Crops with enhanced nutritional content, such as "golden rice", a rice genetically engineered to produce beta-carotene (a precursor of vitamin A), address nutrient deficiencies in regions where

such shortages are prevalent (Figure **1.2.3**). Crops can also be genetically modified to withstand harsh environmental conditions; for instance, drought-resistant and pest-resistant maize varieties are being tested in Ethiopia, and efforts are underway to create genetically-modified indigenous plants with enhanced traits. For more about how biotechnology is used in plants and in agriculture, go to Chapter 9: Agriculture Biotechnology.

Figure **1.2.3**: Golden rice: a genetically-engineered crop. Golden rice has been genetically engineered to biosynthesize beta-carotene, a precursor of vitamin A, providing this essential vitamin in area where there is shortage of it. (Golden rice by IRRI photos, CC BY-NC-SA 2.0)

Biotechnology in Industry and Manufacturing

Industrial biotechnology facilitates the creation of various products, such as biofuels, food and feed ingredients, detergents, and textiles. A significant benefit of industrial biotechnology is its reduced environmental impact. For instance, renewable resources like food waste, animal manure, and algae are now being utilized to produce biofuels, while sugarcane and corn starch are being used to make biodegradable bio-plastics. **Bioengineering** of enzymes are now modifying existing enzymes or developing new ones with enhanced properties for use in laundry detergents, paper production, and food processing. For example, engineered **microbial lipase** is employed in numerous applications. Lipases are enzymes that break down lipids into fatty acids and glycerol, and are important biocatalysts for biotechnology. Microbial lipases are inexpensive to produce, stable and easy to genetically manipulate. As such they are manufactured to be included as additives in the detergent industry, flavor and aroma enhancers in the food industry, replacements for artificial chemicals in cosmetics, and in paper production and leather tanning (Figure **1.2.4**). For more about industrial biotechnology, go to Chapter 11. Industrial Biotechnology.

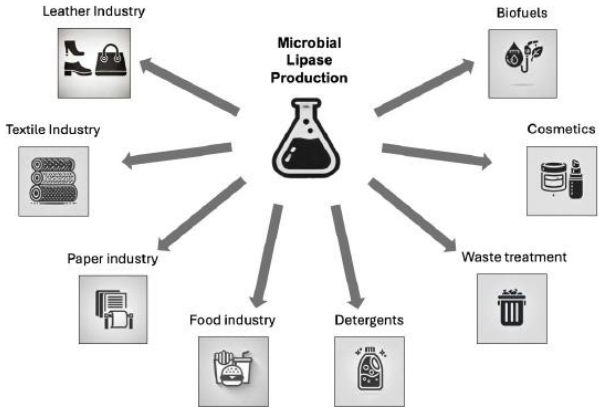


Figure **1.2.4**: Microbial lipase uses. Lipases can be made by bacteria for use the leather, textile, paper, and food industries, in addition to being used in detergents, waste treatment, cosmetics, and biofuels. (Microbial Lipase Uses by Kareen Martin; CC BY 4.0)

Biotechnology and the Environment

The previous sections presented some examples on how biotechnology can benefit the environment: pest resistant plants limit the release of pesticides, or the creation of biofuels reduces greenhouse gases emissions. **Bioremediation** uses biotechnology to clean up polluted areas. Microbes have been genetically engineered to biodegrade contaminants such as oil spills and heavy metals. Biotechnology also provides new technologies to improve waste management, such as microbial digestion and composting, which promote the recycling of organic waste into useful products like compost and bioenergy. Biotechnological innovations in embryo

preservation help conserve biodiversity and protect endangered animal and plant species as already mentioned in the section animal biotechnology. Go to Chapter 12: Environmental Biotechnology to read more about how biotechnology is used to protect the environment.

Biotechnology in Biodefense

Biotechnology also plays a crucial role in the bio-defense sector in numerous ways. Rapid advances in biotechnology bring the alarming potential for developing biological weapons used in **bioterrorism**. Perhaps the best example is the bacteria, *Bacillus anthracis*. *Bacillus anthracis* produces reproductive spores that float in the air and can be inhaled. In 2001, powdered anthrax spores were sent through the mail in a series of attacks that killed five people and infected 17 others (Figure **1.2.5**).

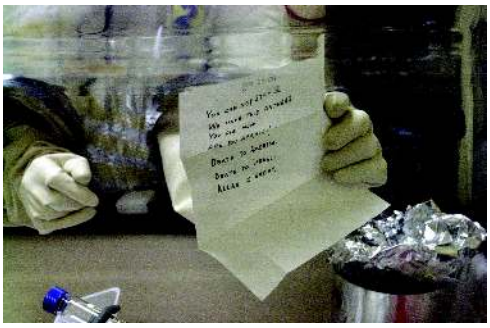


Figure **1.2.5**: Anthrax Letter Sent to Senator Leahy. A laboratory technician's hands is shown holding an anthrax-laced letter addressed to Senator Leahy in December 2001. The letter was safely opened at the U.S. Army's Fort Detrick bio-medical research laboratory. (Anthrax Letter Sent to Senator Leahy by the Federal Bureau of Investigation (FBI), Public Domain)

Such examples make it essential to develop countermeasures against such threats. Biotechnology has been at the leading edge of developing these countermeasures. For example, biotechnology has enabled the creation of multiple vaccines, antibiotics, and anti-viral compounds that protect society against biological agents used in bioterrorism. Finally, the emergence of new biotechnologies is providing governments with critical tools for **bio-surveillance** and early warning of infectious disease events, microbial forensics, decontamination of materials, and risk and threat assessment.

Key Concepts

Biotechnology is the use of biological agents for technological advancement. Some important concepts to remember are:

- Biotechnology can be applied to medicine (e.g. vaccine and antibiotic production), industry (e.g. biofuels, feed, textiles), and agriculture (e.g. crop genetic modification in order to increase yields)
- Genetic engineering allows for the creation of transgenic animals with enhanced growth rates, improved disease resistance, increased milk or meat production
- Somatic cell nuclear transfer (SCNT) technologies help protect endangered animals and improve breeding programs
- Genetically-modified crops are used in agriculture to produce crops with desired traits like resistance to drought, disease, pests, and herbicides, in addition to enhanced nutritional content
- Industrial biotechnology facilitates the creation of various products, such as biofuels, food and feed ingredients, detergents, and textiles.
- Industrial bioengineering of enzymes can modify existing enzymes or develop new ones with enhanced properties for use in industries like detergents, paper production, and food processing
- Bioremediation uses biotechnology to clean up polluted areas

Glossary

Bioengineering - the application of principles from biology, chemistry, physics, mathematics, and engineering to solve problems in biology and medicine; also known as biomedical engineering

Bioremediation - a process that uses microorganisms to clean up contaminated soil, water, and other environments

Biosurveillance - a public health practice that involves the continuous monitoring and detection of biological threats, such as infectious diseases, bioterrorism agents, and emerging pathogens

Bioterrorism - the intentional use of microorganisms to bring about ill effects or death to humans, livestock, or crops

Enucleate - a cell lacking a nucleus

Genetically-modified crop - a crop whose DNA has been altered using genetic engineering techniques to introduce traits like pest resistance, herbicide tolerance, or improved nutritional content

Genetically-modified organism - any organism whose DNA has been altered using genetic engineering techniques

***In vitro* Fertilization** - a type of assisted reproductive technology (ART) where sperm and an egg are fertilized outside of the human body

Somatic Cell Nuclear Transfer (SCNT) - a laboratory technique that creates an embryo from the nucleus of a body cell and the cytoplasm of a enucleate egg; also known as therapeutic cloning

Transgenic organism - an alternate term for a GMO; often used to refer to a genetically-modified animal

---

1.2: Applications of Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

1.3: Common Biotechnology Equipment

Biotech Focus

Believe it or not, without cows, the **centrifuge** used in laboratories all around the world wouldn't have been invented. In 1864, the German engineer Antonin Prandtl created a piece of equipment that separated milk and cream from one another by spinning samples (i.e., centrifuging) at a high speed. Five years later, recognizing its importance, the Swiss physician and biologist Friedrich Miescher applied this same principle to samples in the laboratory. Using a crude, hand-cranked centrifuge like the one shown below (Figure **1.3.1**), Miescher was able to successfully isolate nucleic acids from the nuclei of white blood cells. The early centrifuges of the late 1800s and early 1900s were slow and could only reach revolutions of a few thousand rpm. Theodor Svedberg changed all of that in 1924 with his invention of the first **analytical ultracentrifuge** that could create centrifugal forces of up to 5000-times the force of gravity. For his work, Theodor won the Nobel Prize in 1926 and, today, the **Svedberg unit (S)** is used as a measure of how fast a particle will sediment during centrifugation.

Figure **1.3.1**: The 19th century centrifuge. Up to four samples could be placed in the brass tube holders at the top of the machine and then spun using the hand crank at the base of the centrifuge. (19th century centrifuge by Kierano, CC BY-SA 4.0)

Introduction

The biotech laboratory contains a myriad of equipment (also known as instruments). Some of this equipment is very basic and can be found in other laboratories, like biology, biochemistry, and chemistry labs (e.g. balances, glassware, pipettes). The basic types of lab equipment include:

1. **Equipment for Measuring (i.e. Metrology Equipment)**
    1. Balances
    2. pH Meters
    3. Spectrophotometers
2. **Equipment for Measuring and Transferring Liquids**
    1. Pipettes
    2. Graduated cylinders
3. **Equipment for Storing**
    1. Glassware
    2. Refrigerators and Freezers
4. **Equipment for Safety and Sterility**
    1. Personal Protective Equipment (PPE)
    2. Biosafety cabinets
    3. Fume Hoods
    4. Autoclaves
5. **Equipment for Analysis**
    1. Electrophoresis

2. Centrifuge

Other equipment is more specialized and is usually found in biology, biotechnology, and biochemistry labs. This equipment can include several types of microscopes, electrophoresis equipment for DNA or protein analysis, PCR machines for DNA amplification, DNA sequencers, and microscopes. To learn about these more specialized pieces of equipment, read Chapter 1.4: Advanced Biotechnology Equipment.

Learning Objectives

By the end of this page, you will be able to:

- List and describe the different types of balances used in a lab
- Describe the different ways to measure the pH of a solution
- Describe the use of a serological pipette
- Describe the use of a micropipette
- Describe the different centrifuges available in a lab
- Know how to properly load a centrifuge

Equipment for Measuring

Metrology is the science of measurement. The most common types of measurements: are **weight, distance, volume, temperature, pH**. The scientific system of measurement is the **metric system.** A strong foundation in the metric system is critical to metrology. To review the metric system, go to Chapter 14: Metric System Fundamentals.

Two important components of metrology are accuracy and precision. **Accuracy** is the degree to which a measurement, calculation, or specification complies to the correct value. Accuracy in measurement is key to the generation of a consistent product in biotechnology. In the biotech lab, accuracy is affected by errors. There are two types of errors that can affect accuracy: **random errors**, which are fluctuations from measurement to measurement and **systematic errors**, which are consistent errors due to the state of a piece of equipment. Eliminating systematic errors relies upon a regular process of testing and calibrating (adjusting) equipment so that they fall within an established margin of error. In a biotech lab, testing and calibration typically follows a **standard operating procedure or SOP**. To learn about what an SOP is and why it is used, go to Chapter 12.2: QA/QC Documentation - SOPs, Batch Records, and COAs.

Weighing: Balances & Scales

Instruments for weighing materials are called **balances** and **scales**. While they are often used interchangeably, they are not the same thing. A balance *measures the mass of an object by comparing it to a known reference mass*. A scale *measures the weight of an object* (i.e., the force exerted by gravity on the object) by using a spring loaded cell or other force-measuring technology.

As defined above, *balances are used for the measurement of mass*. Mass is measured in grams (or multiples or fractions thereof). Biotechnology procedures require mass measurements ranging from kilograms (1000 grams) to micrograms (10^{-6} of a gram). Most biotech laboratories will have more than one type of balance, depending on the amount of material being measured and the degree of accuracy required. The simplest type of balance is a **triple-beam balance.** The triple-beam balance has a pan where the object to be weighed is placed and **three beams**, each with a sliding reference weight (i.e. riders) that can be moved along the beam to balance the object (Figure **1.3.2**). The middle beam has the largest (or heaviest) reference weight; the far beam has a reference weight of medium size and weight, and the front beam has the smallest (or lightest) reference weight. To use a triple-beam balance, the object to be weighed is placed on the pan and the reference weights on the beams are moved until the object's mass is "balanced" against the reference weights. The mass is then read.

Figure **1.3.2**: The triple-beam balance. Left image: The triple-beam balance has a stainless steel weighing pan attached to three beams, each with a sliding reference weight or rider. Placing an object on the weighing pan lifts the beams. The riders are moved along the beam until they balance the mass of the object on the pan. (Triple Beam Balance by Patricia Zuk, CC BY-SA 4.0) Right image: The balanced riders indicate a mass of 300 + 90 + 9.4 = 399.4 g (Triple Beam Balance Display by Patricia Zuk, CC BY 4.0)

Concept in Action

Interactive Exercise: Reading a Triple Beam Balance

To measure accurately, most labs use **electronic balances**. An electric balance measures an object's mass using electronic sensors to compare to a reference weight rather than physical reference weights. The mass is displayed using an electronic readout. In general, the more places an electronic balance can read to the right of the decimal point in the balance's readout, the more accurate it is. This means that a balance that can measure to the nearest gram is less accurate than one that can measure to the nearest 100th of a gram. Most electronic balances have a sensitivity of +/- 0.01 g, but **analytical balances** can be sensitive to +/- 0.01mg or less. Balances also have a maximum weight capacity. It is important to never exceed the maximum capacity of a balance or it may be irreparably damaged.

Both the electronic and analytical balance have a stainless steel plate (i.e., a weigh plate) on its weighing surface. This plate is where the sample to be weighed is placed. Because chemicals can damage the balance, they are never placed directly onto the weigh plate. Instead, the sample to be weighed is usually placed in a very light, plastic **weigh boat** (Figure **1.3.3**). Alternatively, **weigh paper** may also be used. The balance will have several operational buttons that can power the balance on and off or change its mode. Another button will be the **"Tare"** button. This "zeroes" the balance before weighing. If you place an empty weigh boat on the plate, the balance will weigh it. If you press the tare button, the balance readout will return to zero and the mass of the weigh boat will be eliminated from what you are about to measure. The analytical balance is very similar to the standard electronic balance. However, its weighing surface is surrounded by a glass enclosure in order to prevent air flow over the weighing surface. This air flow can alter the readout and diminish the precision of the measurement. As such, an analytical balance is used to precisely measure the mass of very small objects.

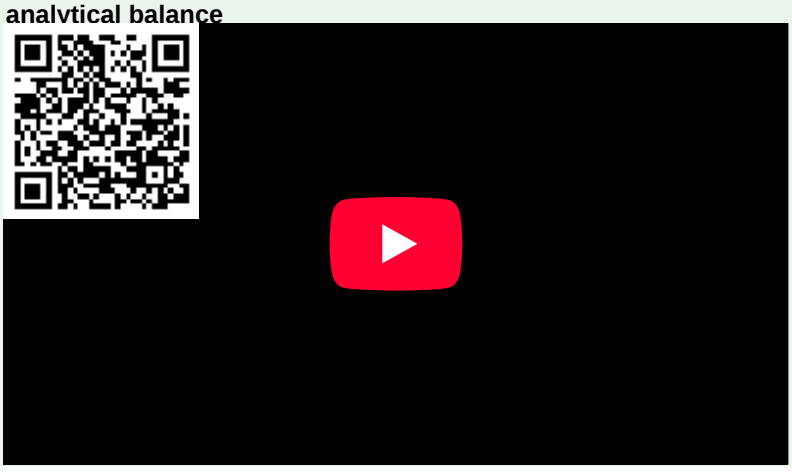
Figure 1.3.3: Measuring mass with electronic and analytical balances. Left image: Objects are placed onto the balance using plastic weigh boats in order to protect the weigh pan of the balance. (Disposable weigh boats by Lilly_M; CC BY-SA 3.0)
Middle image: The electronic balance has a stainless steel weigh pan for the object, a digital readout, and several operational buttons that control the operation of the balance. (Electronic scale by rebcenter; CC BY-SA 4.0)
Right image: The analytical balance is an electronic balance with a weighing chamber that can be opened to insert the object onto the weigh pan and then closed to eliminate air flow over it. (Analytical scales by rebcenter; Public Domain)

Concept in Action

How to use an Electronic Balance:

1. Turn the balance on
2. If weighing an object:
    1. Press the "Tare" button and allow the readout to "zero"
    2. Place the object on the weigh pan
    3. Allow the readout to stabilize
    4. Record the mass of the object
3. If weighing a chemical:
    1. Place an empty weigh boat or paper on the weigh pan
    2. Press the "Tare" and allow the readout to "zero"
    3. Add the chemical to the weigh boat/paper using a spatula.
    4. Allow the readout to stabilize
    5. Record the mass of the chemical
    6. Repeat steps 3 through 5 until the desired mass of the chemical is obtained

Video: How to use an analytical balance



pH: pH Paper & pH Meters

Most solutions prepared in a biotech laboratory must have a carefully controlled **pH**. pH is the measure of how acidic or basic a solution is and is a measure of the concentration of H+ ions in a liter of solution.

The formula for pH is: **pH=−log[H+]**

pH is a scale that ranges from 0 to 14. By definition, the pH value of **neutral solution** is 7.0; any solution with a pH < 7 is considered **acidic;** any solution with a pH > 7 is considered **basic**. pH can be measured in the laboratory using pH indicators, pH paper or a pH meter.

A **pH indicator** is *a chemical added to a solution that changes color when it experiences different pH conditions*. Common pH indicators are phenol red and phenolphthalein. At neutral pH, phenol red is red, but turns yellow in an acidic environment. As a solution becomes more basic, the intensity of the red color will increase. Phenolphthalein becomes colorless at pH values below 8.3 (i.e. neutral and acidic solutions) and turns a bright pink in basic solutions with a pH above 8.3. While lacking in precision and accuracy, a pH indicator can be used to give a quick assessment of pH.

A ph estimate can also be achieved using **pH paper**. pH paper is a special type of paper that will change color when dipped in a solution. pH paper has an array of pH-sensitive chemicals that exhibit a specific color when exposed to a particular pH Figure **1.3.4**.

Figure **1.3.4**: pH indicator paper. A single pH paper strip is coated with a mixture of pH-sensitive dyes that change color when exposed to a solution (four colored squares). pH can be determined by comparing the colors on the pH strip to a reference chart., allowing users to determine the pH by comparing the color change to a reference chart. (pH paper by Kareen Martin, CC BY 4.0)

More accurate and precise measurements of pH is achieved using a **pH meter.** A pH meter is a scientific instrument that measures the hydrogen-ion activity in aqueous solutions, indicating its acidity or alkalinity expressed as pH. A pH meter uses two electrodes: a reference electrode and an electrode that is sensitive to H+ concentration. These two electrodes are frequently housed in a single unit as a combination electrode. Because there is a linear relationship between electric potential and pH, a pH meter is actually a voltmeter that *reads the difference in electric potential between the two electrodes and converts this difference into a pH reading*. As such, the pH meter is sometimes referred to as a "potentiometric pH meter." pH meters can be small and handheld or stationary with arms to hold the electrode apart from the base unit (Figure **1.3.5**). The small handhelds are convenient but sacrifice accuracy for that convenience. The electrodes of the pH meter need to be stored properly – either capped or placed in a storage solution – to prevent them from drying out. A concentrated solution of 3M KCl is frequently used. This solution needs to be rinsed away with distilled water before the electrode is used. Prior to use, the meter also must be calibrated using **buffers** of fixed pH. The three most common buffers are pH 4.0, 7.0 and 10.0. The pH meter should be calibrated using two of these three buffers and the buffers chosen should bracket the desired pH. For example, if a solution is to be adjusted to pH 5.0, then the pH 4.0 and 7.0 buffers should be used to calibrate the meter.

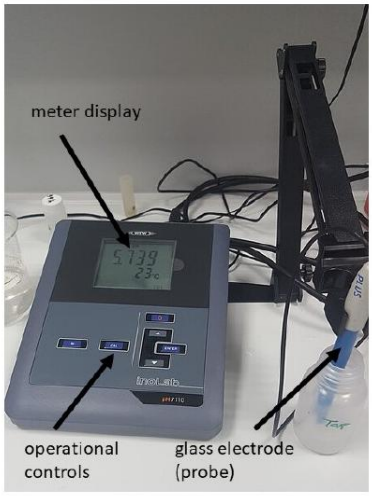


Figure 1.3.5: The digital pH meter. The digital pH meter determines pH using a glass electrode probe that is immersed in the liquid. The pH of the solution is shown on a digital display. Operational controls can be used to calibrate the meter prior to measurement. (Digital pH meter by S Shamima Nasrin; CC-BY-SA 4.0)

Concept in Action

Interactive Exercise: pH Scale Simulation

Spectrophotometer

A **spectrophotometer** measures the concentration of a solution by measuring how much light is absorbed by the solutes within the solution. In a spectrophotometer, a lamp provides the source of light. This beam of light can be separated into specific wavelengths that range from UV to visible to infrared. The spectrophotometer is set to a specific wavelength depending on what solute is being measured, as only that wavelength will be absorbed by the solute within the solution. The light passes through the solution and a detector then measures the amount of light that is transmitted. This transmittance can be converted into a numeric value of absorbance that is used to calculate concentration. There are several types of spectrophotometers based on the wavelengths of light they produce. In the biotech lab, the two most common are the **visible light spectrophotometer** and the **UV/VIS (ultraviolet/visible light) spectrometer**. The visible light spectrophotometer measures absorbance in the visible spectrum (400 to 700 nm), while the UV/VIS spectrophotometer measures absorbance in the visible range and the UV range (200 to 400 nm). The visible spectrophotometer can be used in the biotech lab to measure the concentration of proteins in solution, while the UV/VIS spectrophotometer, because of its low wavelength range, is used for the determination DNA and RNA concentrations.

Concept in action

Video: How to use a spectrophotometer

https://bio.libretexts.org/@go/page/138867

Equipment for Measuring and Transferring Liquids

Measuring and transferring liquids in a laboratory is done using graduated cylinders and pipettes. The choice between graduated cylinder and pipette will depend on the volume of liquid being transferred. Graduated cylinders are generally used to measure and transfer larger volumes of liquid (e.g. over 25 mL), while a pipette is used to transfer smaller volumes (e.g. under 25 mL)

Graduated cylinders

Graduated cylinders are one of the most accurate ways of measuring and transferring moderate amounts of liquid. Composed of either clear plastic or glass, the cylinders have volumetric measurements (in milliliters/mL) printed on the side. To measure volume in a graduated cylinder, it is placed on a flat surface and the liquid is slowly poured into it. The liquid should be allowed to settle before reading its volume so that the **meniscus**, the curved surface of the liquid, can form in the cylinder (Figure **1.3.6**). The meniscus forms due to adherence of the liquid's edges to the graduated cylinder. The volume of the liquid in the cylinder is read using the *bottom of the meniscus*. For an accurate measurement, the meniscus of the liquid should be read at eye level. A graduated cylinder can measure within 1% of the target volume, making it fairly accurate. However, the larger the cylinder, the less accurate it will be. This is due to the large diameter of the meniscus, which is sensitive to atmospheric pressure. The rule of thumb is to use the smallest available cylinder of sufficient capacity. For example, if a 25 mL volume needs to be measured, a 50 mL graduated cylinder should be used, not a 500 mL.

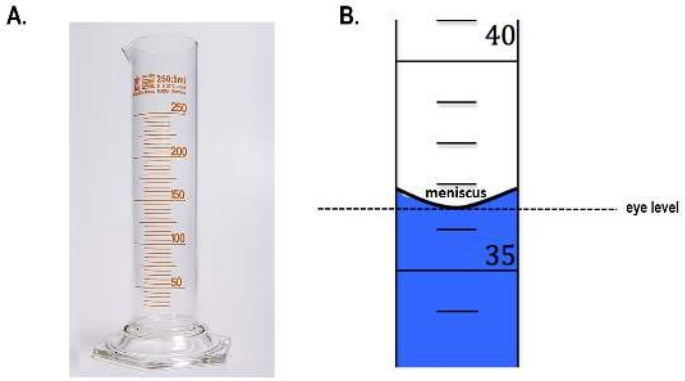


Figure **1.3.6**: The graduated cylinder. (A) A glass graduated cylinder for the measurement of 250 mL of liquid. (B) Volume measurement should be taken at the bottom of the meniscus with the eye placed at the level of the liquid. (Reading a graduated cylinder by Patricia Zuk, CC BY 4.0; adapted from Glass graduated cylinder by Lilly_M, CC BY-SA 4.0 and Graduated Cylinder Illustration by Praphai Donphaimueang, CC BY-SA 4.0)

Serological Pipettes

Serological pipettes are used for dispensing precise volumes that may be too difficult (or small) to measure using a graduated cylinder. A serological pipette is one of several **vacuum-assisted pipettes,** or pipettes that draw up liquids by creating a vacuum. Serological pipettes can be made of plastic or glass and are used to to deliver volumes between 1 and 100 mL. Examples of serological

pipettes include glass Pasteur pipettes, plastic transfer pipettes, and the volumetric serological pipette (Figure **1.3.7**). The vacuum that draws liquid into serological pipettes is created using a "pipet-aid" that can be as simple as a rubber bulb or an electronic pipette pump.

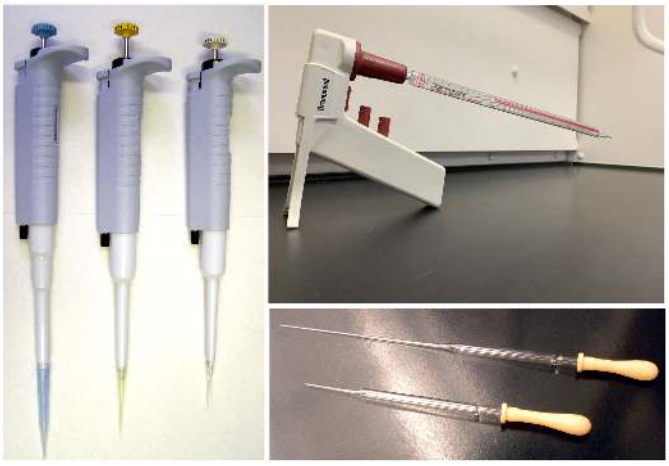


Figure **1.3.7**: Pipettes. The types of pipettes used in the biotech lab range from micropipettes (left) to volumetric serological pipettes (top right; shown with an electronic pipette pump) and glass Pasteur pipettes (bottom right; shown with pipette bulb). (Pipette gallery by Squidonius, CC BY-SA 4.0)

The volumetric serological pipette is used to precisely deliver liquids into small containers where spilling from a beaker or cylinder would be likely. Like all volume measuring devices, there are gradations on the side (Figure **1.3.8**). Many pipettes will have gradations on both sides with one side increasing in volume the other side decreasing in volume. Smaller volume pipettes like the 5 and 10 mL have major gradations indicating milliliter (mL) marks (e.g. 1, 2 mL), in addition to minor gradations of 0.1 mL volume. The larger volume pipettes (e.g. 25 mL) will have 1 mL major gradations, but have larger volume minor gradations (e.g. 0.2 mL). Like the graduated cylinder, the volume in a volumetric pipettes should be read using the meniscus held at eye level. All serological volumetric pipettes are designed to hold liquid in their tips. This volume may or may not be taken into account when measuring and dispensing a specific volume. Pipettes in which this residual volume is taken into account are known as "TD-Ex" ("to deliver") pipettes. The TD-Ex pipette will hold a small amount of liquid after dispensing that should not be delivered. Those pipettes in which the residual volume is not taken into account are "TC-In" ("to contain") pipettes, also known as "blow out" pipettes. This means that the liquid must be "blown out" by the pipet-aid in order to dispense the correct volume. To learn more about using a serological pipette, go to Chapter 13.3 Lab Technique: Pipetting.

Figure **1.3.8**: Volumetric serological pipettes. Serological pipettes measure and transfer liquids from 1 mL to 100 mL, although 5, 10 and 25 mL volumes are the most commonly used in the biotech lab. The volumetric serological pipette will have volume gradations on one side and may even have gradations on both sides. At the top of these pipettes is information detailing its maximum volume (e.g. 25 mL), the smallest gradation (e.g. 0.2 mL), the temperature it is to be used (e.g. 20°C) and whether it is a "TD-Ex" (T.D.) or "TC-In" (T.C.) pipette. Cell culture serological pipettes will have a cotton plug at the top of the pipette (not shown). (Serological Pipettes by Patricia Zuk, CC BY 4.0)

Pipette Pumps (Pipet-Aids)

The drawing of fluid into a serological pipette and the dispensing of it can be done with a device called a **pipette pump** or "**pipet-aid**". A pipette pump creates a vacuum that "sucks" fluids up into a pipette. There are many types of pipette pumps, ranging from a simple rubber squeeze bulb to an electronic pump (Figure **1.3.9**). Most biotech labs use a rechargeable, battery-driven **electronic pipette pump** is to dispense liquids via volumetric serological pipettes. The electronic pipette pump has a plastic nose cone where the pipette is inserted, and a handle with two buttons. The nose piece will have a filter inside of it to absorb volatile compounds and stop liquids from being aspirated into the pump. The top button on the handle aspirates the liquid into the pipette. The bottom button dispenses the liquid. To learn more about using a pipette pump, go to Chapter 13.3 Lab Technique: Pipetting.

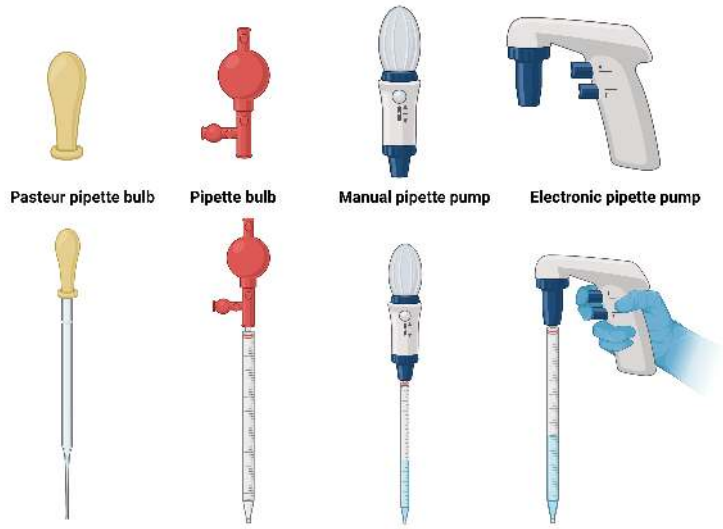
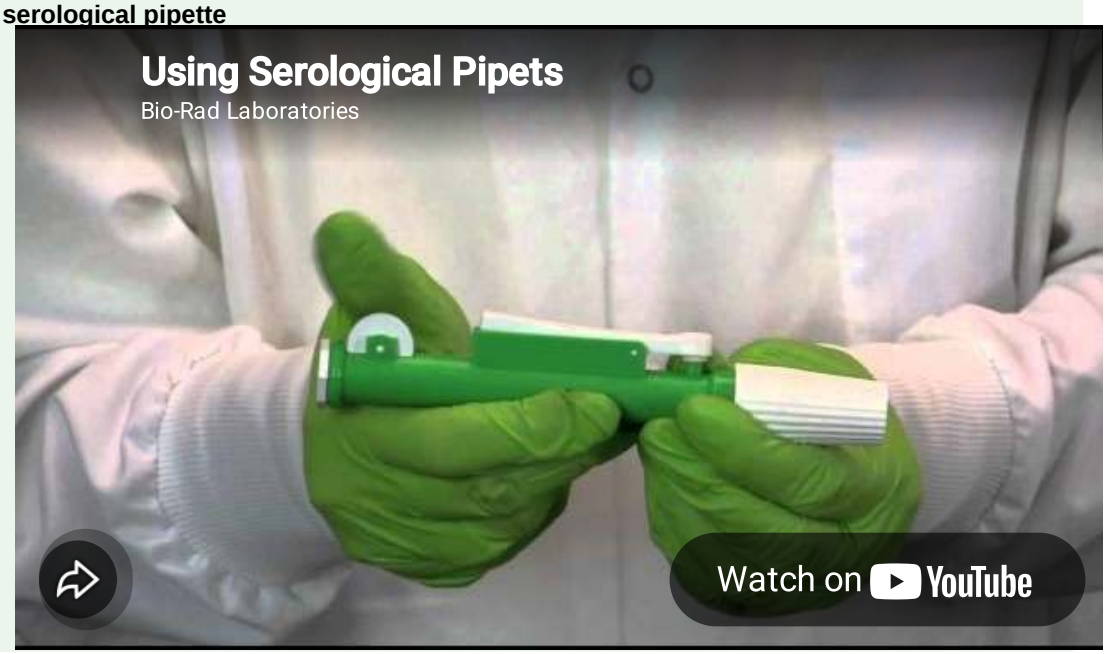


Figure **1.3.9**: Pipette pumps. Common pipette pumps used in the lab include (left to right) the rubber Pasteur pipette bulb, the manual pipette pump, and the electronic pipette pump. The pipette bulb and the manual and electronic pipette pumps are used for serological pipettes, while the Pasteur pipette bulb is used with glass Pasteur pipettes. (Pipet Aids by Patricia Zuk; figure created in BioRender. Zuk, P. (2025))

Concept in Action

Video: How to use a serological pipette



Micropipettes

Micropipettes are an essential part of any biotech lab. They are used for dispensing volumes less than 1 mL. Each micropipette has the same basic component parts: a **plunger** for aspirating and dispensing fluids, a **volume adjustment knob**, a **volume display** in the **body** of the micropipette, a **shaft** for the attachment of a **pipette tip** and a **tip ejector** (Figure **1.3.10**).

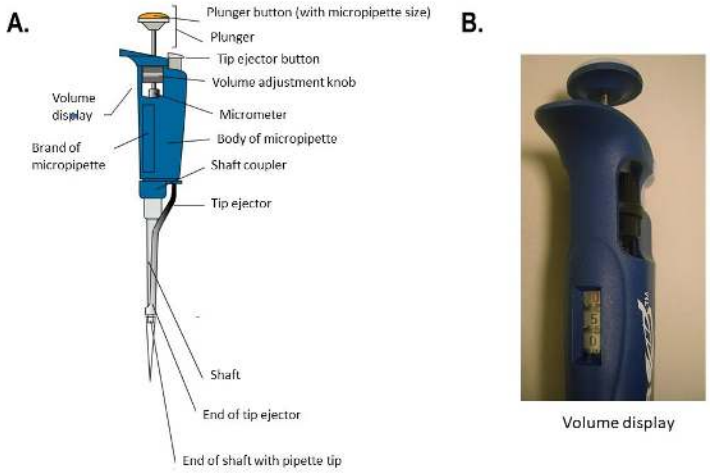


Figure **1.3.10**: The micropipette is used to dispense volumes less than 1 mL. (A) The basic components of the micropipette are a plunger for aspirating and dispensing fluids, a volume adjustment knob, a volume display in the body of the micropipette, a shaft for the attachment of a pipette tip and a tip ejector. (B) The volume display is found in the body of the micropipette and indicates the volume being dispensed. (The micropipette by Patricia Zuk, CC BY 4.0; (adapted from Gilson schematic by Squidonius, CC BY-SA 3.0 and Micropipette part by Superchilum, CC BY-SA 3.0)

There are several sizes of micropipettes depending on the maximum volume they can dispense:

1. a **P2** that accurately dispenses volumes from 0.2 uL to 2.0 uL
2. a **P10** that accurately dispenses volumes from 1 uL to 10 uL
3. a **P20** that accurately dispenses volumes from 2 uL to 20 uL
4. a **P50** that accurately dispenses volumes from 5 uL to 20 uL
5. a **P100** that accurately dispenses volumes from 10 uL to 100 uL
6. a **P200** that accurately dispenses volumes from 20 uL to 200 uL
7. a **P1000** that dispenses volumes from 100 uL to 1000 uL

The range of a micropipette is very important. Working outside of the range of a micropipette will decrease its accuracy and precision and can even damage or break it. Because they are very precise pieces of lab equipment, micropipettes are calibrated on a regular basis to ensure that they are precise and accurate. For more information about how to use a micropipette, go to Chapter 13.13 Lab Technique: Pipetting.

Concept in Action

Video: How do you use a micropipette?

https://bio.libretexts.org/@go/page/138867

Equipment for Storing

Beakers

A **beaker** is a simple container for making solutions, stirring, heating liquids, and adjusting pH. Beakers are generally cylindrical in shape, with a flat bottom (Figure **1.3.11**). Most have a small spout (or "beak") to aid in pouring. Most will also have measurement gradations printed on the side. Beakers are available in a wide range of sizes, from 1 mL up to several liters. The graduations on the side are not to be used for volumetric measurement as they are usually only within ±5% of the actual volume.

Erlenmeyer flasks

An **Erlenmeyer flask** has slanted sides and a narrow opening (Figure **1.3.11**). The flask's design facilitates swirling and mixing of the contents without spilling. The narrow opening of the flask can be covered with Parafilm or another impermeable cover or a glass stopper allowing for the temporary storage of liquids inside the flask. Some flasks come with threaded openings and screw cap or culture tops that "snap" onto the opening. These flasks are used to grow liquid cultures of micro-organisms like bacteria and yeast. Like beakers, the Erlenmeyer flask should not be used for measuring, although the gradations on the outside provide a rough guide for measuring a volume. A 100 mL Erlenmeyer flask will be accurate to within ±0.2 mL. Erlenmeyer flasks with capacities from 25 mL to 4 L are commonly found in laboratories.

Volumetric Flasks

Volumetric flasks are used to make solutions for analytical assays. There are no graduated markings in a volumetric flask (Figure **1.3.11**). When liquids are brought to the indicator line, they have the highest degree of volumetric accuracy for glassware. For example, a 100 mL volumetric flask can have an accuracy of +/- 0.1 mL.

Reagent Bottles

Reagent bottles are used to store solutions and are available in many shapes and sizes (Figure **1.3.11**). The bottles can be made of plastic or glass, depending on the solution that is being stored and how it is to be stored. For example, 1M NaCl can be stored in a plastic reagent bottle at room temperature whereas HCl needs to be stored in a glass bottle. It is important to realize that while strong acids need to be stored in glass bottles, a strong base will react with the glass and must be stored in a plastic bottle. A chemical's **MSDS (Material Data Safety Sheet)** will have information on how to properly store the chemical when in solution. Many reagent bottles are made of Pyrex glass so that they are heat resistant and can be heated or autoclaved.

Figure 1.3.11: Laboratory glassware. Glass 2000 mL beaker (by Lilly_M, CC BY-SA 3.0), Erlenmeyer flask (by Hannes Grobe CC BY-SA 2.5), Brand volumetric flask (by Lucasbosch, CC BY-SA 3.0) and Laboratory glass bottles (by Lilly_M, CC BY-SA 3.0).

Refrigerators and Freezers

Many reagents used in biotech laboratories require specific storage temperatures to prevent their degradation, maintain their stability, and prevent contamination. While the use of household refrigerators and freezers may be attractive due to their costs, laboratory refrigerators and freezers are more durable and provide better insulation and more precise temperature control. In addition, many lab refrigerator and freezers come with monitoring systems to warn users when the temperature deviates from its set range.

Different types of refrigerators can be found in a biotech lab, including the:

- **General laboratory refrigerator (4°C):** used for storing chemicals and reagents such as culture media and molecular and cell biology reagents that are sensitive to extreme cold (e.g. some antibodies and enzymes); can come equipped with either glass doors or a metal door for light-sensitive reagents
- **Pharmaceutical refrigerator (4°C):** used for storing vaccines, medicines, and blood and plasma samples used for diagnostic tests
- **Explosion-proof refrigerators (4°C):** used for the storage of flammable or volatile substances (e.g. ethanol)

Different types of freezers can be found in a biotech laboratory, including the:

- **Standard laboratory freezer (-20°C to -30°C):** used for storing common reagents for molecular biology procedures (e.g. PCR, DNA sequencing), chemicals (e.g. ethanol, phenol), and biological samples (e.g. DNA, proteins, enzymes, antibodies)
- **Ultra-low temperature freezer (-80°C):** used for long-term storage of biological samples, such as bacteria cultures, tissues, RNA, and proteins
- **Cryogenic freezer (-150°C to -196°C):** also known as liquid nitrogen storage; used for long-term storage of eukaryotic cell cultures and other highly temperature-sensitive biological materials (e.g. viruses)

It is important to stress to those working in the lab that laboratory refrigerators and freezers are to be used for lab samples and reagents and not for food and drink storage.

Equipment for Safety and Sterility

Maintaining safety and ensuring the quality of experiments are paramount in biotechnology labs. Several pieces of equipment in the biotech lab can provide users with the ability to perform experiments under safe conditions and decrease the chance of microbial contamination.

Personal Protective Equipment (PPE)

Personal protective equipment (PPE) protects the user against specific hazards in the laboratory. Safety glasses, lab coats and gloves are examples of PPE that are commonly used in biotech labs. The type of glove chosen will depend on the potential hazard that might be encountered in the lab. Most gloves used in biotech labs are either **latex, nitrile, or vinyl**. Latex is the simplest material and the most common kind of glove. However, many people may exhibit skin sensitivity to latex. Some disposable latex gloves are also flammable, which would prevent their use when working with bacteria. Nitrile gloves are used a common alternative to latex. Nitrile is thicker than latex, does not irritate most user's skin and provides a more substantial barrier. The use of a lab coat in a biotech lab may be mandatory or optional, depending on the lab. However, wearing a lab coat is recommended as it protects clothing and skin. Finally, while not necessary for every lab protocol, safety glasses are important pieces of PPE and should be selected based on the specific risks anticipated when working in a biotech lab (e.g. splash risk, shortwave, ultraviolet radiation).

Biological Safety Cabinet (BSC)

The **Biological Safety Cabinet (BSC)** is an enclosed, ventilated workspace for safely working with biological materials that *require a defined biosafety level*. Provide a controlled environment to handle pathogenic organisms and prevent contamination of samples and exposure to researchers. There are several types of BSCs, with each providing different levels of protection to the user, the environment, and the biological product. To read more about the BSC, go to Chapter 6.3: Cell Culture Equipment and Chapter 13.12: Lab Technique - Cell Culture.

Fume Hood

A **fume hood** is a ventilated enclosure that is used in many biotech labs to safely handle hazardous chemical, vapors, and fumes. The fume hood works by pulling air in from the room, therefore pushing the contaminated air away from the user. The contaminated air is either expelled directly to the outside environment through a ventilation system or by filtered through a specialized system before expelling it. It is important to remember that a fume hood is not a BSC. While both protect the user in some manner, the fume hood does not prevent microbial contamination and the BSC does not provide protection from hazardous vapors and fumes.

Autoclave

An **autoclave** is a high-pressure steam sterilizer that uses *moist-heat sterilization* to sterilize equipment, liquid reagents, and media (Figure 1.3.12). Charles Chamberland (1851–1908) designed the modern autoclave in 1879 while working in the laboratory of Louis Pasteur. The autoclave produces high-temperature steam to sterilize items such as surgical equipment and laboratory glassware, without damaging them. This high temperature also sterilizes liquids, killing bacteria, viruses, and fungi, without degrading the components of the liquid media or reagent. Standard operating temperatures for autoclaves are 121°C or, in some cases, 132°C, typically at a pressure of 15 to 20 pounds per square inch (psi). The length of exposure depends on the volume and nature of material being sterilized, but is typically 20 minutes or more, with larger volumes requiring longer exposure times to ensure sufficient heat transfer to the materials being sterilized. The steam must directly contact the liquids or dry materials being sterilized, so containers are left loosely closed and instruments are loosely wrapped in paper or foil.

Figure 1.3.12: An autoclave. Autoclaves are designed to sterilize liquids and solid materials using pressurized steam. This autoclave contains several pieces of equipment ready to be sterilized. (Sterilisator by KOchstudioO; CC BY-SA 3.0)

In general, the air in the chamber of an autoclave is removed and replaced with increasing amounts of steam, resulting in increased interior pressure and temperatures above the boiling point of water. The two main types of autoclaves differ in the way that air is removed from the chamber. In gravity displacement autoclaves, steam is introduced into the chamber from the top or sides. Air, which is heavier than steam, sinks to the bottom of the chamber, where it is forced out through a vent. The steam replaces the air. However, complete displacement of air is difficult, so longer cycles may be required for the type of autoclave. In pre-vacuum sterilizers, air is removed completely using a high-speed vacuum before introducing steam into the chamber. Because air is more completely eliminated, the steam can more easily penetrate wrapped items. Many autoclaves are capable of both gravity and pre-vacuum cycles, using the former for the decontamination of waste and sterilization of media and unwrapped glassware, and the latter for sterilization of packaged instruments. The autoclave is still considered the most effective method of sterilization and is crucial for maintaining aseptic conditions in the laboratory.

The Centrifuge

A **centrifuge** is used to separate or collect materials of different densities by spinning them at high speeds. Through the generation of **centrifugal force**, denser materials, such as cells, subcellular organelles, and insoluble particles, are pushed to the bottom of the centrifuge tube, while the lighter components remain at the top. The centrifuge works by spinning a **rotor**, which is designed to hold tubes of specific volumes. Biotech centrifuges will typically have a **fixed-angle rotor** and a **swinging-bucket rotor** (Figure **1.3.13**). The fixed-angle rotor holds the sample tubes at a specific angle, allowing for faster sedimentation rates of dense materials. A swinging-bucket rotor holds the tubes vertically at rest but allows the tubes to "swing-out" to a horizontal position when spinning. Such a rotor allows for better separation between materials of different densities. The choice of rotor will depend on what the centrifuge is being used for. The speed of the centrifuge can be measured as **revolutions per minute (rpm)** or **relative centrifugal force (RCF)**. These two measurements are not the same but are related to one another. RCF is the actual centrifugal force exerted on the sample, while rpm is the actual spinning speed of the centrifuge. Two centrifuges spinning at the same rpm with different rotors will have two different RCF measurements. RCF can be converted into rpm using a mathematical formula that takes the size of the rotor into account.

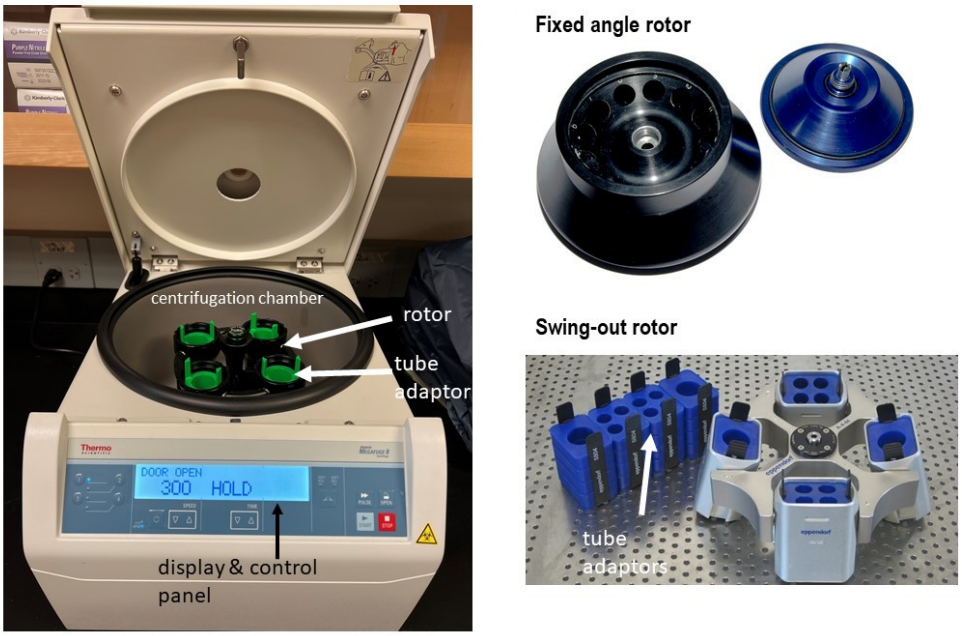


Figure **1.3.13**: The centrifuge. Left image: the centrifuge has a centrifugation chamber into which the rotor and samples are placed. Rotors can be "fixed angle" or "swing-out". A control panel controls centrifuge speed and temperature (if possible). (Tabletop centrifuge by Patricia Zuk, CC BY 4.0)
Top right image: fixed angle rotors hold centrifuge tubes at a specific angle during centrifugation. (Beckman TLA55 by Nadine90; CC BY-SA 3.0)
Bottom right image: swing-out rotors spin centrifuge tubes in a horizontal position. Rotors often come equipped with tube adaptors to allow for the centrifugation of many tube sizes. (Eppendorf centrifuge rotor by Kitmondo Marketplace; CC BY 2.0)

Centrifuges are used in biotech labs to pellet cells within a liquid, separate liquid components, purify proteins, and isolate DNA. There are several types of centrifuges (Figure **1.3.14**), including the:

- **Preparative centrifuge:** used to isolate and purify substances (e.g. subcellular organelles, DNA, proteins); spins at higher speeds (up to 30,000 rpm); different sized rotors can hold vessels as small as a few milliliters to as large as a liter; often refrigerated so that heat-sensitive material, such as cells and proteins, are not damaged due to the high heat generated during centrifugation
- **Tabletop centrifuge:** used to pellet cells and separate liquids; a small centrifuge that spins at slower speeds (less than 5,000 rpm); rotors hold tubes from a few milliliters to 50 mL; typically use fixed angle and swing-out rotors; clinical versions use swing-out rotors to separate biological fluids and pellet cells; refrigerated options available
- **Micro-centrifuge:** used to separate liquids and to pellet materials like cells, insoluble proteins, and DNA; a compact centrifuge that spins from 1,000 rpm to 14,000 rpm; uses a fixed-angle rotor that holds 2.0 mL (or smaller) micro-centrifuge tubes;

refrigerated options available

- **Ultra-centrifuge:** used to isolate and study extremely small materials like ribosomes, DNA, RNA, proteins, and viruses, like DNA; spins at very high speeds (up to 100,000 rpm) within a vacuum chamber to prevent overheating; preparative versions spin a higher speeds versus analytical versions; uses fixed angle rotors only and specialized tubes designed to withstand high RCF.

Figure **1.3.14**: Types of centrifuges. The tabletop centrifuge (left) and microcentrifuge, also called a "microfuge" (right), are two common types of centrifuges found in biotech laboratories. (Centrifuges by Kareen Martin; CC BY 4.0)

To centrifuge, the materials are placed into the appropriately sized centrifuge tube or bottle. The specific tube or bottle used will depend on the volume of the sample and the speed of centrifugation. Centrifuge tubes and bottles are created to withstand centrifugal forces. Higher speed/centrifugal force will require thicker plastic. The samples are loaded into the centrifuge, the lid closed, and the samples are spun at the required speed and temperature. However, because of their high rotation rates, samples must be loaded correctly in order to prevent injury or breaking the centrifuge. Specifically, the samples must be loaded such that they are *balanced within the rotor* (Figure **1.3.15**).

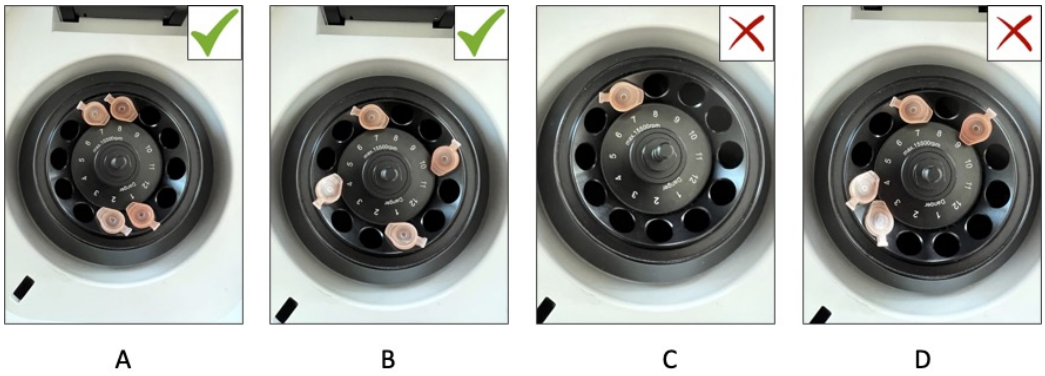


Figure **1.3.15**: Correct and incorrect loading of samples in a centrifuge. The samples shown in A and B are loaded into the rotor correctly and are balanced. Balanced samples will have the same number of empty spaces between them. The samples shown in C and D have been loaded incorrectly. The centrifuge in C has only one tube, another one with the same amount should be added on the opposite side. D has 4 tubes, 2 side by side and 2 others randomly placed. (Centrifuge Loading by Kareen Martin; CC BY 4.0)

Concept in Action

Video: How to use a centrifuge

https://bio.libretexts.org/@go/page/138867

Electrophoresis Equipment

Electrophoresis is a laboratory technique that uses an electric field to separate fragments of DNA, RNA, or proteins according to their sizes and electric charges. This basic lab technique is known as **electrophoresis**. In electrophoresis, samples containing fragments of nucleic aids or proteins are loaded into wells created in a polymeric gel (i.e. agarose or acrylamide) and an electric field applied. The negatively charged nucleic acids or proteins will move through the gel towards the positive electrode (anode). The smaller fragments will move faster through the gel and travel further towards the anode. While the larger fragments will move slower. In order to perform electrophoresis, a biotech lab will have several kinds of electrophoresis "tanks" or "rigs" that are designed for either nucleic acids (i.e. DNA and RNA) or protein. Nucleic acid electrophoresis is typically done using a horizontal tank, while protein electrophoresis is done in a vertical tank (Figure **1.3.16**). To learn about electrophoresis of DNA, go to Chapter 13.1: Lab Technique - Agarose Gel Electrophoresis of DNA. To learn about electrophoresis of proteins, go to Chapter 13.10: Lab Technique - SDS-PAGE.

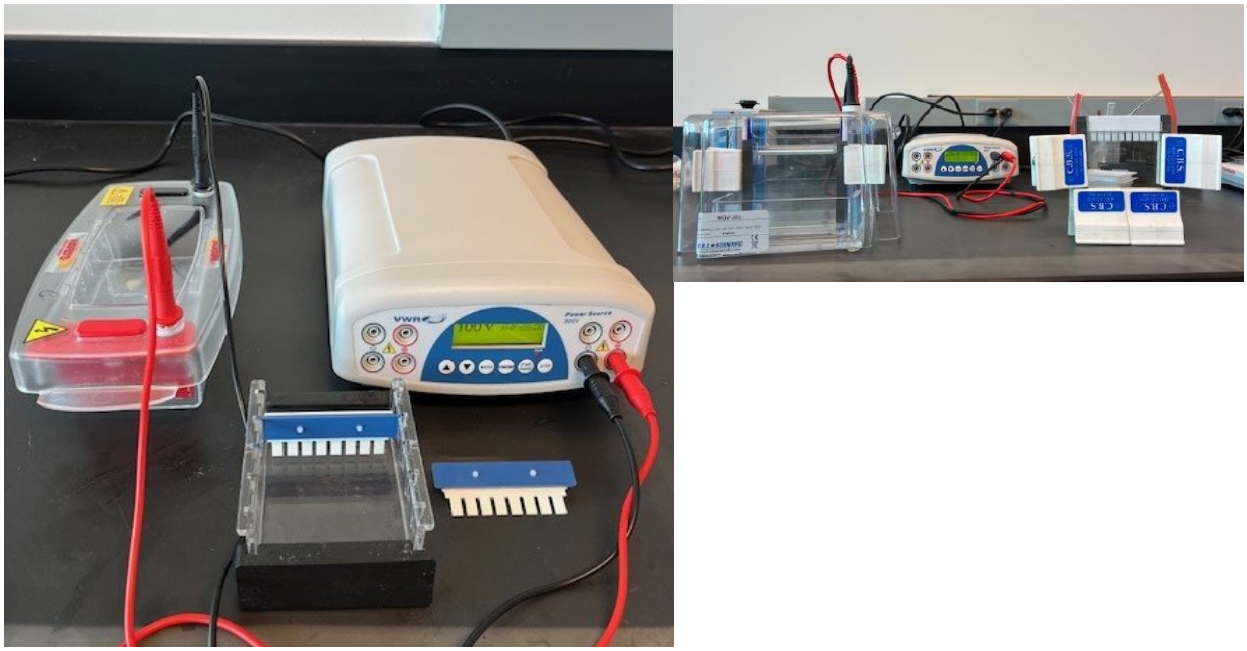


Figure **1.3.16**: Electrophoresis equipment. Left image - an electrophoresis system for DNA and RNA analysis, including (clockwise from the left) the horizontal electrophoresis tank, the power supply, and the gel tray with inserted comb. Right image - an electrophoresis system for protein analysis, including (clockwise from the left) the vertical electrophoresis tank, the power supply, and the gel plates with comb inserted. (DNA and Protein Electrophoresis Equipment by Patricia Zuk, CC BY 4.0)

Molecular Protocols

- Lab Technique: Pipetting
- Lab Technique - Agarose Gel Electrophoresis of DNA
- Lab Technique - SDS-PAGE

Key Concepts

The equipment used in biotechnology is diverse and specialized, reflecting the complexity and precision required in this field. Understanding the functions and applications of these tools is essential for anyone working in or studying biotechnology, as they form the foundation for scientific discoveries and technological advancements. Some important concepts to remember are:

- Basic laboratory tools include equipment for measuring, transferring liquids, storing, safety and sterility, and analysis
- Equipment for measuring includes balances and scales, pH meters, pH paper, and spectrophotometers
- Equipment for transferring small amounts of liquids include serological pipettes and micropipettes
- Serological pipettes require vacuum-producing devices like manual and electronic pipette pumps
- Equipment for transferring larger volumes of liquids includes graduated cylinders
- Equipment for storing liquids includes beakers, flasks, and reagent bottles
- Equipment for storing materials at controlled temperatures include refrigerators and freezers
- Freezers can range from standard models (-20°C to -30°C), to ultra-low freezers (-80°C), to cryogenic freezers, also known as liquid nitrogen containers (-150°C to -196°C)
- Safety equipment includes personal protective equipment (PPE) like gloves, biosafety cabinets, fume hoods, and autoclaves
- Equipment for basic lab analysis includes electrophoresis equipment for DNA, RNA, and protein separation, and centrifuges
- Centrifuges can range from low-speed table-top models to high-speed "ultracentrifuges"

Glossary

Autoclave - a high-pressure steam sterilizer that uses heat and pressure to sterilize equipment, liquid reagents, and media

Balance - a piece of equipment to measure the mass of an object

Buffer - a solution that resists changes in pH

Biological Safety Cabinet (BSC) - an enclosed, ventilated workspace for safely working with biological materials that require a defined biosafety level

Centrifuge - a piece of equipment that uses centrifugal force to separate materials of different densities

Electrophoresis - a laboratory technique that uses an electric field to separate fragments based on their size and charge

Fume Hood - a ventilated enclosure that is used to safely handle hazardous chemical, vapors, and fumes

Mass - the quantity of matter within an object

Meniscus - the curved surface of a liquid

Metrology - the science of measurement

Metric System - a standardized system of measurement that uses a set of base units a nomenclature based on decimal-based prefixes

Personal Protective Equipment (PPE) - lab equipment worn to protect a user against chemical and biological hazards

Pipette - a piece of equipment used to measure and transfer small amounts of liquid; found as types including Pasteur pipettes, serological pipettes, and micropipettes

pH - a logarithmic scale used to measure how acidic or basic a solution is

pH Indicator - a chemical added to a solution that changes color based on the pH of a solution

pH Meter - a piece of equipment that measures the pH of a solution

pH Paper - a type of paper that changes color based on the pH of a solution

Random Error - fluctuations from measurement to measurement

Relative Centrifugal Force (RCF) - the amount of force applied to an object using a centrifuge

Revolutions per minute (rpm) - a unit of rotational speed for rotating machines like centrifuges

Scale - a piece of equipment to measure the weight of an object

Spectrophotometer - a piece of equipment that measures the amount of photons of light absorbed by the solutes in a solution; used to determine the concentration of a solution

Systemic Error - consistent errors due to the state of a piece of equipment

Tare - a function on a balance or scale that removes the weight of a container holding the object being weighed

Weight - the force exerted on an object due to gravity

1.3: Common Biotechnology Equipment is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

- **2.3: Instruments of Microscopy** by OpenStax is licensed CC BY 4.0. Original source: https://openstax.org/details/books/microbiology.

1.4: Advanced Biotechnology Equipment

Biotech Focus

A **biofilm** is a complex community of one or more microorganism species, typically forming as a slimy coating attached to a surface. In nature, biofilms are abundant and frequently occupy complex niches within ecosystems (Figure **1.4.1**). In medicine, biofilms can coat medical devices and exist within the body. Because they possess unique characteristics, such as increased resistance against the immune system and to antimicrobial drugs, biofilms are of particular interest to microbiologists and clinicians alike. Because biofilms are thick, they cannot be observed very well using conventional light microscopy or electron microscopy. This is because slicing a biofilm to create a thinner specimen might kill or disturb the microbial community. Confocal microscopy, in combination with fluorescent dyes or antibodies, is often used to image biofilms because it scans the film on one z-plane at a time and produces a three-dimensional image of a thick specimen.

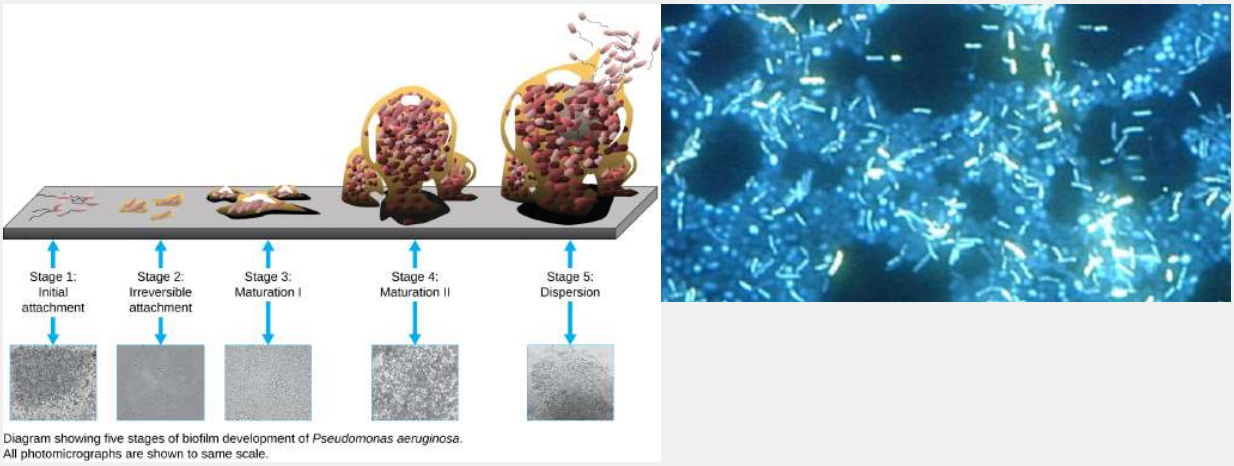


Figure **1.4.1**: Biofilms and microscopy. Left image - a biofilm forms when planktonic (free-floating) bacteria of one or more species adhere to a surface, produce slime, and form a colony. Right image - multiple species of bacteria growing as a biofilm on stainless steel stained with the UV fluorescent dye, DAPI, for fluorescence microscopy. (Biofilms by OpenStax Microbiology, CC BY-SA 4.0).

Introduction

The biotech laboratory often requires specialized equipment to conduct experiments and analyze results. This equipment can include several types of microscopes, PCR machines for DNA amplification, and DNA sequencers.

Learning Objectives

By the end of this page, you will be able to:

- Identify and describe the parts of a brightfield microscope
- Calculate total magnification for a compound microscope
- Describe the distinguishing features and typical uses for various types of light microscopes and electron microscopes
- Describe the thermocycler or PCR machine
- Describe how flow cytometers can analyze cells

The Microscope

A **microscope** is an instrument used to magnify an object. The technique of using a microscope is called **microscopy**. Through their work, the early pioneers of microscopy, including Zacharias Janssen (1590s) and Anton von Leeuwenhoek (1670s), opened a window into the invisible world of microorganisms. These microscopes were rudimentary, using a light source and simple glass magnifying lenses. However, microscopy continued to advance in the centuries that followed. In 1830, Joseph Jackson Lister essentially created the first modern **compound light microscope**, a microscope that combines multiple lenses for magnification,

together with a lamp for visible light. The 20th century saw the development of more advanced microscopes, such as the **fluorescent microscope**, which uses an ultraviolet light source, and the **electron microscope**, which uses short-wavelength electron beams. These advances led to major improvements in magnification, resolution, and contrast.

The two major categories of microscopes are light microscopes and electron microscopes. However, advancements in microscopy has created a third category, the atomic microscope (i.e., scanning probe microscope) which uses physical probes to examine objects at the atomic level.

While differences exist between the types of microscopes, most microscopes have the same basic components. These components are best exemplified in the compound light microscope shown in Figure 1.4.2.

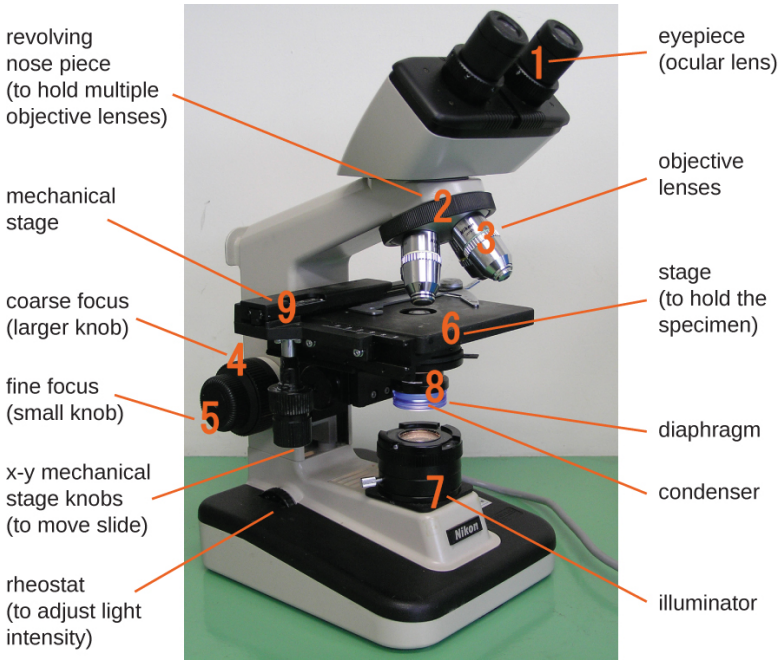


Figure 1.4.2: Components of a typical compound light microscope. The parts of the compound light microscope includes the coarse and fine focus knobs, the mechanical stage and the mechanical stage knobs, the illuminator which is the light source, the rheostat to adjust light intensity, the condenser to focus the light, the objective lenses, and the eyepieces. (image from OpenStax Microbiology, CC BY-SA 4.0)

The components of a compound light microscope include the:

- **mechanical stage:** the platform of the microscope where the specimen is placed and secured
- **x-y mechanical stage knobs:** for movement of the stage in the x-y plane
- **eyepiece:** used to view the specimen; in light microscopes it contains an ocular lens
- **ocular lens:** found in the eyepiece of light microscopes; typically magnify images 10x to 15x
- **objective lens:** provide different levels of magnification (i.e. "power"); made of glass in light microscopy; in electron microscopy, the objective lenses are electromagnetic
- **objective lens nosepiece:** a rotating nosepiece containing all objective lenses
- **coarse focus knob:** used for large-scale movements of the stage up and down in order to focus on the specimen
- **fine focus knob:** used for small-scale movements of the stage up and down in order to focus on the specimen; used for low power objective lenses (e.g. 4X and 10X)
- **illuminator:** provides an energy source in the form of visible light, ultra-violet light, or electrons for viewing the specimen
- **condenser:** focuses the illuminator on specimen to improve image clarity; the position can be optimized
- **rheostat:** a dimmer switch that controls the intensity of the illuminator
- **diaphragm (i.e., iris):** controls the amount of energy (e.g. light) passing through the condenser

In microscopy, **magnification** is how much larger the object appears compared to the size of the actual specimen. In electron microscopy, magnification is determined by the interaction between the electron beam, the electromagnetic objective lenses and the image projection system. In light microscopy, magnification is provided by both the objective lens and the ocular lens.

The total magnification is the product of the ocular magnification and objective magnification and is calculated using this formula:

$$\text{total magnification} = \text{ocular magnification} \times \text{objective magnification}$$

For example, if a 40X objective lens is selected and the ocular lens is 10X, the total magnification would be:

$$(40\times)(10\times) = 400\times$$

Contrast is the difference in light intensity between an object and its background, allowing for better detail when observing the object. Contrast can be enhanced in microscopy though the use of stains or the use of microscope filters. **Resolution** is the ability to distinguish two objects as separate entities. The higher the resolution, the clearer the image. In light microscopy, resolution may be compromised at very high magnification. When light passes through the small amount of air between the specimen and the lens, the differences in their refractive indices scatters the light rays before they can be focused by the objective lens. To solve this problem, a drop of **immersion oil** can be used to fill the space between the specimen and the objective lens. Since immersion oil has a refractive index very similar to that of glass, it decreases light scattering and increases the amount of light collected by the objective lens (Figure **1.4.3**). A variety of oils can be used for different types of light. However, only one type of objective lens can be used. This lens, known as an **oil immersion lens**, is a special lens designed to be used with immersion oils.

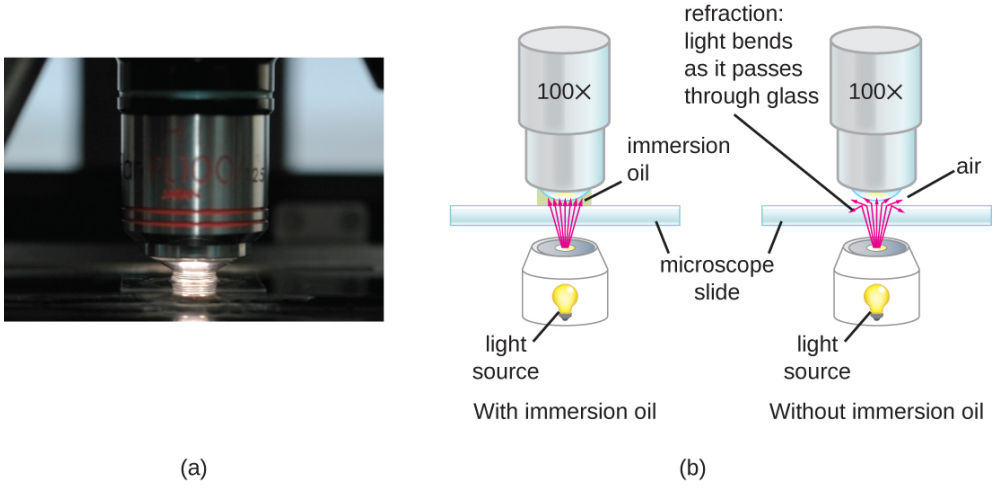


Figure **1.4.3**: Oil Immersion Microscopy. (a) The oil immersion lens works in concert with immersion oil to improve the resolution of an object at higher magnifications. (b) Because immersion oil and glass refract (i.e. bend) light rays to a similar degree, light passing through a microscope slide and into the air does not bend significantly before it reaches the objective lens. Without immersion oil, light passes through the microscope slide and then bends and scatters as it passes through the air. This decreases the resolution of the image captured by the objective lens. (images from OpenStax Microbiology, CC BY-SA 4.0)

Light microscopy

The concept of **light microscopy** is relatively straightforward. The specimen is processed and secured to the stage. The illuminator is turned on and the condenser focuses the light onto the specimen. The intensity of the light from the illuminator can be adjusted using the diaphragm. The specimen must be thin enough for the light to pass through. Staining techniques may be used to enhance the contrast of the specimen. The light passes through the specimen and is collected by the objective lens. Modern objectives are actually several glass lenses stacked on top of one another that act to collect the light passing through the specimen and focus it up towards the body of the microscope towards the ocular lens. The ocular lens will have an additional glass lens that magnifies the image further. Most light microscopes will have several objective lenses of increasing power (4X, 10X, 20X, 40X, 100X) on a rotating nosepiece. The revolving nosepiece of the microscope allows for objective lenses of different magnification or "power" to be chosen. As the power of the objective lens is increased, the amount of light passing through it decreases. This will require increased illumination, which is controlled by the rheostat. The coarse focus knob is adjusted to bring the specimen into rough focus, while the fine focus knob can fine tune the focus for finer details. e;

Some of the major types of light microscopes are:

1. **Brightfield microscope**
2. **Darkfield microscope**

3. **Phase-contrast microscope**
4. **Fluorescence microscope**
5. **Confocal Laser Scanning microscope**
6. **Polarizing microscope**

Today, a biotech lab might use several types of light microscopes to advance the progress of their research.

The Brightfield Microscope

The **brightfield microscope** is perhaps the most common type of light microscope used in a biotech lab. The brightfield microscope (often just called a light microscope) is a compound light microscope that uses its lenses to produce a dark image on a bright background. Older brightfield microscopes are monocular (having a single eyepiece), while most newer brightfield microscopes are binocular (having two eyepieces). As described above, the condenser of the brightfield microscope directs light from the illuminator up through the specimen into the objective lens and then the ocular lens to produce a magnified image. Structures in the specimen will appear darker, to various extents, than the bright background, creating maximally sharp images at total magnifications up to about 1000x. Further magnification using a brightfield microscope would create a larger image, but with poor resolution.

The Darkfield Microscope

The **darkfield microscope** is a brightfield microscope that has a small but significant modification to the condenser. A small, opaque disk, called an **light stop** or **illumination block**, is placed between the light source and the condenser lens. As light passes through the condenser on its way to the objective lens, most of it is blocked by the light block. This produces a hollow cone of light that passes through the specimen. The only light that reaches the objective lens is light that has been refracted or reflected by structures in the specimen. The resulting image typically shows bright objects on a dark background (Figure **1.4.4**).

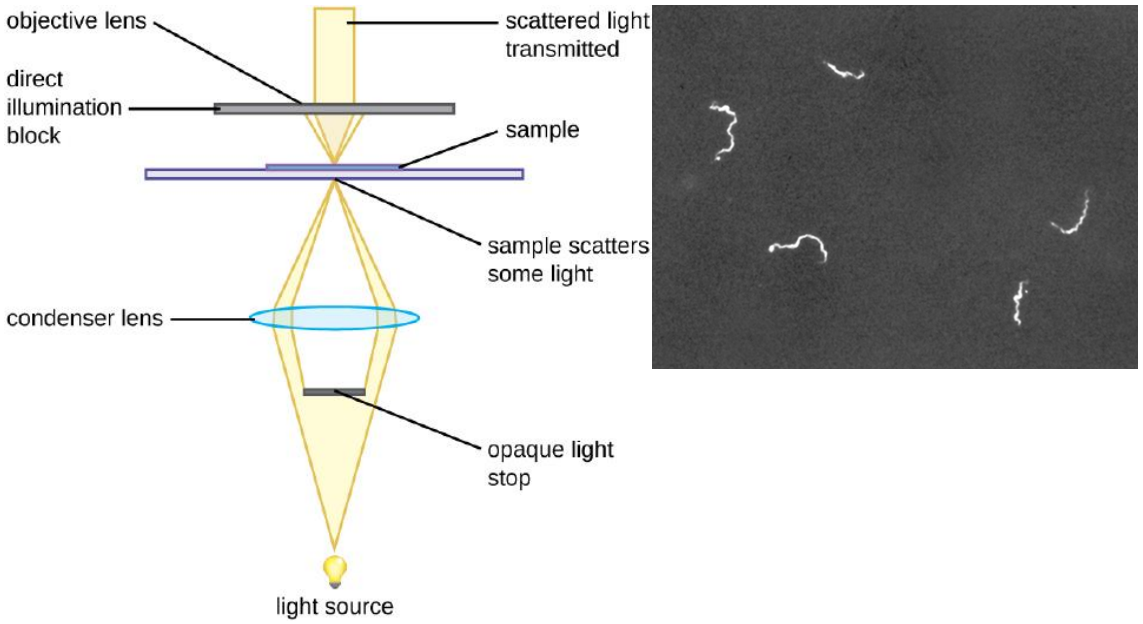
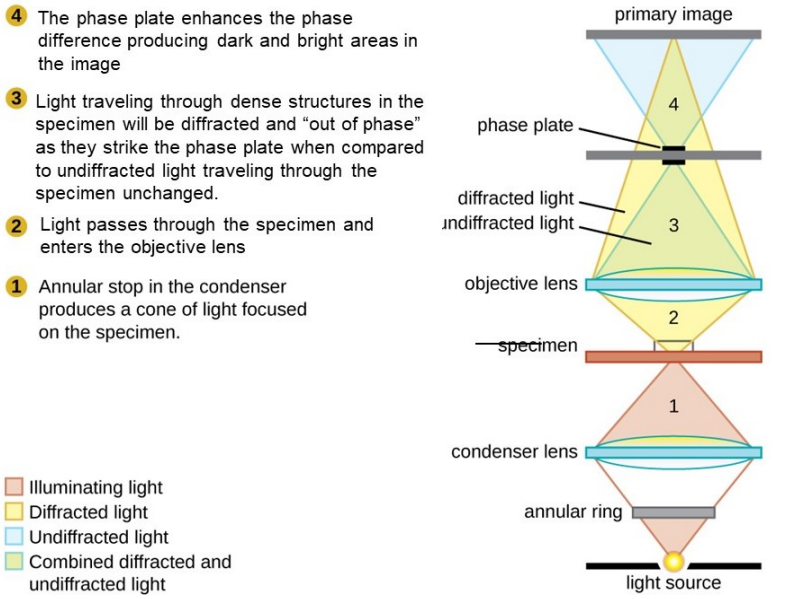


Figure **1.4.4**: Darkfield microscopy. Left image - Light, from a light source, passed through the condenser and then passes through a specimen. The light travels through the specimen and then passes through an illumination block (gray rectangle). The majority of the light rays from the specimen are blocked. Only those light rays that have been refracted and reflected by the specimen will reach the objective lens. The magnified specimen seen through the eyepiece will be bright against a dark background. Right image - A darkfield microscope image of live, unstained samples of the spirochete *Treponema pallidum*. Similar to a photographic negative, the spirochetes appear bright against a dark background. (images from OpenStax Microbiology, CC BY-SA 4.0)

Darkfield microscopy can often create high-contrast, high-resolution images of specimens without the use of fixatives and stains, which is particularly useful for viewing live specimens that might be killed or otherwise compromised by the stains.

The Phase-Contrast Microscope

The **phase-contrast microscope** is actually the oldest and simplest type of light microscope. The phase-contrast microscope creates a high-contrast, high-resolution image by altering the wavelengths of light rays passing through the specimen. To create these altered wavelength paths, an **annular stop** is used in the condenser. The annular stop produces a hollow cone of light that is is focused on the specimen. Some of this light will enter the objective lens having passed through the specimen unchanged (ie. undiffracted), whereas some of the light will be slowed and shifted by dense structures in the specimen (i.e. diffracted). The difference between undiffracted and diffracted light within the objective lens is known as a **phase shift**. The objective lens contains a **phase plate,** with a **phase ring**. As the diffracted light enters the phase plate, its phase shift is increased in comparison to the undiffracted light (Figure **1.4.5**). Areas of high phase shift are seen as dark regions in the specimen, with the undiffracted light showing as brighter areas.



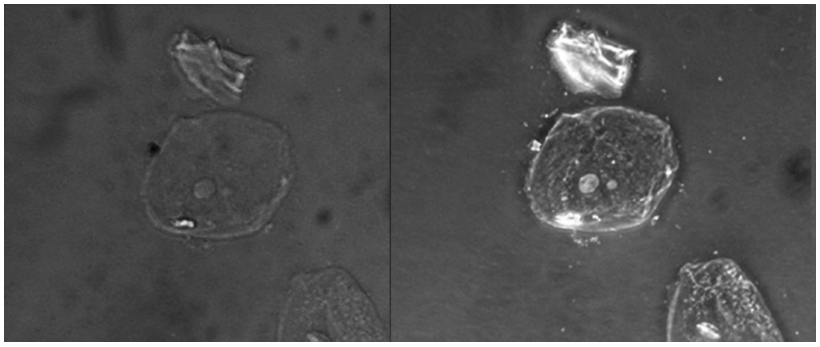


Figure **1.4.5**: Phase contrast microscopy. Top image - A schematic showing how a phase-contrast light microscope works. (1) An annular stop in the condenser produces a cone of light focused on the specimen. (2) The light passes through the specimen and enters the objective lens. (3) Light traveling through dense structures in the specimen will be diffracted and "out of phase" as they strike the phase plate when compared to undiffracted light traveling through the specimen unchanged. (4) The phase plate enhances this phase difference, producing dark and bright areas in the image. Bottom image - A brightfield image of unstained simple squamous epithelial cells (left) next to a phase-contrast image (right). Notice that the unstained cells in the brightfield image are almost invisible against the background, whereas the cells in the phase-contrast image appear to glow against the background, revealing far more detail. (images from OpenStax Microbiology, CC BY-SA 4.0)

Because it increases contrast without requiring stains, phase-contrast microscopy is often used to observe live specimens. Certain structures, such as organelles in eukaryotic cells and endospores in prokaryotic cells, are especially well visualized with phase-contrast microscopy (Figure **1.4.7**) .

The Fluorescence Microscope

A **fluorescent microscope** is a type of light microscope that uses fluorescent dyes or proteins to visualize specific structures. These fluorescence dyes or proteins are known as **fluorochromes**, which are capable of absorbing energy from a light source and then emitting this energy as visible light. Fluorochromes include naturally fluorescent substances (such as chlorophyll), as well as fluorescent stains that are added to the specimen to create contrast. Dyes such as Texas Red and Fluorescein Isothiocyanate (FITC) are examples of fluorochromes. Other examples include the nucleic acid dyes 4',6'-diamidino-2-phenylindole (DAPI) and acridine orange. Fluorescence microscopes can be used to identify pathogens, to find a particular species within an environment, or to find the locations of particular molecules and structures within a cell. Approaches have also been developed to distinguish living from dead cells using fluorescence microscopy based upon whether they take up particular fluorochromes. Sometimes, multiple fluorochromes are used on the same specimen to show different structures or features.

In fluorescence microscopy, the microscope transmits light of short wavelength excitation, such as ultraviolet or blue light, towards the specimen; the fluorochromes absorb this excitation light and emit visible light with longer wavelengths. This emission light is directed towards the ocular lenses using a dichrotic mirror located below the ocular. The emitted light is filtered (in part because ultraviolet light is harmful to the eyes) so that only visible light (in the form of fluorescence) reaches the ocular lens (Figure **1.4.6**). This produces an image of the specimen in bright colors against a dark background.

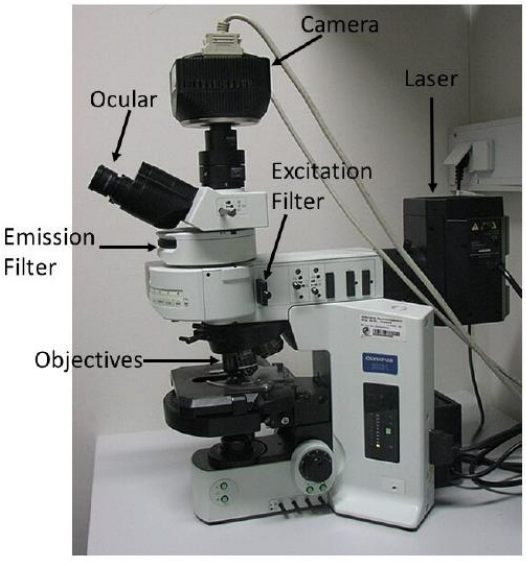


Figure **1.4.6**: The fluorescent microscope. A fluorescent microscope uses a laser and excitation filters to excite fluorochromes bound to a specimen. Fluorescence being emitted by the specimen is directed up towards the ocular using a mirror (not shown). Emission filters screen out unwanted fluorescent wavelengths prior to the light reaching the ocular. (The Fluorescent Microscope by Patricia Zuk, CC BY 4.0; modified from Fluorescence Microscopy by Masur, CC BY-SA 2.5)

One of the most important applications of fluorescence microscopy is a technique called **immunofluorescence**, which is used to identify certain structures or organisms using **fluorescently-labeled antibodies**. There are two approaches to this technique: direct immunofluorescence and indirect immunofluorescence (Figure **1.4.7**) . In direct immunofluorescence, antibodies known as **primary antibodies** are coupled to a fluorochrome. The binding of this labeled primary antibody to its target antigen (usually a protein) permits visualization of the structure expressing this antigen. In indirect immunofluorescence, antibodies known as **secondary antibodies** are coupled to the fluorochrome, rather than primary antibodies. The unlabeled primary antibody binds to its antigen, followed by binding of the labeled secondary antibody to the primary antibody. Since multiple secondary antibodies can often attach to a single primary antibody, IFA amplifies the fluorescence being emitted, making it easier visualize features in a specimen.

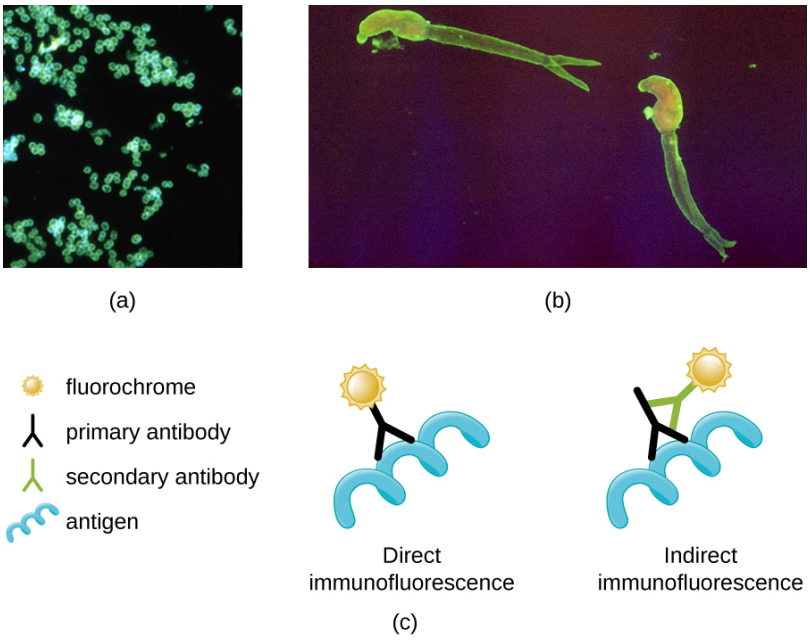
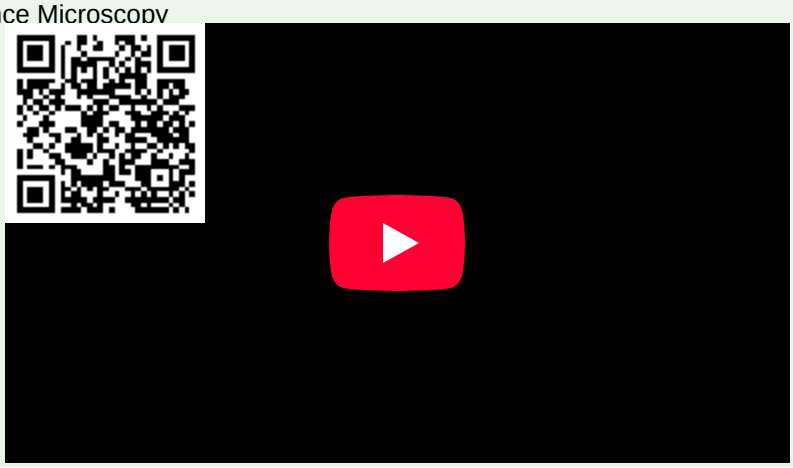


Figure **1.4.7**:(a) Direct immunofluorescence is used to visualize *Neisseria gonorrhoeae*, the bacterium that causes gonorrhea. b) Indirect immunofluorescence is used to visualize larvae of *Schistosoma mansoni*, a parasitic worm that causes schistosomiasis, an intestinal disease common in the tropics. (c) In direct immunofluorescence, a fluorochrome is coupled to the primary antibody, which binds to the antigen. In indirect immunofluorescence, the fluorochrome is coupled to the secondary antibody, which binds to a primary antibody, which, in turn, binds to the antigen. (images from OpenStax Microbiology, CC BY-SA 4.0)

Concept in Action

Animation: Fluorescence Microscopy



The Confocal Microscope

A **confocal microscope** is a specialized fluorescent microscope that uses a laser to excite the fluorochromes and a pinhole aperture to remove out of focus fluorescence and enhance image clarity. Whereas a conventional fluorescent microscope creates an image by focusing on the image at a single depth (or z-plane), a confocal microscope uses a laser to scan multiple z-planes within the object. This produces numerous two-dimensional, high-resolution images at various depths, which can be constructed into a three-dimensional image by a computer (Figure **1.4.8**). Because of this, confocal microscopy is also known as **confocal laser scanning microscopy.**

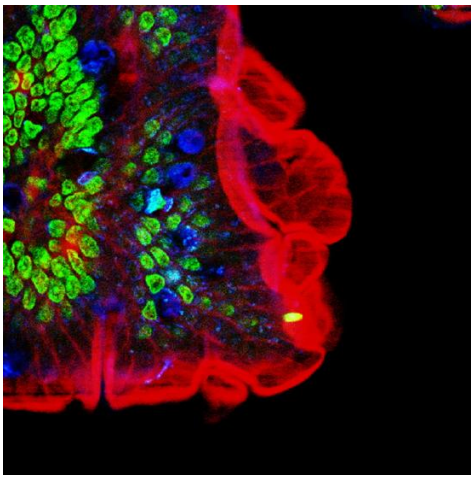
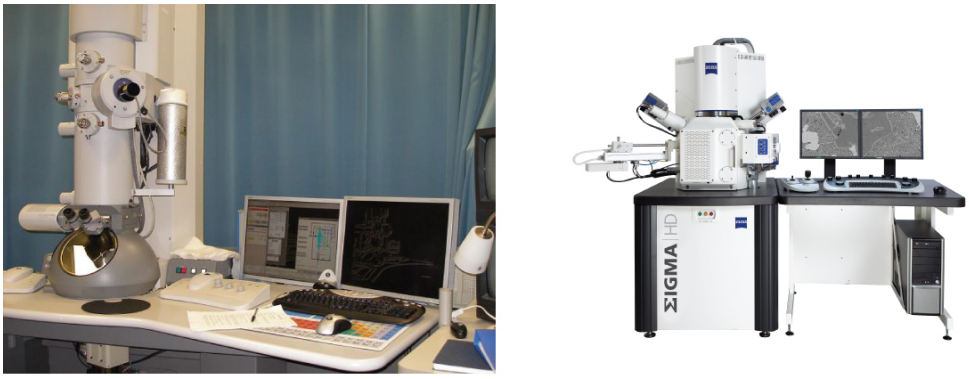


Figure 1.4.8: Confocal microscopy. A confocal image of a section of mouse intestine showing the staining of goblet cells (blue), the brush-border (red), and nuclei (green). (Multi-photon excitation by Alberto Diaspro et al., CC BY 2.0)

Electron Microscopy

The maximum theoretical resolution of images created by light microscopes is limited by the longer wavelengths of visible light. Most light microscopes can only magnify 1000X, and a few can magnify up to 1500X, but this does not begin to approach the magnifying power of an **electron microscope (EM).** The EM is an imaging tool that *uses short-wavelength electron beams rather than light to increase magnification and resolution*. An EM can produce a sharp image that is magnified up to 100,000X. Thus, EMs can resolve subcellular structures as well as some molecular structures (e.g., single strands of DNA); however, electron microscopy cannot be used on living material because of the methods needed to prepare the specimens. There are two basic types of EM: **the transmission electron microscope (TEM)** and **the scanning electron microscope (SEM)**(Figure 1.4.10). However, there is a third type of EM, the scanning transmission electron microscope (STEM), which combined TEM and SEM for advanced analysis of atomic structure, biological structures, and biomaterials.



(a) (b)

Figure 1.4.10: Electron microscopes. (a) A transmission electron microscope (TEM). (b) A scanning electron microscope (SEM). (Electron microscopes by LibreText, CC BY-SA 4.0)

The TEM is somewhat analogous to the brightfield light microscope in terms of the way it functions (Figure 1.4.11); a beam of energy passes through a specimen and is collected by an objective lens. However, rather than a lamp generating visible light focused by a glass lens (i.e., a condenser), the TEM uses an "electron gun" focused by magnetic lenses to project an electron beam through the specimen and onto a detector which captures the image. The image can then be visualized on a computer screen. In TEM, the electron gun, magnetics and specimen are all placed under vacuum. Prior to visualization with a TEM, the specimen must be processed to be extremely thin (20–100 nm thick) and stained with an electron dense material, such as a metal like lead, osmium, or tungsten. Those parts of the specimen stained with these materials will absorb electrons and produce dark regions on the image. Areas of the image where the electron beam passes through can be seen as lighter.

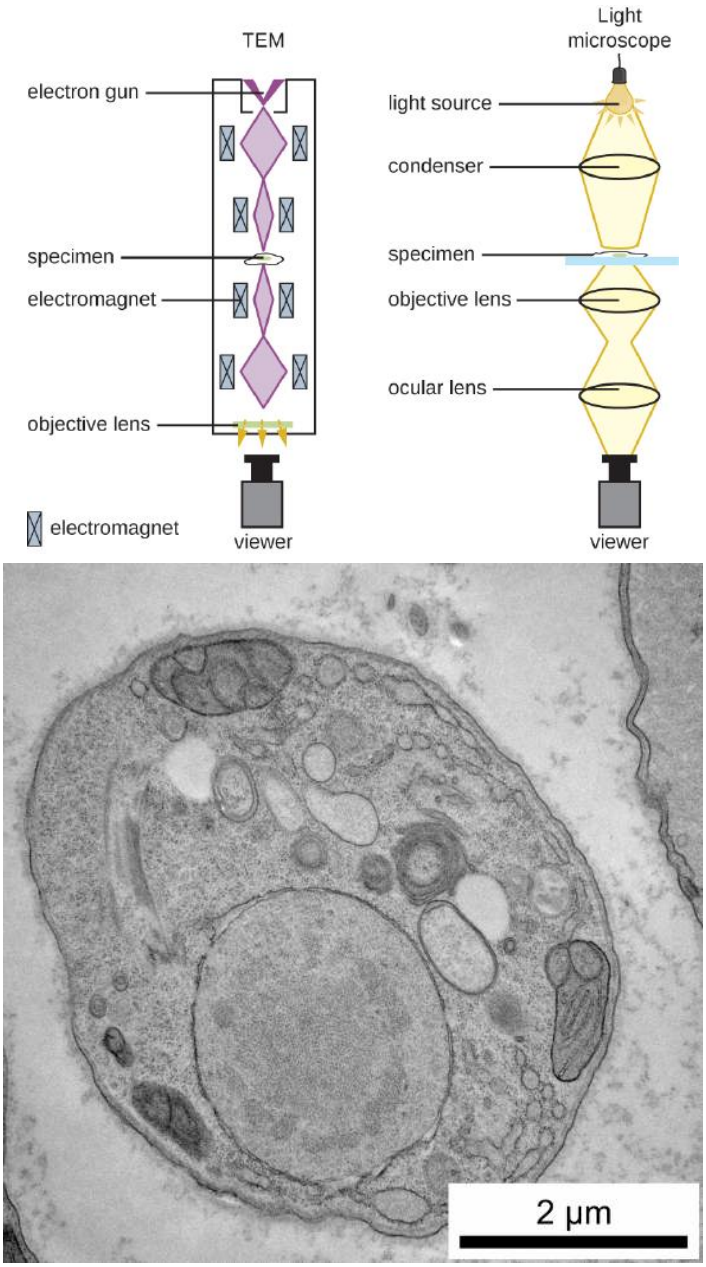


Figure **1.4.11**: TEM microscopy. Top image - A schematic comparing the designs of the electron and light microscopes. Electron microscopes use magnets to focus an electron beam onto a specimen similarly to the way that light microscopes use glass lenses to focus light. (images from OpenStax Microbiology, CC BY-SA 4.0)
Bottom image - a transmission electron microscope (TEM) image of a eukaryotic cell. (TEM Cell by Klingm01, CC BY-SA 4.0)

Whereas TEM requires very thin sections and allows one to see internal structures such as organelles, the SEM is used to view the surface of a specimen. To do this, an electron beam produces electrons that scan the surface of a sample, creating a highly detailed image with a three-dimensional appearance that is displayed on a monitor (Figure **1.4.12**). In SEM, specimens are dried and prepared with fixatives before being "sputter-coated" with a thin layer of metal such as gold. The atoms in this gold layer interact with the electron beam to produce backscattered electrons, secondary electrons, and X-rays. These signals are processed by the microscope to generate a gray-scale image of the specimen surface that can be left as is, or colorize if desired.

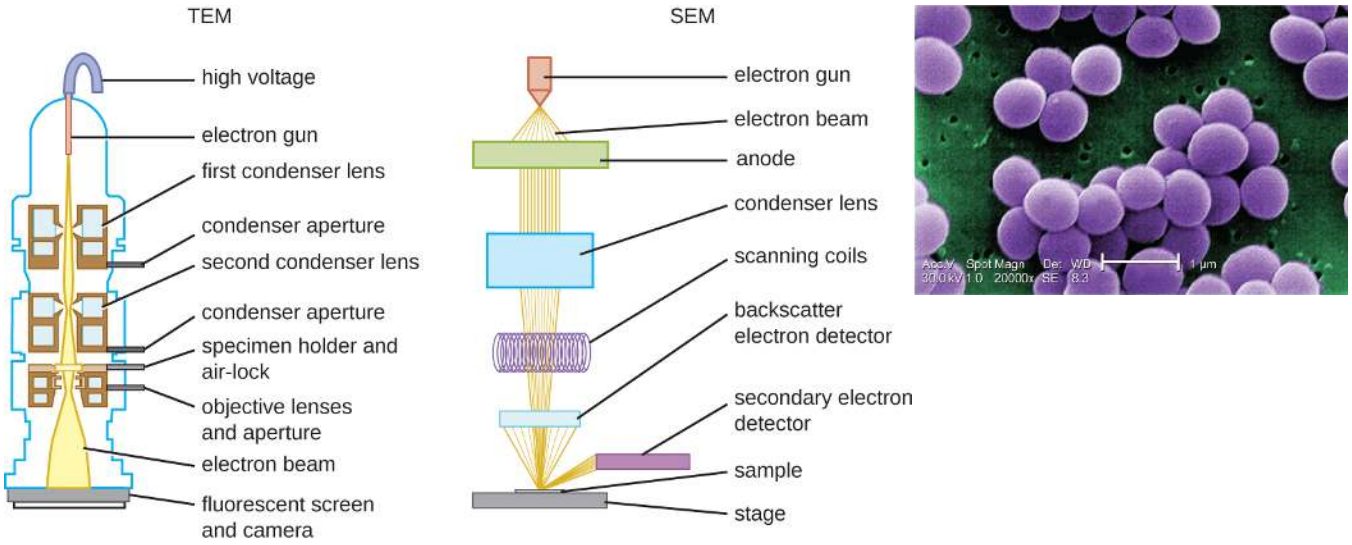


Figure 1.4.12: The TEM versus the SEM. Top image - A schematic comparing the parts of the transmission electron microscope (TEM) and scanning electron microscope (SEM) shows how a electron gun is used in both microscopes to create an electron beam that is directed towards a specimen using condenser lenses. In the TEM the electron beam passes through the specimen and strikes a fluorescent screen and camera. In the SEM, the electron beam is reflected off the specimen's surface and is directed towards electron detectors. In both electron microscopes, images are rendered using these screens and detectors. Right image - a colorized, 3D image of *Staphylococcus aureus* produced using an SEM. (TEM vs. SEM by Patricia Zuk, CC BY 4.0; modified from OpenStax Microbiology, CC BY-SA 4.0)

The PCR Machine

A **PCR machine**, also known as a **thermocycler**, is a piece of equipment used to amplify DNA through the process called **Polymerase Chain Reaction (PCR)**. The PCR machine cycles through three different temperatures in order to facilitate denaturation of the DNA helix into single strands, anneal primers to the single strands, and extend from the primers to create strands of daughter DNA. Tubes are placed into a metal tube holder designed to quickly and efficiently transfer heat to the tubes. A front display allows for easy creation of modification of the PCR program (Figure 1.4.13). For more about PCR, go to Chapter 13.2: Lab Technique - Polymerase Chain Reaction.

Figure 1.4.13: The PCR machine or thermocycler. (PCR machine by Patricia Zuk, CC BY 4.0)

The Flow Cytometer

A **flow cytometer** is used to analyze and sort cells in a fluid using specific physical or chemical characteristics. Flow cytometry is essential for cell sorting, cell counting, and biomarker detection. Cells or particles are suspended in a liquid. Fluorescent dyes or antibodies can be added to the sample to label specific cell markers. The flow cytometer focuses the cells in a "single-file stream" that passes in front of a laser. Detectors in the cytometer then gather specific properties of light that is then used to analyze and sort

the cells into distinct populations (Figure **1.4.14**). **Forward Scatter (FSC)** can be used to measure cell size and **Side Scatter (SSC)** can be used to measure cell granularity. If fluorescent dyes or antibodies are used, fluorescent emissions can be also used to identify specific cell markers. Using these parameters, the flow cytometer can produce graphs plotting SSC vs FSC, SSC vs fluorescence, or FSC vs. fluorescence. Fluorescence can also be used to physically separate the cells into samples for additional analysis through a technique called **Fluorescence-Activated Cell Sorting or FACS**.

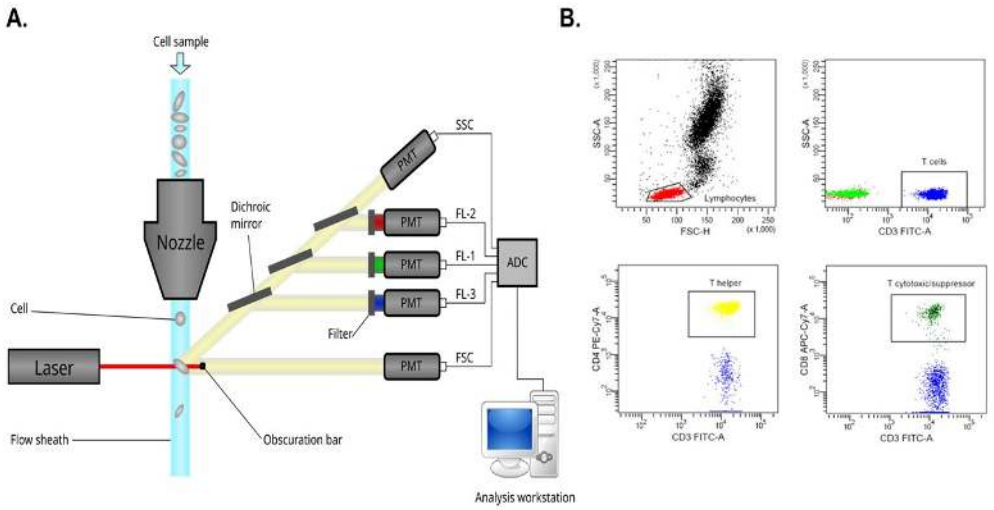


Figure **1.4.14**: Flow cytometry. (A) The flow cytometer is comprised of a nozzle which creates a fluidic stream of single cells, a laser to excite the cells and several dichrotic mirrors that divert the information to specific detectors (PMT; photomultiplier tubes) that collect information on SSC (side scatter), FSC (forward scatter) and fluorescence (red/FL-2, green/FL-1, and blue/FL-3). (Flow cytometry by Kierano, CC BY 3.0)
(B) The information obtained by these detectors is processed to produce plots. Specific populations can be isolated through "gating" (black boxes) and the cells inside the gate further analyzed for cell number, size, and fluorescent intensity. (Flow cytometry plots by Patricia Zuk, CC BY 4.0; modified from Flow Cytometry Chart by Charlotte Hayden, CC BY-SA 4.0)

Key Concepts

The equipment used in biotechnology is diverse and specialized, reflecting the complexity and precision required in this field. From basic laboratory tools to advanced and specialized machinery, each piece of equipment plays a crucial role in enabling researchers to conduct experiments, analyze data, and produce biotechnological products. Understanding the functions and applications of these tools is essential for anyone working in or studying biotechnology, as they form the foundation for scientific discoveries and technological advancements.

Glossary

Antibody - a Y-shaped protein produced by B cells of the immune system that recognizes and binds to a specific antigen; also called an immunoglobulin

Antigen - a molecule that triggers an immune response

Condenser - a lens system located beneath the microscope stage that focuses and directs light from the microscope's light source onto the specimen in order to enhance resolution and contrast

Contrast - the difference in light intensity between the specimen and the background; makes structures more visible under the microscope

Electron gun - an instrument used by an electron microscope to create a focused beam of electrons

Flow cytometry - a lab technique used to analyze and measure the physical and chemical characteristics of a cell population; uses a piece of equipment called a flow cytometer

Fluorescence - the property of a substance to absorb light at one wavelength and emit it at a longer wavelength

Forward scatter - a flow cytometry parameter that refers to the light scattered in the same direction as the laser beam; generally proportional to the size of the cell or particle and is used to help distinguish large cells from small cells

Magnification - how much larger an object is compared to its actual size

Microscope - an instrument used to magnify an object; classified into two major types - light and electron

Microscopy - the technique of using microscopes to observe objects and structures that are too small to be seen with the naked eye

Resolution - the ability of a microscope to distinguish two close points as separate; higher resolution means finer detail can be observed

Side scatter - a flow cytometry parameter that refers to the light that is scattered at a 90° angle to the laser beam; it reflects the granularity or internal complexity of the cell, such as the presence of granules or organelles

Thermocycler - an instrument used to amplify a fragment of DNA; also called a PCR machine

Attributions

Figures adapted from OpenStax Microbiology, CC BY 4.0

- Figure 1.4.1
- Figure 1.4.2
- Figure 1.4.3
- Figure 1.4.4(in part)
- Figure 1.4.5
- Figure 1.4.7
- Figure 1.4.10
- Figure 1.4.11(in part)
- Figure 1.4.12

1.4: Advanced Biotechnology Equipment is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

- **2.3: Instruments of Microscopy** by OpenStax is licensed CC BY 4.0. Original source: https://openstax.org/details/books/microbiology.

CHAPTER OVERVIEW

2: The Molecules of Life

2.1: Water
2.2: Carbon
2.3: Macromolecules
2.4: Carbohydrates
2.5: Lipids
2.6: Proteins
2.7: Nucleic Acids

2: The Molecules of Life is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.1: Water

Biotech Focus

Strategies for Cleaner Water

Industrial activities produces a large amount of wastewater containing pollutants such as pesticides or industrial dyes. Using biotechnology, wastewater treatment can be more efficient, sustainable and cost effective. Several strategies have been developed, using microorganisms, enzymes, or plants specifically engineered to break down pollutants and contaminants, ultimately making the water safer for consumption.

For more information about the different approaches to treat water, read Biological wastewater treatment technology: Advancement and drawbacks

Introduction

Do you ever wonder why scientists spend time looking for water on other planets? It is because water is essential to life; even minute traces of it on another planet can indicate that life could or did exist on that planet. Water is one of the more abundant molecules in living cells. Approximately 60–70 percent of the human body is made up of water. It is critical our viability by serving many essential functions (Figure **2.1.1**). In humans, water forms saliva and keeps mucosal membranes moist. It allows the body's cells to grow, reproduce and survive. Water flushes out wastes through urination. It provides lubrication to joints. It is needed by the brain to manufacture hormones and neurotransmitters and acts as a shock absorber for the brain, spinal cord, and fetus. Without water, life simply would not exist.

Figure **2.1.1**: The functions of water. Water is the major component of most body parts and serves a number of essential functions like keeping membrane moist, flushing wastes from the body, lubricating joints, and regulates body temperature. (The water in you: What water does for your body by USGS.gov, public domain)

Water has several important properties, including:

1. **it is polar**
2. **it has the ability to form hydrogen bonds**
3. **it is a solvent**
4. **it has high cohesion**
5. **it has high adhesion**
6. **it moderates temperature**

Learning Objectives

Water is one of the most abundant molecules on Earth. By the end of this section, you will be able to:

- Describe the properties of water that are critical to maintaining life
- Explain why water is an excellent solvent
- Provide examples of water's cohesive and adhesive properties
- Discuss the role of acids, bases, and buffers in homeostasis

The Polarity of Water & Hydrogen Bonding

The bonds between the oxygen (O) and hydrogen (H) atoms in the water molecule are known as **covalent bonds.** *Electrons are shared between two atoms in a covalent bond*. Oxygen has 6 electrons in its outermost shell and hydrogen has only 1 electron. As a result, oxygen shares each of its outer electrons with a hydrogen atom. While there is no overall charge to a water molecule, the shared electrons are found more closely associated with the center of the oxygen atom than they are with the center of a hydrogen atom. This results in "unequal sharing" of electrons within the covalent bond and produces a slight positive charge on each hydrogen atom and a slight negative charge on the oxygen atom. Because of these charges, the slightly positive hydrogen atoms repel each other and form the unique shape (Figure **2.1.2**).

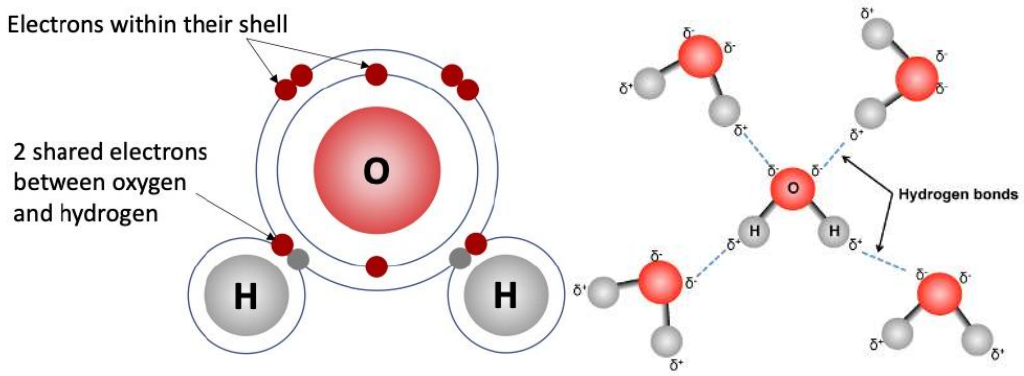


Figure **2.1.2**: Left image: The electron configuration in a molecule of water produces polar covalent bonds. The two hydrogen electrons are "pulled" closer to the center of the oxygen atom, making the hydrogen atoms slightly positive in charge and the oxygen atom slightly negative. This "unequal sharing" of electrons is known as a polar covalent bond. (Electron sharing by Kareen Martin, CC BY-NC 4.0)
Right image: Hydrogen bonds form and reform between the partial positive end of one water molecule (i.e., the hydrogen atom) and the partial negative charge of an adjacent water molecule (i.e. the oxygen atom.) The partial charge in a polar bond is denoted with the Greek letter "delta". A molecule of water can interact with 4 hydrogen bonds with 4 other molecules of water. (Hydrogen bonds by Kareen Martin, CC BY-NC 4.0)

As a result of the positive and negative charges in the different parts of the water molecule, each water molecule attracts other water molecules (Figure **2.1.2**). These attractions create **hydrogen bonds** between water molecules. Hydrogen bonds are weak interactions that form between *a hydrogen with a partial positive charge of one molecule and another atom with a partial negative charge*. The partial charge in a polar bond is denoted with the Greek letter "delta". Hydrogen bonds can break and reform easily giving water's fluidity.

Concept in Action

Animation: How polarity makes water behave strangely

Water is an Excellent Solvent

The slight positive and negative charges of water molecules enables them to easily dissolve ionic compounds and polar molecules through their formation of hydrogen bonds with surrounding water molecules. A compound that dissolves in water is referred to as **hydrophilic** ("water-loving"). Because nonpolar molecules do not have partial positive or partial negative charges, they are not attracted to water molecules, explaining why oil separates when added to water. Or vinegar, a polar substance, does not mix with olive oil (a non-polar substance) as seen in Figure **2.1.3**. These nonpolar compounds are called **hydrophobic** ("water-fearing").

Figure **2.1.3**: Balsamic vinegar (black), a polar compound and olive oil (yellow), a non polar one, do not mix. (Plate with vinegar by Tim Ereneta, CC BY-NC 2.0)

Because water is capable of dissolving another substance, it is considered to be a **solvent**. The compounds dissolved in a solvent are called **solutes**. When polar and ionic compounds are dissolved in water, hydrogen bonds will form between the charged compounds and adjacent water molecules, resulting in the compound becoming surrounded by a layer of water molecules. This layer is called a **hydration shell** and it serves to keep solutes separated or dispersed in the water. For example, when table salt (NaCl) is dissolved in water (Figure **2.1.4**), the sodium and chloride ions separate, or dissociate, in the water, and hydration shells form around each ion. Each positively-charged sodium ion is surrounded by negatively-charged oxygen atoms in water molecules. Each negatively-charged chloride ion is surrounded by positively-charged hydrogen atoms.

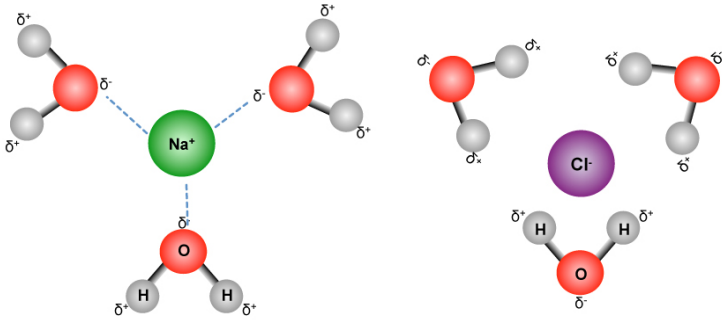


Figure **2.1.4**: When table salt (NaCl) is mixed in water, each ion becomes surrounded by a hydration shell of water molecules. (Hydration shell by Kareen Martin, CC BY-NC 4.0)

The polarity of the water molecule makes it an effective solvent and is important in its many roles in living systems. However, one common mistake or misconception about water is that it dissolves everything because it is the “universal solvent." Water has the ability to dissolve many substances but the term “universal solvent" is misleading. *Water is able to dissolve other polar molecules and ions but cannot dissolve non-polar compounds.*

Water Moderates Temperature

Temperature is defined as a measure of the motion, or the kinetic energy of molecules. There is a direct relationship between kinetic energy and temperature. As kinetic energy increases, temperature increases. However, water is able to moderate changes in temperature within organisms and in the environment. This is because water has a high **specific heat capacity. Specific heat** is defined as the *amount of energy it takes to raise 1 gram of water by 1 degree Celsius*. Water's high specific heat capacity means that it takes a lot of energy to raise the temperature of water by a single degree, and it is the result of hydrogen bonding. Increasing kinetic energy disrupts the hydrogen bonds found between water molecules. Because these bonds can break and re-form rapidly, water can absorb large increases in energy before its temperature will change. In fact, water has the highest specific heat capacity of any liquid.

As energy input continues, the balance between hydrogen-bond formation and destruction swings toward the destruction side. More bonds are broken than are formed. This process results in the release of individual water molecules in gas form in a process called **evaporation**. As with specific heat capacity, it takes a significant amount of energy to convert liquid water to gaseous water (i.e. steam). This is because water has a **high heat of vaporization**. Evaporation of sweat, which is 90 percent water, allows for cooling of an organism, because breaking hydrogen bonds requires an input of energy and takes heat away from the body.

As kinetic energy decreases and temperatures drop, less energy is present to break the hydrogen bonds between water molecules. At a specific temperature, these hydrogen bonds will remain intact and begin to form a rigid, lattice-like structure as the water solidifies into ice (Figure **2.1.5**). When water freezes, the distance between the water molecules increases, making ice less dense then liquid water. This means that ice floats (Figure **2.1.5**). In lakes, ponds, and oceans, ice will form on the surface of the water, creating an insulating barrier to protect the animal and plant life beneath from freezing in the water. If this did not happen, plants and animals living in water would freeze in a block of ice and could not move freely, making life in cold temperatures difficult or impossible.

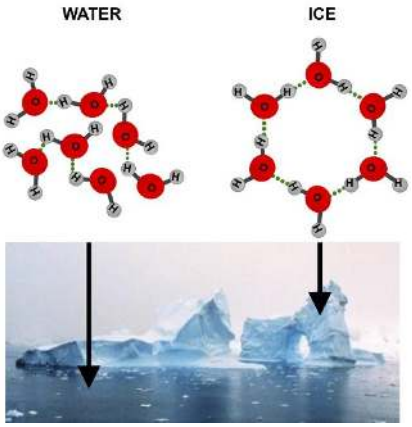


Figure **2.1.5**: Hydrogen bonding makes ice less dense than liquid water. The lattice structure of ice, seen on the right side, makes it less dense than the freely flowing molecules of liquid water, (left side) enabling it to float on water. (Icebergs by Ben-Zin, public domain, Water and Ice Structure by Kareen Martin, CC BY-NC 4.0)

Water Is "Sticky" (Cohesion and Adhesion)

Water has both high cohesion and high adhesion. In **cohesion**, water molecules at a liquid-air interface are attracted to each other (because of hydrogen bonding), keeping the molecules close together and well organized (Figure **2.1.6**). The hydrogen bonds found between water molecules at the surface are stronger than those below them, giving rise to **surface tension**, the capacity of a substance to withstand rupture when placed under tension or stress. Because of cohesion and surface tension, it is possible to “float” a steel needle on top of a glass of water if the needle is placed gently and does not break the surface tension. This is possible

even though the needle is denser than the water. Small insects such as the water strider can walk on water because their weight is not enough to break the surface tension. (Figure **2.1.6**).

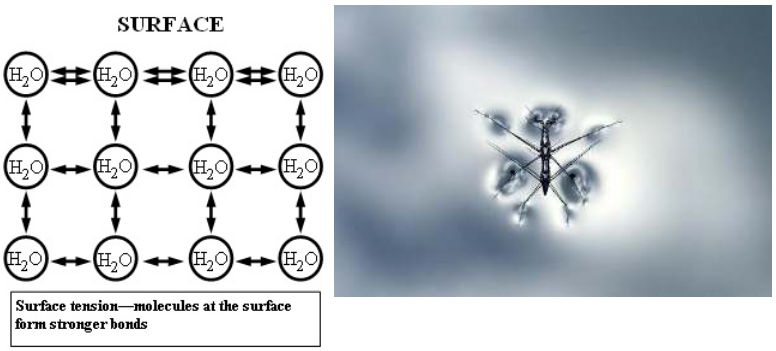


Figure **2.1.6**: Left image - surface tension in water is due to cohesion (i.e. attraction) between water molecules at the surface of the water as adjacent water molecules form strong hydrogen bonds between one another. (Surface tension by USGS.gov, public domain)
Right image - a "water strider" is able to walk on top of water due to a combination of high surface tension of water and long, hydrophobic legs to help them stay above water. (Water strider by Unsplash, CCO)

Water also exhibits **adhesion**, which is the attraction of *two different molecules to one another*. This can be observed when water "climbs" up a straw placed in a glass of water. Because of adhesion, the water molecules are attracted to the plastic of the straw and form bonds with it. It is important to distinguish that adhesion and cohesion are related to one another *but are not the same thing*. Adhesion takes place because of the interaction between water molecules and another molecule; cohesion takes place because of the interaction between water molecules. Cohesive and adhesive forces are important for sustaining life. For example, because of these forces, water can flow up from the roots to the tops of plants to feed the plant (Figure **2.1.7**).

Figure **2.1.7**: Left: Cohesion between water molecules allows a drop to form and cohesion results in the drop clinging to the tip of a leaf. Right: With the help of adhesion and cohesion, water can work its way all the way up to the branches and leaves at the top of trees. (Cohesion and Adhesion by Kareen Martin, CC BY-NC 4.0)

Buffers, pH, Acids, and Bases

The **pH** of a solution is a measure of its acidity or basicity and is determined by the concentration of hydrogen ions in that solution. The overall concentration of hydrogen ions is inversely related to its pH. The more H+ present, the lower the pH the more acidic the solution is; conversely, the fewer hydrogen ions, the higher the pH and the more basic (i.e. alkaline) the solution is. Acidity and basicity is measured using the **pH scale** (Figure **2.1.8**). The pH scale ranges from 0 to 14. A change of one unit on the pH scale represents a change in the concentration of hydrogen ions by a factor of 10; a change in two units represents a change in the hydrogen ion concentration by a factor of 100. Thus, small changes in pH represent large changes in the concentrations of hydrogen ions. Pure water is neutral. It is neither acidic nor basic, and has a pH of 7.0. Anything below 7.0 (from 0.0 to 6.9) is acidic, and anything above 7.0 (from 7.1 to 14.0) is alkaline. Most cells operate within a very narrow window of the pH scale, typically ranging only from 7.2 to 7.6.

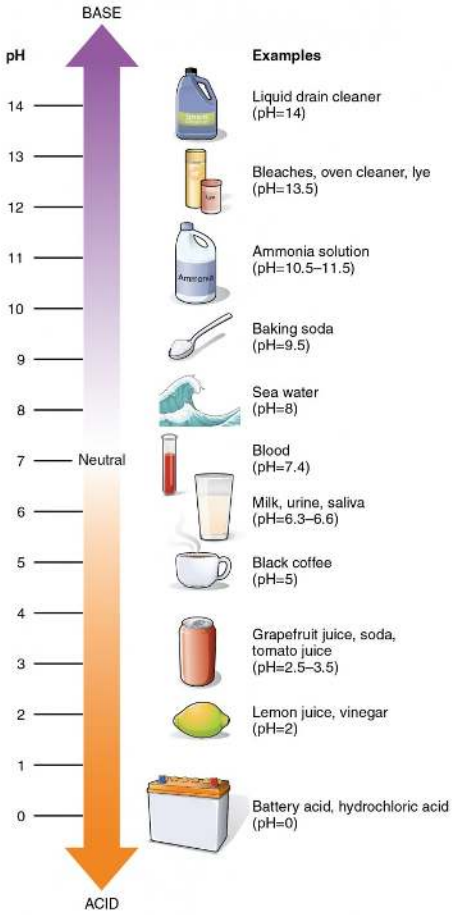


Figure **2.1.8**: The pH scale ranges from 0 to 14, and most solutions fall within this range. Anything below 7.0 is acidic, and anything above 7.0 is alkaline, or basic. The pH values of common substances like milk, saliva, coffee and blood are shown. (pH Scale by OpenStax College, CC BY 3.0)

An **acid** is a substance that donates hydrogen ions (H+) and lowers pH. (Figure **2.1.9**). The stronger the acid, the more readily it donates H+. For example, hydrochloric acid and lemon juice are very acidic and readily give up H+ when added to water. Conversely, a **base** is a substance that accepts H+ ions and raises pH. A base can also be defined as a substance that donates hydroxide ions (OH–). The OH– ions from the base combine with H+ from the acid to produce water, which raises a substance's pH. Sodium hydroxide (NaOH) and many household cleaners are very alkaline and give up OH– rapidly when placed in water, thereby raising the pH.

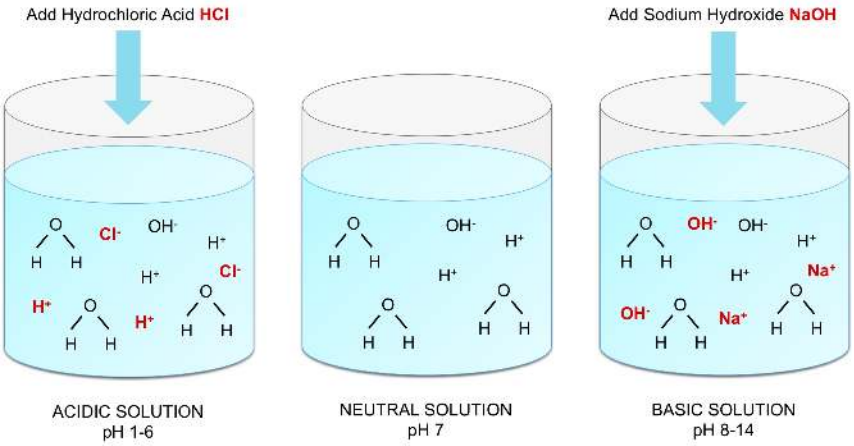


Figure **2.1.9**: Left image - in an acidic solution (e.g. hydrochloric acid/HCl), ionic dissociation gives rise to an excess of H^+. The pH of an acidic solution ranges between 1 and 6. Center image - in a neutral solution, water molecules dissociate into an equal number of hydrogen ions (H^+) and hydroxide ions (OH^-) and recombine to reform water. The resulting pH is 7.0. Right image - in a basic solution (e.g. sodium hydroxide/NaOH), ionic dissociation gives rise to an excess of OH^- that can combine with H^+ to produce water molecules. As a result, the number of H^+ decreases and the pH of the solution becomes basic. The pH of a basic solution ranges comprised between 8 and 14. (pH Solutions by Kareen Martin, CC BY-NC 4.0)

Organisms can maintain the pH of their cells and fluids within a relatively narrow range using buffers. A **buffer** is a solution that resists pH changes when small amounts of acid or base are added. In other words, buffers readily absorb excess H+ or OH–, maintaining the pH of the organism at a desired level. **Carbon dioxide (CO_2)** is part of a prominent buffer system used by many organisms called the **carbonic acid-bicarbonate buffer system**. This buffer system involves **carbonic acid (H_2CO_3)** and **bicarbonate (HCO_3^-) anion** (Figure **2.1.10**). If too a solution has too much H+, bicarbonate will combine with the H+ to create carbonic acid and limit the decrease in pH of the solution. Likewise, if too much OH– is introduced into a solution, carbonic acid will rapidly dissociate into bicarbonate and H+ ions. The H+ ions can combine with the OH– ions, limiting the increase in pH.

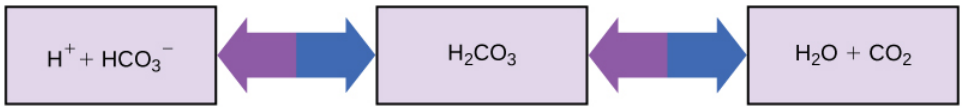


Figure **2.1.10**: Carbonic acid acts as a buffer. Water and carbon dioxide (CO_2) combine to form carbonic acid (H_2CO_3). Carbonic acid dissociates to form H+ and bicarbonate ions (HCO_3^-). When an acid is added to a fluid, HCO_3^- react with the excess H^+ to form carbonic acid and prevent a drop in pH. When a base is added, carbonic acid donates H+ to neutralize the excess OH- and form more carbonic acid (modified from Buffer by CNX OpenStax; CC BY 4.0).

Many organisms rely upon this buffering system to maintain the pH of their blood plasma (and tissue fluids) between 7.2 and 7.4. If the blood becomes too acidic, the organism will increase its respiration rate to remove more carbon dioxide and decrease the concentration of carbonic acid. This will decrease the amount of H+ in the blood produced by carbonic acid dissociation. If the blood becomes too basic, the organism can slow its breathing rate, increasing carbonic acid levels and lowering pH through the production of more H+.

Key Concepts

Water has many properties that are critical to maintaining life. These properties of water are intimately connected to the biochemical and physical processes performed by living organisms, and life would be very different if these properties were altered, if it could exist at all.

Some important concepts to remember:

- Water is a polar molecule, allowing for the formation of hydrogen bonds with other molecules, including other water molecules
- Water is an excellent solvent. Its hydrogen bonds allow ions and other polar molecules to dissolve in water
- Water stabilizes temperature. The hydrogen bonds between water molecules cause the water to have a high heat capacity, meaning it takes considerable added heat to raise its temperature.
- The evaporation of water takes considerable amounts of energy; this allows organisms to cool their internal temperatures

- Water is less dense when in solid form; this means that ice floats
- The attraction of water molecules to one another at the surface of a volume of water is called cohesion; cohesion produces surface tension
- The attraction between a water molecule and another molecule is called adhesion
- The pH of a solution is a measure of hydrogen ion concentration
- Acids and bases can change pH values, but buffers tend to moderate the changes they cause

Glossary

Acid - a substance that increases the concentration of hydrogen ions (H^+) in a solution (i.e. a H+ donor)

Adhesion - the attraction between molecules of different substances; caused by intermolecular forces such as hydrogen bonding, van der Waals forces, or dipole interactions

Base - a substance that increases the concentration of hydroxide ions (OH^-) or accepts protons (H^+) in a solution (i.e. an OH- donor)

Buffer - a solution that resists changes in pH when small amounts of acid or base are added; helps to maintain a stable pH in biological and chemical systems

Cohesion - the attraction between molecules of the same substance due to intermolecular forces like hydrogen bonding, van der Waals forces, or dipole interactions

Evaporation - the process by which a liquid turns into a gas at a temperature below its boiling point; it occurs at the surface of the liquid, where molecules with sufficient kinetic energy escape into the air as vapor

Hydrophilic - a substance that is attracted to and dissolves in water

Hydrophobic - a substance that repels water and does not dissolve in it

Kinetic energy - the energy of motion

pH scale - a measurement system used to express how acidic or basic a solution is; ranges from 0 to 14

Solute - a substance that dissolves in a solvent

Solvent - a solution that dissolves a solute

Surface tension - a property of liquids that causes the surface to act like a membrane; a result of strong cohesive attraction between water molecules at the surface of the liquid

Temperature - a measure of the average kinetic energy of the particles in a substance

Attributions

text adapted from Open Stax Biology 2e, licensed CC BY-4.0

2.1: Water is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.2: Carbon

Biotech Focus

Greenhouse gas emissions, like carbon dioxide (CO_2), represent one of the major reasons behind climate change. The technology of **carbon capture** aims to reduce CO_2 emissions from industrial processes before it can even enter the environment. Today, biotechnology is playing an increasingly important part in this technology through its genetic engineering of organisms that can pull large amounts of CO_2 from the environment for photosynthesis; organisms like "supertrees" that grow bigger and faster or genetically-engineered bacteria and algae. However, nature may already have created such an organism without the need for the lab.

Meet **"Chonkus",** a new cyanobacteria (i.e. photosynthetic bacteria)

Chonkus was so big, round, and heavy, it reminded the researchers of animals that were overweight or "chonk". Discovered off the coast of Italy, Chonkus was found to grow faster and faster the more CO_2 it encountered. Plus, they discovered a unique quality. Once it sequesters enough CO_2, Chonkus sinks, enabling it to be easily collected and removed. These two qualities might make this "fat" cyanobacteria a viable method for carbon capture.

To read more about Chonkus, check out the researchers' article in Applied and Environmental Microbiology.

Introduction

It is often said that life is “carbon-based.” This means that carbon atoms, bonded to other carbon atoms or other elements, form the fundamental components of many, if not most, of the molecules found uniquely in living things. Other elements play important roles in biological molecules, but carbon certainly qualifies as the “foundation” element for molecules in living things. It is the bonding properties of carbon atoms that are responsible for its important role. Before moving on to the different types of organic molecules or molecules of life, it is important to understand why carbon atoms are an essential part of organic molecules.

Learning Objectives

Carbon is the "foundational" atom of all molecules on Earth. By the end of this section, you will be able to:

- Explain why carbon is important for life
- Describe the role of functional groups in biological molecules

Carbon

Carbon has an atomic mass of 12 and an atomic number of 6. This means it has 6 protons, 6 neutrons and 6 electrons. The first 2 electrons are distributed as follows: 2 are found in the first electron shell (1s), with the 4 remaining electrons will be found in the second and outermost **valence shell** (2 electrons in 2s, 2 electrons in 2p). Therefore, to complete its outer shell, the carbon atom needs to share *4 electrons in 4 covalent bonds* to satisfy the octet rule (Figure **2.2.1**).

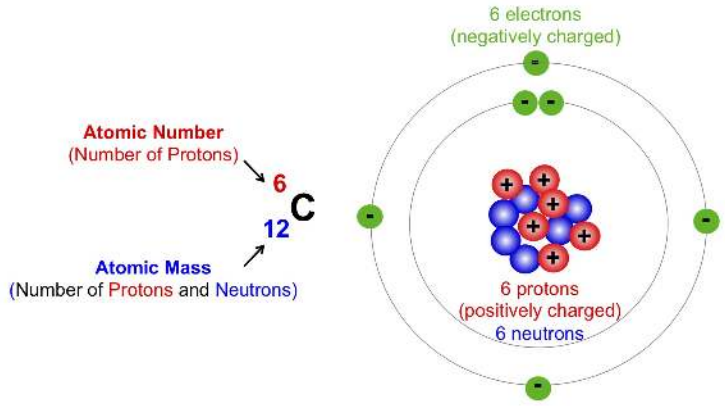


Figure **2.2.1**: The carbon atom. Left image - the carbon atom is represented using the symbol "C". Its atomic number is 6 meaning it has 6 protons. Its atomic mass is 12, meaning it has 6 protons and 6 neutrons. Right image - the 6 positively charged protons and the 6 neutral neutrons are found in the carbon atom's nucleus. The 6 negatively charged electrons are distributed as follows: 2 in the first electron shell and 4 in the second electron shell. The 4 single electrons in the outermost electron shell are available for covalent bonding with other atoms. (The Carbon Atom by Kareen Martin; CC BY-NC 4.0)

Carbon Bonding

The four electrons found in the outermost shell of the carbon atom are available for covalent bonding with additional carbon atoms or atoms other than carbon (e.g. hydrogen, nitrogen, oxygen, phosphorus, sulfur). In most biological organisms, carbon forms bonds with itself, linking together to form long chains, branched structures, and rings. These molecules form the basis of the organic compounds known as proteins, lipids, carbohydrates, and nucleic acids.

The Hydrocarbon

Hydrocarbons are organic molecules consisting of carbon and hydrogen. The carbon atoms link together to form a "backbone" onto which the hydrogen atoms bind. (Figure **2.2.2**). Occasionally, a carbon atom within the hydrocarbon might be substituted for by another element such as nitrogen or oxygen. Hydrocarbons with other elements in their carbon backbone are called **substituted hydrocarbons**. Hydrocarbons are often used as fuels—like the propane in a gas grill or glucose in a cell. The many covalent bonds found in hydrocarbons store a great amount of energy, which is released when these molecules are broken (i.e. oxidized). The most basic hydrocarbon is **methane (CH_4)** (Figure **2.2.2**). Methane has a central carbon atom bonded to four different hydrogen atoms. The geometry of the methane molecule, where the atoms reside in three dimensions, is determined by the shape of its electron orbitals. The carbons and the four hydrogen atoms form a shape known as a **tetrahedron**, with four triangular faces; for this reason, methane is described as having tetrahedral geometry.

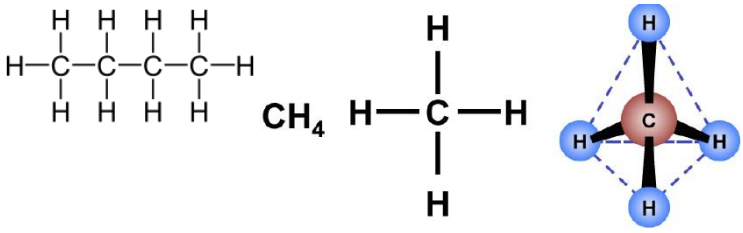


Figure **2.2.2**: The Hydrocarbon. Left image - the hydrocarbon molecule known as butant is made of 4 carbons linked to one another in a chain. Each carbon is also bound to hydrogen atoms so that each carbon has four covalent bonds. (Butane Lewis Structure, public domain)
Right image - the different representations of methane (CH_4). The molecular formula of methane is shown on the left as CH_4. The structure of methane is shown in the middle with the single carbon atom joined to four hydrogen atoms by a single covalent bond. The 3 dimensional representation of methane is shown on the right, with the carbon at the center and the 4 hydrogen positioned to form a tetrahedron (Methane by Kareen Martin; CC BY-NC 4.0)

As the backbone of the large molecules of living things, hydrocarbons may exist as linear carbon chains, carbon rings, or combinations of both. Furthermore, individual carbon-to-carbon bonds may be single, double, or triple covalent bonds, and each type of bond affects the geometry of the molecule in a specific way. This three-dimensional shape or conformation of the large molecules of life (macromolecules) is critical to how they function.

Some examples of different "carbon skeletons" for simple hydrocarbons are shown below. Some of the molecules shown have the same chemical formula, yet their atoms are arranged differently, or the double bonds are located in different positions. Due to these differences, these molecules have different properties. These are simple hydrocarbons, but organic molecules are usually very large, which leads to an immense variety of possible structures and properties.

- The hydrocarbon skeleton can vary in **length**, with some molecules having just a few carbons to some having hundreds (Figure **2.2.3**).

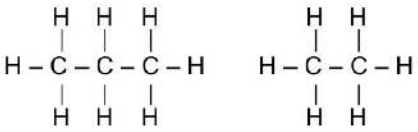


Figure **2.2.3**: Propane and ethane are both linear hydrocarbons. Left image - propane is made of 3 carbons with 8 hydrogens. Right - ethane is made of 2 carbons and 6 hydrogens. (Propane and Ethane by Kareen Martin; CC BY-NC 4.0)

- Hydrocarbon skeletons may have **double bonds** at different locations within the molecule (Figure **2.2.4**).

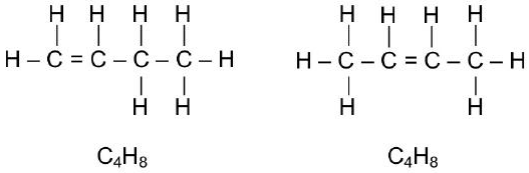


Figure **2.2.4**: Butene is a molecule with the chemical formula C_4H_8. The structure and function of butene depends upon the location of the carbon-carbon double bond. There are four different possible locations for this double bond. Two are shown in this figure. Left image - 1-butene has a double bond between the two first carbons. Right image - 2-butene has a double bond between the second and third carbons. (Butene Structures by Kareen Martin; CC BY-NC 4.0)

- Hydrocarbon skeletons can be **linear** or **branched** (Figure **2.2.5**).

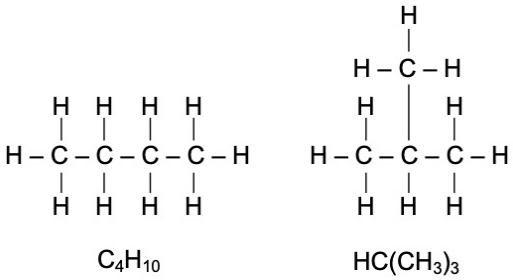


Figure **2.2.5**: Butane and its isomer isobutane have the chemical formula C_4H_{10}. Left image - Butane is a linear molecule. Right image - isobutane is a branched molecule with a central carbon bonded to 1 Hydrogen and 3 methyl groups ($-CH_3$). (Butane and Isobutane by Kareen Martin; CC BY-NC 4.0)

- The hydrocarbon skeleton can form **rings** when placed in a aqueous solution (Figure **2.2.6**)

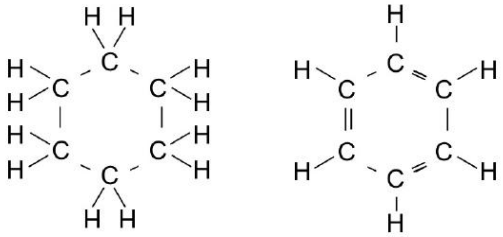


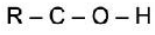
Figure **2.2.6**: Two examples of hydrocarbons arranged as rings. Left image - Cyclohexane with the chemical formula C_6H_{12} is made up of 6 carbon atoms arranged in a hexagonal ring, where each carbon atom is bound to two other carbon atoms and two hydrogen atoms. Right image - Benzene with the chemical formula C_6H_6 is made up of six carbon atoms arranged in a hexagonal ring with alternating single and double bonds between the carbons. Each carbon atom is bound to two other carbons and one hydrogen atom. (Ring hydrocarbons by Kareen Martin; CC BY-NC 4.0)

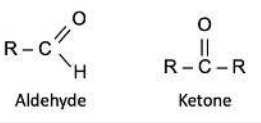
Functional Groups Modify Hydrocarbons

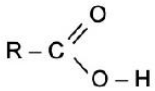
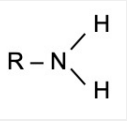
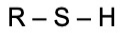
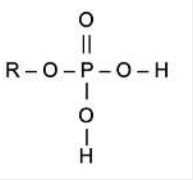
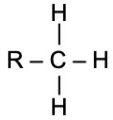
A functional group is a group of atoms that binds to a molecule and give it specific chemical properties. In biology, functional groups are bound to the hydrocarbon "backbone" of carbohydrates, proteins, lipids, and nucleic acids. The nature, number and location of a functional group gives a molecule it characteristic properties. For example, the addition of a certain functional group can make a molecule acidic whereas another can make it basic. Some groups can make a region of a molecule hydrophilic (reacts with water), whereas others could be hydrophobic (repels water). Some of the important functional groups in biological molecules are shown in Table **2.2.1**. They include: hydroxyl, methyl, carbonyl, carboxyl, amino, phosphate, and sulfhydryl.

Table **2.2.1**: **The 7 most important functional groups in biological molecules**

https://bio.libretexts.org/@go/page/138621

Functional group	Structure (R denotes the remaining molecule)	Properties
Hydroxyl (-OH)		Polar (Hydrophilic) • the H+ dissociates in aqueous environments leaving an O atom with a slight negative charge • found in alcohols and sugars

Carbonyl (-CO)		Polar (Hydrophilic)
		• 2 types depending on the location of the oxygen:
		  ◦ Aldehydes have the oxygen located at the end of the hydrocarbon
		  ◦ Ketones have the oxygen located in the middle
		• found in sugars

Carboxyl (-COOH)		Charged and acidic. Ionizes to release H^+ into solution • the H+ dissociates in aqueous environments leaving an O atom with a slight negative charge • found in amino acids and fatty acids
Amino (-NH2)		Charged and basic. Accepts H^+ to form NH^{3+} • found in amino acids and nucleotide bases
Sulfhydryl (-SH)		Polar (Hydrophilic) • the H+ dissociates in aqueous environments leaving an S atom with a slight negative charge • found in the amino acid cysteine • found in acetyl-co-A
Phosphate ($-PO_4$)		Charged and acidic. Ionizes to release H^+ into solution • the H+ dissociates in aqueous environments leaving an O atom with a slight negative charge • found in nucleotides, phospholipids
Methyl ($-CH_3$)		Non polar (Hydrophobic)

The functional groups shown in Table **2.2.1** play an important role in the formation of molecules like DNA, proteins, carbohydrates, and lipids and will be discussed in the remaining sections in this chapter.

Key Concepts

Carbon is the backbone of many inorganic and organic molecules found in nature.

Some important concepts to remember:

- Carbon forms four covalent bonds
- Carbon can bond with other carbon atoms and with hydrogen atoms to form hydrocarbons
- Carbon atoms can bind to one another through single, double, and triple bonds
- Hydrocarbons can be found in linear, branched and ring form
- Functional groups can attach to the hydrocarbon and can determine its properties and functions

Glossary

Atom - the smallest unit of an element, consisting of protons, neutrons, and electrons

Atomic number - the number of protons in an atom's nucleus

Atomic symbol - the abbreviation used to denote an chemical element; usually one or two letters

Bonding - the process by which atoms join together to create a chemical bond

Covalent bond - type of chemical bond where two atoms share electrons in order to achieve a more stable electron configuration; categorized as either polar or non-polar

Electron - a subatomic particle with a negative charge that orbits the nucleus of an atom

Electron shell - a region around the nucleus of an atom where electrons are most likely found; can hold a specific number of electrons; also called an orbital

Element - a substance made up of only one type of atom; distinguished by its atomic number and atomic symbol

Functional group - a group of atoms attached to a carbon chain that determines the chemical properties of the molecule

Molecule - a group of atoms joined by chemical bonds

Neutron - a subatomic particle found in the nucleus of an atom that is neutral (has no charge)

Non-polar molecule - a molecule in which there is an equal sharing of electrons between atoms

Polar molecule - a molecule in which there is an unequal sharing of electrons between atoms

Proton - a positively charged subatomic particle found in the nucleus of an atom

Valence - the ability of an atom to form a bond with other atoms based on the number of electrons it can gain, lose, or share; determined by the number of valence electrons

Valence electrons - the number of electrons in the outermost electron shell of an atom

---

2.2: Carbon is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.3: Macromolecules

Biotech Focus

The study of how macromolecules are made can be difficult. Initial studies in this area were made possible through radiolabeling. In **radiolabeling**, radioactive isotopes, like carbon-14 (^{14}C), sulfur-35 (^{35}S) and phosphorus-32 (^{32}P), are added to cells so that these isotopes are incorporated into the macromolecules being made. For DNA synthesis, researchers will rely upon ^{32}P incorporation into nucleotides. Protein synthesis uses ^{35}S, while carbohydrate synthesis can be studied using ^{14}C. Thanks to its sensitivity, radiolabeling allows researchers to track, quantify, and analyze the behavior of macromolecules in biological systems.

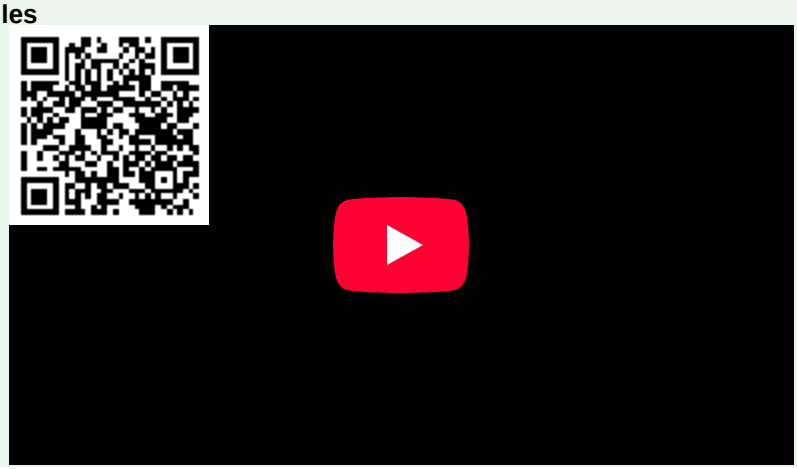
Introduction

A **macromolecule** is a large, complex molecule that is essential to the viability and function of cells. There are four major classes of biological macromolecules, **carbohydrates, lipids, proteins, and nucleic acids**, and each is an important component of the cell. Combined, these molecules make up the majority of a cell's dry mass. They perform a wide variety of functions, including energy storage, structural support, cellular communication, and genetic information transfer. Understanding the structure of a macromolecule is key to comprehending complex biological processes, from cellular metabolism, to the transfer of genetic information.

Figure **2.3.1**: Portrait of a man made out of fruits and vegetables by Giuseppe Arcimboldo. (Arcimboldo, Public Domain)

Concept in Action

Video: Macromolecules



Learning Objectives

By the end of this section, you will be able to:

- Describe the relationship between a monomer and a polymer
- Understand the synthesis of macromolecules

- Explain dehydration and hydrolysis reactions

Synthesis of macromolecules

Biological macromolecules are large molecules, necessary for life, that are built from smaller organic molecules. Biological macromolecules are organic, meaning they contain carbon and are bound to hydrogen, and may contain oxygen, nitrogen, and additional minor elements. Most (but not all) biological macromolecules are polymers. A **polymer** is a molecule made up of smaller subunits, called **monomers**. Think of a polymer as being like a necklace, a series of beads strung together. Typically all the monomers in a polymer tend to be the same, or at least very similar to each other, linked to one another over and over again to build up the larger macromolecule. The monomer used depends on the macromolecule. Table **2.3.1** outlines the types of monomers used to construct a carbohydrate, protein, and nucleic acid.

Table **2.3.1**: Types of monomer forming organic molecules

Monomer	Polymer (Macromolecule)
"Bead"	*"Necklace"*
Monosaccharide	Carbohydrate
Amino Acid	Protein
Nucleotide	Nucleic Acid (DNA and RNA)

Monomers combine with each to make the polymer through the formation of covalent bonds. In doing so, the monomers release water molecules as byproducts. This type of reaction is known as **dehydration synthesis**, which means “to put together while losing water.” When performed in a test tube, the release of water results in condensation on the side of the test tube. As such, dehydration synthesis reactions are also known as condensation reactions.

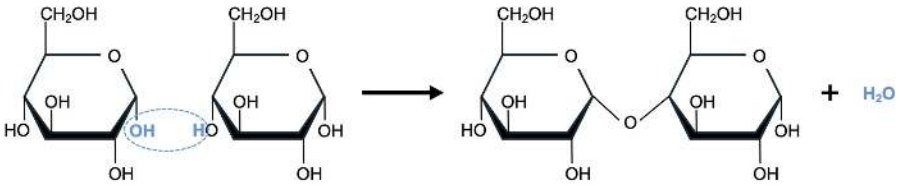


Figure **2.3.2**: Dehydration synthesis reactions create bonds through the removal of a water molecule. In the dehydration synthesis reaction depicted above, two molecules of glucose combine together to form the disaccharide maltose. A hydroxyl (OH) is removed from carbon 1 of one glucose and combines with a hydrogen (H) removed from carbon 4 of the second glucose, to create a molecule water (dotted circle). A new covalent bond forms between the two glucose molecules. Additional monomers may join by the same process, growing the chain and forming a polymer. (Dehydration synthesis by Kareen Martin; CC BY 4.0)

In a dehydration synthesis reaction (Figure **2.3.2**), the hydrogen of one monomer combines with the hydroxyl group of another monomer, releasing a molecule of water. At the same time, the monomers share electrons and form covalent bonds. As additional monomers join using dehydration synthesis, the growing chain of repeating monomers forms the polymer. Since dehydration reactions involve the formation of new bonds, they require an input of energy. Different types of monomers can combine in many configurations, giving rise to a diverse group of macromolecules. Even one kind of monomer can combine in a variety of ways to form several different polymers: for example, glucose monomers are the constituents of starch, glycogen, and cellulose.

Because polymers form through the removal of water molecules, water is required to break them down. The bonds found within a polymer can be broken through a reaction called **hydrolysis**, which means “to split with water”. The hydrolysis reaction is a reaction in which a water molecule is added to a bond, resulting in the breaking of the bond (Figure **2.3.3**); one product of the reaction gains a hydrogen atom (H+) and the other gains a hydroxyl molecule (OH–). Because hydrolysis reactions break bonds, they will release energy.

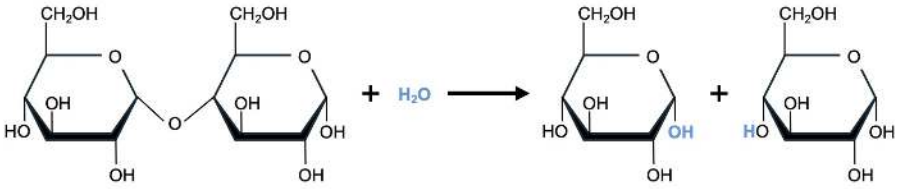


Figure **2.3.3**: Hydrolysis reactions break bonds through the addition of a water molecule. In the hydrolysis reaction shown here, the addition of water breaks down the disaccharide maltose to form two glucose monomers. One of the new molecules gains a hydrogen atom, while the other gains a hydroxyl (-OH) group. Note that this reaction is the reverse of the dehydration synthesis reaction. (Hydrolysis by Kareen Martin; modified from Synthesis of Biological Macromolecules; CC BY 4.0)

Dehydration and hydrolysis reactions are catalyzed, or "sped up," by specific **enzymes**. In biological organisms, these enzymes are specific to the macromolecule. For instance, the carbohydrates amylose, sucrose, lactose, and maltose are broken down by the enzymes amylase, sucrase, lactase, and maltase, respectively. DNA polymers are created by a class of enzymes called DNA polymerases. A discussion of each of the four biological macromolecules, how they are made and how they are degraded, can be found in the remaining pages of this chapter.

Key Concepts

Biological macromolecules are important cellular components and perform a wide array of functions necessary for the survival and growth of living organisms.

Some important concepts to remember are:

- a polymer is a large macromolecule made up of subunits called monomers
- the monomers of a polymer are joined together through a dehydration synthesis reaction, which removes a molecule water to form the bond
- a monomer can be removed from a polymer through a hydrolysis reaction, which breaks a bond through the addition of a water molecule
- the four major classes of biological macromolecules are carbohydrates, lipids, proteins, and nucleic acids
- most biological macromolecules can be considered polymers

Glossary

Catalyst - a substance that speeds up chemical reactions without being consumed, often refers to enzymes in biological systems

Dehydration - to remove water

Dehydration synthesis - a chemical reaction in which two molecules combine to form a larger molecule, while removing a water molecule (H_2O); also known as a condensation reaction

Enzyme - a biological catalyst that speeds up a chemical reaction; not consumed by the chemical reaction

Hydrolysis - to break with water ("hydro" = water; "lysis" = to break)

Macromolecule - a large molecule; often used interchangeably with polymer

Monomer - a small molecule that when combined with one another forms a polymer

Polymer - a large molecule made up of repeating units called monomers

2.3: Macromolecules is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.4: Carbohydrates

Biotech Focus

Carbohydrates are not only important components of foods, but they are essential macromolecules that perform a range of critical biological functions in cells. In addition to providing structure to cells (in plants) and being a source of cellular energy (in all cells), some carbohydrates have been approved for the treatment of various diseases. However, their use is very limited. To learn why and read about the future potential of carbohydrates as medicine, check out the article Exploring Carbohydrates for Therapeutics: A Review on Future Directions.

Introduction

Carbohydrates play essential roles in the human body as well as in other organisms. They are an important source of energy to cells and also provide structural support.

Learning Objectives

By the end of this section, you will be able to:

- Discuss the role of carbohydrates in cells and in the extracellular materials of animals and plants
- Explain the basic composition of a carbohydrate
- List the major types of carbohydrates
- Explain how monosaccharides are classified
- Explain the major forms of monosaccharides
- Explain the different types of common monosaccharides, disaccharides, and polysaccharides
- Explain the difference between a dehydration synthesis reaction and a hydrolysis reaction

Molecular Structures

Carbohydrates are biological molecules made of carbon, hydrogen, and oxygen. This composition gives carbohydrates their name: they are made up of carbon (carbo-) plus water (-hydrate). Carbohydrates can be represented by the stoichiometric formula $\mathbf{(CH_2O)_n}$, where n is the number of carbons in the molecule. In other words, there is as number of carbon atoms in a carbohydrate molecule equals that of oxygen atoms and there are twice as many hydrogen atoms as carbon.

Carbohydrates can be classified into three categories:

1. **monosaccharides**
2. **disaccharides**
3. **polysaccharides**

Monosaccharides

Monosaccharides (mono- = “one”; sacchar- = “sugar”) are the simplest form of a carbohydrate and are often referred to as **simple sugars**. They typically range in size from three to seven carbons (Figure **2.4.1**). Most monosaccharide names end with the suffix "-ose" (e.g. glucose). They can be classified in several ways, one of which is based on the number of carbons.

Examples of carbohydrates classified using this method are:

- **triose sugars** (three carbons)
- **pentose sugars** (five carbons)
- **hexose sugars** (six carbons)

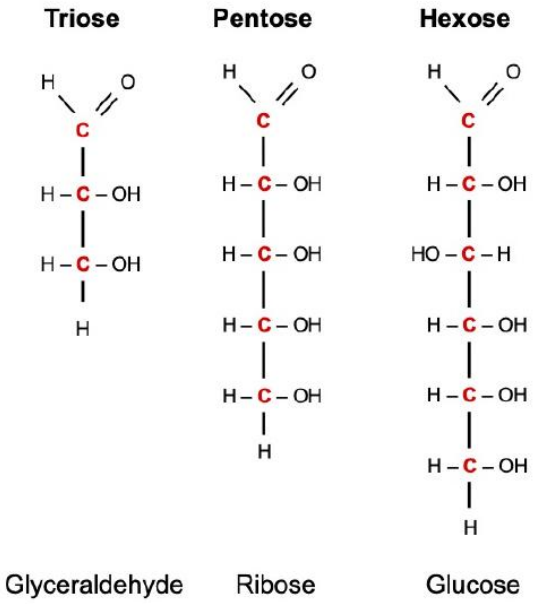


Figure 2.4.1: Monosaccharides can be classified based on the number of carbons in the backbone. The triose sugar, glyceraldehyde, ($C_3H_6O_3$) has three carbons (carbons are highlighted in red). The pentose sugar, ribose, ($C_5H_{10}O_5$) has five sugars. The hexose sugar, glucose, ($C_6H_{12}O_6$) is made of 6 carbons. (Monosaccharides by Kareen Martin; CC BY 4.0)

A monosaccharide is composed of carbon, oxygen, and hydrogen atoms. Most of the oxygen atoms in the monosaccharide can be found in hydroxyl (OH) groups. However, one oxygen atom is found as part of a functional group called a **carbonyl (C=O)** (Figure 2.4.2). The position of the carbonyl group is another way to classify a monosaccharide.

Using this method, there are two types of monosaccharides:

1. **Aldose:** the carbonyl (C=O) is located at the end of the sugar, forming an **aldehyde** group
2. **Ketose:** The carbonyl (C=O) is located within the molecule, forming a **ketone** group

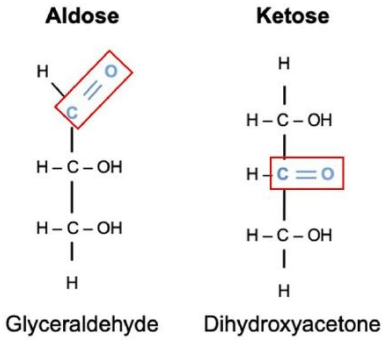


Figure 2.4.2: Monosaccharides can be classified based on the position of their carbonyl group (C=O) in the carbon chain (red boxes). Aldose sugars (e.g. glyceraldehyde) have a carbonyl group at the end of the carbon chain, and ketose sugars (e.g. dihydroxyacetone) have a carbonyl group in the middle of the carbon chain. (Aldose vs Ketose by Kareen Martin; CC BY 4.0)

The most common monosaccharides found in cells are the hexose sugars **glucose, fructose, and galactose** and the pentose sugars **ribose and deoxyribose.** Both ribose and deoxyribose are found in nucleic acids. Galactose can be found as part of lactose (i.e. milk sugar) and fructose is a component of the disaccharide in sucrose, which is found in fruit. Glucose is an important source of energy to eukaryotic cells. During cellular respiration, energy is released from glucose, and that energy is used to help make adenosine triphosphate (ATP). Plants synthesize glucose using carbon dioxide and water, and glucose in turn is used for energy requirements for the plant. In plants and certain algae excess glucose is stored as starch that is broken down by humans and other animals that feed on these materials. In animals and certain fungi, excess glucose is stored as glycogen.

The chemical formula for glucose, galactose, and fructose is $C_6H_{12}O_6$. While their chemical formulae are identical, their structures are different (Figure **2.4.3**). Compounds that have the same molecular formulae, but have different structures are known as **structural isomers.** Therefore, *glucose, galactose and fructose are structural isomers of one another.*

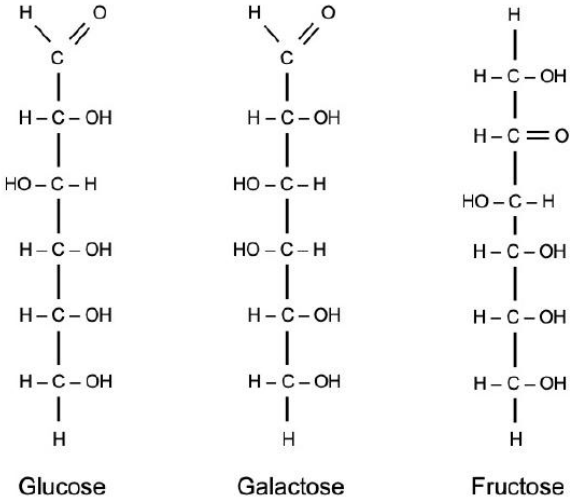


Figure **2.4.3**: Hexose sugars. Glucose, galactose, and fructose are all hexose sugars with the same chemical formula ($C_6H_{12}O_6$). They are known as structural isomers, meaning they have the same chemical formula but a different arrangement of atoms. (Structural Isomers of Glucose by Kareen Martin; CC BY 4.0)

When placed into aqueous solutions, monosaccharides form **ring-shaped molecules** (Figure **2.4.4**). The ring form of a monosaccharide is more stable than its linear counterpart. This is because the reactive carbonyl function group found in the linear form is no longer exposed and becomes "trapped" in the ring.

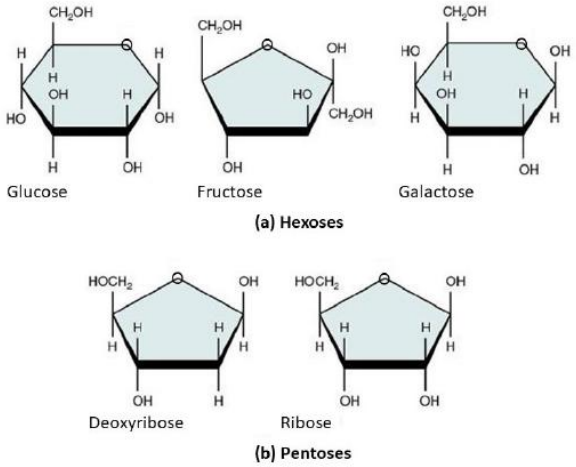


Figure **2.4.4**: Monosaccharides can be found in ring forms. The ring forms of the hexose sugars glucose, fructose, and galactose are shown at the top of the figure and the pentose sugars deoxyribose and ribose are shown below. (Carbohydrate Ring Forms by Kareen Martin; CC BY 4.0)

The linear molecule is converted into a ring form when the hydroxyl group on the fifth carbon reacts with the carbonyl group on the first carbon. Numbering of the carbons in the resulting ring-shaped monosaccharide begins with the carbon to the right of the oxygen and proceeds in a clock-wise fashion (Figure **2.4.5**).

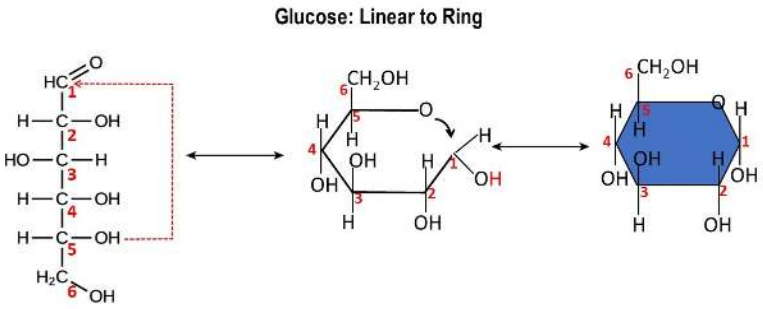


Figure **2.4.5**: Transformation of linear glucose to the ring form. Conversion of a linear glucose molecule to its ring form occurs when the hydroxyl group at the 5th carbon reacts with the carbonyl group on the 1st carbon (left image, dotted red arrow). The linear form begins to convert to its ring conformation, with a bond forming between the 5th and 1st carbon (middle image, arrow). The hydrogen from the 5th carbon (shown in red) bonds to the oxygen at the 1st carbon to form a hydroxyl group. The ring formation of glucose has a specific numbering scheme with the 1st carbon located to the right of the oxygen (right image). (Glucose Linear to Ring by Patricia Zuk, CC BY 4.0)

Glucose and galactose in their ring structure, differ in the orientation of the hydroxyl group (OH) at the fourth carbon, with glucose having this functional group located below the plane of the ring. Furthermore, glucose can have two different arrangements of the hydroxyl group around the first carbon. If the hydroxyl group at carbon 1 found is below the plane of the ring, it is said to be in the **alpha (α) position**, and if it is above the plane, it is said to be in the **beta (ß) position** (Figure **2.4.6**). The beta-form is more stable than the alpha form and is typically found in large polymers like cellulose, whereas the alpha form can be found in plant starches like amylose. Fructose, with its unique shape does not have alpha and beta conformations.

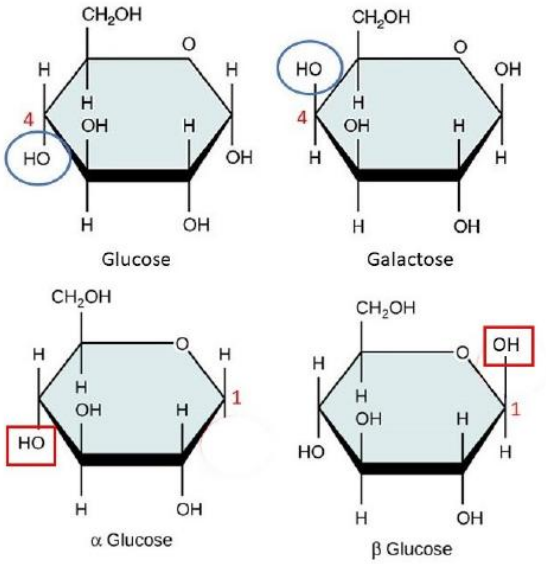


Figure **2.4.6**: Monosaccharide conformations. Glucose and galactose can be distinguished using the hydroxyl groups located at carbon four (blue circles). The alpha and beta forms of glucose can be distinguished using the hydroxyl groups located at carbon one (red squares). (Glucose and Galactose Forms by Kareen Martin, CC BY 4.0)

Disaccharides

The joining of two monosaccharides forms a **disaccharide** (di- = “two”; sacchar- = “sugar”). Disaccharides are formed through **dehydration synthesis** and result in the formation of a **glycosidic bond** between the two monosaccharide subunits. During this process, the hydroxyl group (–OH) of one monosaccharide combines with a hydrogen atom of another monosaccharide, releasing a molecule of water (H_2O) and forming a covalent bond between atoms in the two sugar molecules (Figure **2.4.7**).

Common disaccharides are:

- **sucrose** - formed by joining glucose to fructose

- **lactose** - formed by joining glucose to galactose
- **maltose** - formed by joining glucose to glucose

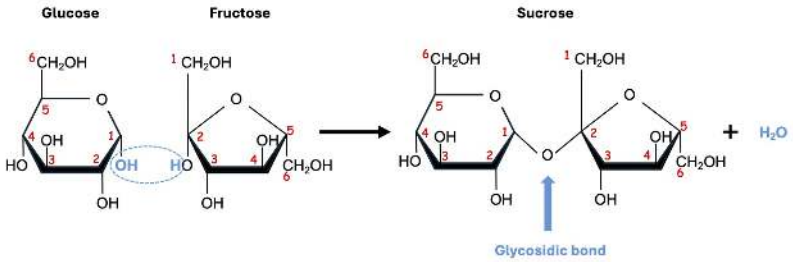


Figure **2.4.7**: Sucrose is a disaccharide made of a monomer of glucose and a monomer of fructose. Sucrose is formed through a dehydration reaction that forms a glycosidic linkage between carbon 1 in glucose and carbon 2 in fructose. The components of the water molecule removed to make the bond is shown with a dotted circle. (Sucrose Formation by Kareen Martin; CC BY 4.0)

The breakdown of these disaccharides is done through a **hydrolysis reaction** and will yield glucose plus either galactose or fructose. In cells, this hydrolytic breakdown is catalyzed by enzymes. For example, the enzyme **sucrase** catalyzes the breakdown of sucrose into glucose and fructose Figure **2.4.8**).

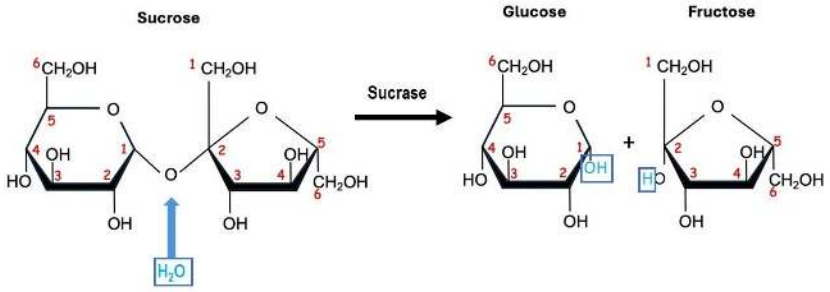


Figure **2.4.8**: Sucrose breakdown by sucrase. The enzyme sucrase catalyzes the hydrolytic breakdown of sucrose into the monomers glucose and fructose. The location of the incorporated water molecule is shown with blue boxes. (Sucrose Breakdown by Patricia Zuk, CC BY 4.0)

Polysaccharides

The joining of three of more monosaccharides creates a **polysaccharide** (poly- = "many"). Each polysaccharide is made of either a few or hundreds of monosaccharides with each joined through a dehydration synthesis reaction. Unlike simple sugars, polysaccharides are not soluble in water and do not taste very sweet. This is because the majority of polysaccharides found in living organisms are made of glucose monomers. Starch, glycogen, cellulose, and chitin are examples of polysaccharides. Starch and glycogen are considered **storage polysaccharides** owing to their roles in energy utilization. Cellulose and chitin are examples of **structural polysaccharides** that are incorporated into the structure of the organism.

Starch is the stored form of glucose made by plants and can be found in two forms: **amylose** and **amylopectin** (Figure **2.4.9**). Amylose is the simplest of these two forms with the alpha form of glucose linked by glycosidic bonds found between the first carbon of one glucose monomer and the fourth carbon of the next monomer (i.e. 1-4 glycosidic linkages). This creates a starch with a helical (i.e. coiled) conformation. Amylopectin has regions of coils, in addition to branch points that are formed by glycosidic bonds that form between the first carbon of one glucose monomer and the sixth carbon of another (i.e. 1-6 glycosidic linkages). Excess glucose in plants is stored as starch in different plant parts, including the roots and seeds. The starch that is consumed by animals is broken down into smaller molecules, such as glucose. The cells can then utilize the glucose.

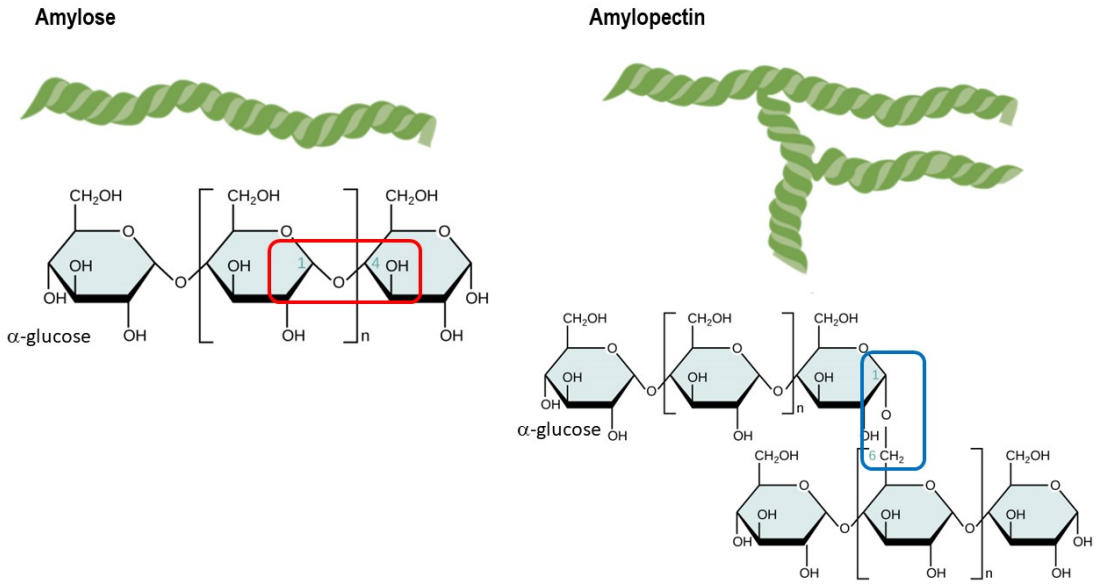


Figure 2.4.9: Amylose and amylopectin. The starch molecules amylose and amylopectin are composed of α-glucose subunits. Amylose (left image) is a simple, coiled starch made of α-glucose monomers joined through 1-4 glycosidic linkages (red box). Amylopectin (right image) is made of α-glucose monomers joined through 1-4 glycosidic linkages, in addition to 1-6 glycosidic linkages (blue box). The 1-6 glycosidic linkages produce a branch point in this starch. (Amylose and Amylopectin by Patricia Zuk, CC BY 4.0; modified from Carbohydrates, CC BY-SA 4.0)

The main polysaccharide of vertebrates is **glycogen.** Glycogen is a complex, branched polysaccharide whose monomers are the beta form of glucose (Figure 2.4.10) Glycogen is the animal equivalent of starch. Branch points are the result of 1-6 glycosidic bonds, with the coiled sections being the result of 1-4 glycosidic bonds. Excess glucose is converted into glycogen by the cells of the liver and skeletal muscle. When glucose levels in an organism drop, glycogen is broken down to release glucose.

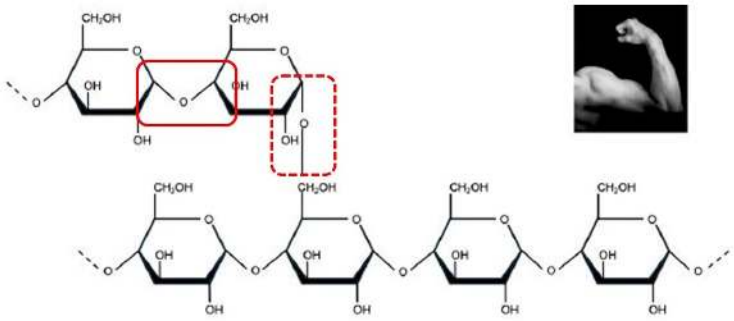


Figure 2.4.10: Glycogen structure. Glycogen is a highly branched molecule made of ß-glucose monomers connected by 1-4 glycosidic linkages (solid ed box) and 1-6 glycosidic linkages (dotted red box). The 1-6 glycosidic linkage will produce a branch point in the molecule. Glycogen is the animal equivalent of starch and usually stored in liver and muscle cells. (Glycogen Structure by Kareen Martin; CC BY 4.0)

Cellulose is one of the most abundant natural biopolymers. The cell walls of plants are mostly made of cellulose, which provides structural support to the cell. Wood and paper are mostly cellulosic in nature. The chemical formula for cellulose is $(C_6H_{10}O_5)_n$, where n is the number of glucose monomers. Cellulose is made up of glucose monomers that are linked by 1-4 glycosidic bonds. (Figure 2.4.11). While this is the same glycosidic linkage found in starch, *in cellulose the glucose monomer is the beta form* and every other beta-glucose monomer in cellulose is flipped in its orientation. This allows for the creation of extremely long chains of glucose that are rigid and have a high tensile strength—two parameters that are important to plant cells.

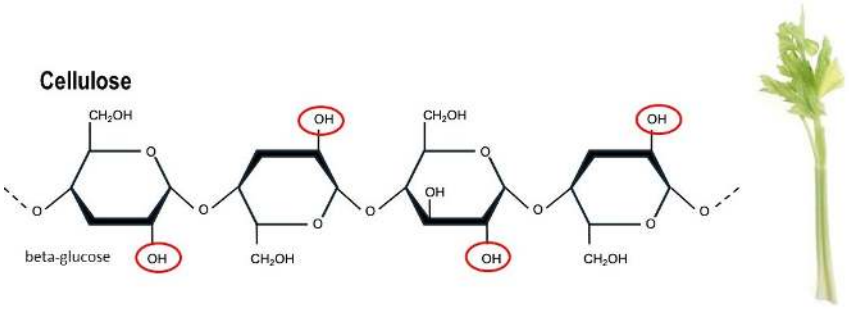


Figure **2.4.11**: Cellulose structure. In cellulose, glucose monomers (beta form) are linked in unbranched chains (left image). Because of the way the beta-glucose subunits are joined, every glucose monomer is flipped relative to the next one (OH groups at carbon 3 shown circled in red). Cellulose is found in the cell walls of plants and gives them rigidity as seen in celery (right image). (Cellulose Structure by Kareen Martin; CC BY 4.0)

Cellulose passing through the digestive system is called dietary fiber. While the glucose-glucose bonds in cellulose cannot be broken down by human digestive enzymes, herbivores such as cows, buffalos, and horses are able to digest grass that is rich in cellulose and use it as a food source. In these animals, certain species of bacteria reside in the digestive system and secrete the enzyme **cellulase.** The appendix in some ruminants also contains bacteria that break down cellulose. Cellulases can break down cellulose into glucose monomers that can be used as an energy source by the animal.

Carbohydrates serve other functions in different animals. Arthropods, such as insects, spiders, and crabs, have an outer skeleton, called the **exoskeleton**, which protects their internal body parts. This exoskeleton is made of the biological macromolecule **chitin**, which is a nitrogenous carbohydrate. Chitin is made of repeating units of **N-acetylglucosamine (GlcNAc)**, a modified sugar derived from glucose and containing nitrogen. (Figure **2.4.12**).

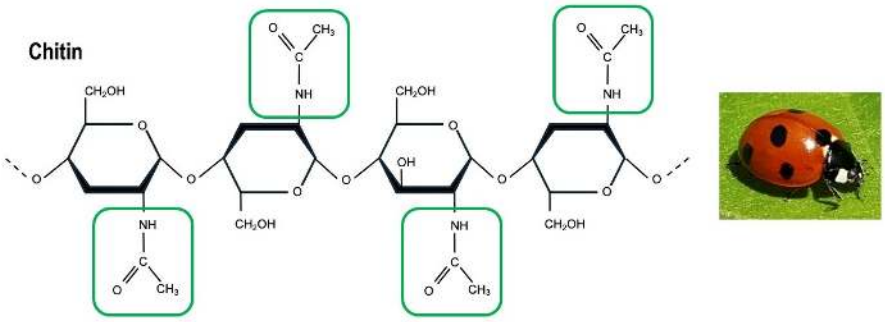


Figure **2.4.12**: Chitin structure. Chitin is a polysaccharide made of repeating units of N-acetylglucosamine (GlcNAc). N-acetylglucosamine is a modified glucose monomer that has an amine and acetyl group (shown in the green box) bound to the 2nd carbon. Insects like the ladybug shown on the right, have a hard outer exoskeleton made of chitin. (Chitin Structure by Kareen Martin; CC BY 4.0)

While the polysaccharides discussed in this page are all built of glucose monomers, differences in their molecular structure allow for the different functions of energy storage (starch and glycogen) and structural support and protection (cellulose and chitin).

Key Concepts

Carbohydrates are a group of macromolecules that are a vital energy source for the cell and provide structural support to plant cells, fungi, and all of the arthropods that include lobsters, crabs, shrimp, insects, and spiders.

Some important concepts to remember are:

- Carbohydrates are classified as monosaccharides, disaccharides, and polysaccharides depending on the number of monomers in the molecule
- The monomer of a carbohydrate is the monosaccharide
- Monosaccharides are linked together by glycosidic bonds that are formed as a result of dehydration synthesis reactions
- Disaccharides are formed by joining two monosaccharides by a glycosidic bond

- Polysaccharides are formed by joining numerous monosaccharides by glycosidic bonds
- Glucose, galactose, and fructose are common monosaccharides
- Common disaccharides include lactose, maltose, and sucrose
- Starch and glycogen are storage polysaccharides, made of glucose and found in plants and animals, respectively.
- Cellulose and chitin are structural polysaccharides

Glossary

Carbohydrate - a macromolecule composed of carbon, hydrogen, and oxygen; primarily used for energy storage and structural functions.

Cellulose - a structural polysaccharide found in the cell walls of plants; composed of glucose molecules linked by glycosidic bonds; indigestible by humans but important as dietary fiber.

Disaccharide - a carbohydrate composed of two monosaccharides linked by a glycosidic bond, such as sucrose, lactose, and maltose.

Fiber - a type of carbohydrate that is not digested by human enzymes but aids in digestion and overall health; found in plant-based foods.

Fructose - a monosaccharide found in fruits and honey; often referred to as fruit sugar.

Glucose - a monosaccharide that serves as the primary source of energy for cells; often referred to in humans as as blood sugar.

Glycogen - a polysaccharide that serves as the primary storage form of glucose in animals and humans; stored mainly in the liver and muscles.

Glycosidic bond - a covalent bond that links carbohydrate molecules together; formed between the hydroxyl groups of two sugars.

Lactose - a disaccharide composed of glucose and galactose; commonly found in milk and dairy products.

Monosaccharide - the simplest form of carbohydrate, consisting of a single sugar unit, such as glucose, fructose, or galactose.

Polymer - a large molecule made up of repeating units called monomers

Polysaccharide - a complex carbohydrate composed of multiple monosaccharide units, such as starch, cellulose, and glycogen.

Starch - a polysaccharide found in plants that serves as their main form of energy storage; examples include amylose and amylopectin.

Sucrose - a disaccharide made up of glucose and fructose; commonly known as table sugar.

Attribution

text modified from OpenStax Biology 2e carbohydrates and OpenStax Biology 2e Biological Molecules

2.4: Carbohydrates is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.5: Lipids

Biotech Focus

Statins are among the most widely-prescribed medications in the United States due to their ability to decrease LDL-cholesterol and lower the risk of developing cardiovascular diseases. Their development started with the works of Drs. Brown and Goldstein on the underlying mechanisms of cholesterol metabolism, for which they were awarded the 1985 Nobel Prize in Physiology or Medicine. Their findings eventually led to the discovery of the first statins by the Japanese biochemist Akira Endo and the subsequent development of statin drugs.

Learn more about the history, mechanism, and side effects of statins by watching the video below.

Introduction

Lipids include a diverse group of compounds that are largely non-polar in nature. This is because they are hydrocarbons that include mostly non-polar carbon–carbon or carbon–hydrogen bonds. Non-polar molecules are hydrophobic (“water fearing”), or insoluble in water. Lipids perform many different functions in a cell. Cells store energy for long-term use in the form of fats. Lipids also provide insulation from the environment for plants and animals. For example, they help keep aquatic birds and mammals dry when forming a protective layer over fur or feathers because of their water-repellant hydrophobic nature. Lipids are also the building blocks of many hormones and are an important constituent of all cellular membranes.

Lipids include the following:

1. **triglycerides (fats, oils)**
2. **phospholipids**
3. **steroids (cholesterol, bile salts, hormones)**
4. **fatty acids (omega family)**
5. **fat-soluble vitamins**
6. **eicanosoids (prostaglandins, leukotrienes)**
7. **lipoproteins (HDL, LDL)**
8. **waxes**
9. **pigments**

Learning Objectives

Lipids are a diverse group of non-polar macromolecules. By the end of this section, you will be able to:

- Describe the four major types of lipids
- Explain the role of fats in storing energy
- Differentiate between saturated and unsaturated fatty acids
- Describe phospholipids and their role in cells

- Define the basic structure of a steroid and some functions of steroids
- Explain the how cholesterol helps to maintain the fluid nature of the plasma membrane

Figure **2.5.1**: The hydrophobic lipids found in the fur of aquatic mammals, such as this river otter, protect them from the elements by repelling water.

Triglycerides: Fats and Oils

A **triglyceride** molecule consists of two main components: **glycerol and fatty acids**. A solid triglyceride is referred to as a fat. A liquid triglyceride is known as an oil

- **Glycerol** is an organic compound (alcohol) with three carbons, five hydrogens, and three hydroxyl (OH) groups.
- **Fatty acids** have a long chain of hydrocarbons to which a carboxyl group is attached, hence the name “fatty acid.” The number of carbons in the fatty acid may range from 4 to 36; most common are those containing 12–18 carbons.

To form a triglyceride, three fatty acids attach to the glycerol molecule through three separate dehydration synthesis reactions. The bond that forms between a fatty acid and the glycerol is called an **ester linkage or ester bond** (Figure **2.5.2**). To form an ester bond, a hydrogen from the glycerol's hydroxyl group and a hydroxyl group from the fatty acid's carboxyl group are removed to form a molecule of water. During triglyceride synthesis a total of three water molecules are formed and released and three ester bonds are created.

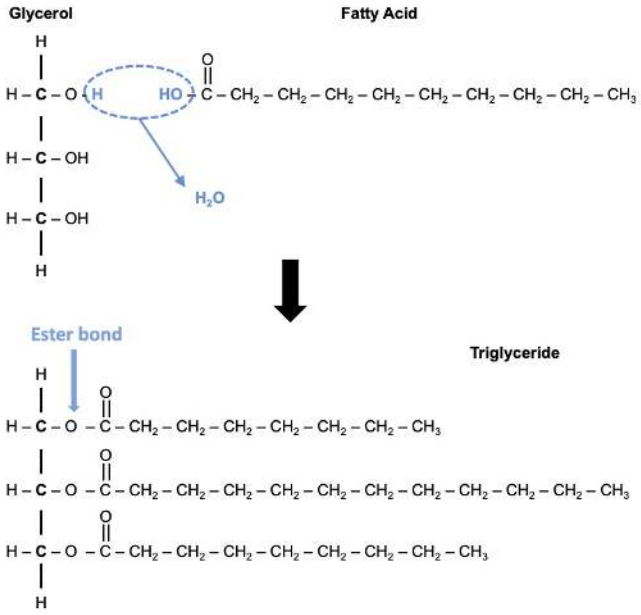


Figure **2.5.2**: The triglyceride. A triglyceride is composed of three fatty acids and a glycerol backbone. Glycerol is a 3-carbon molecule with each carbon bound to a hydroxyl group. A fatty acid is a long chain of carbon and hydrogen with a carboxylic acid (-COOH) on one end. In triglyceride synthesis, the hydroxyl group (OH) fatty acid's carboxyl group combines with a hydrogen (H) from the glycerol's hydroxyl group (dotted circle), forming a molecule of water and resulting in the formation of an ester bond between the fatty acid and the glycerol (see arrow). Two more fatty acids bind to the remaining two hydroxyl groups of the glycerol. A total of three water molecules are formed. (Triglyceride Formation by Kareen Martin; CC BY 4.0)

The three fatty acids in the triglyceride may be similar to or different from one another. Some fatty acids have common names that specify their origin. For example, palmitic acid, is a fatty acid derived from the palm tree. Arachidic acid is derived from *Arachis hypogea*, the scientific name for peanuts. Fatty acids can be classified as saturated and unsaturated. In a **saturated fatty acid**, all carbons in the hydrocarbon chain are linked to each other through single bonds. Because of this, saturated fatty acids are said to be "saturated" with hydrogen; in other words, the number of hydrogen atoms attached to the carbon skeleton is maximized and no more hydrogen may be added to the carbon atoms of the chain. The saturated fatty acid is a long, linear molecule. Stearic acid is an example of a saturated fatty acid (Figure **2.5.3**).

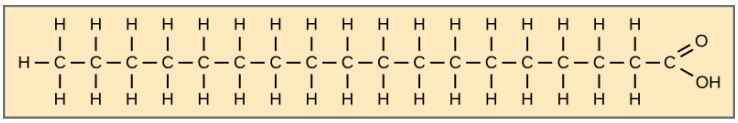


Figure **2.5.3**: The saturated fatty acid. Stearic acid is a saturated fatty acid with an 18-carbon chain. All bonds between the carbons are single bonds. (Stearic acid by Openstax; CC BY 4.0)

In an **unsaturated fatty acid**, the hydrocarbon chain contains one or multiple carbon-carbon double bonds. In other words, carbons are not saturated with hydrogens. Oleic acid is an example of an unsaturated fatty acid (Figure **2.5.4**). If there is one double bond in the molecule, then it is known as a **monounsaturated fatty acid** (e.g., found in olive oil), and if there is more than one double bond, then it is known as a **polyunsaturated fatty acid** (e.g., found in canola oil). Only one of the three fatty acids needs to be unsaturated for the triglyceride to be classified as unsaturated.

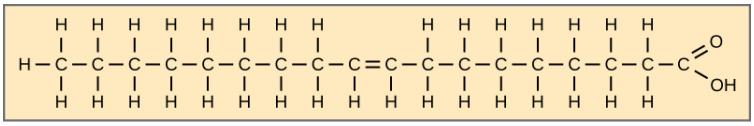


Figure **2.5.4**: The unsaturated fatty acid. Oleic acid is an unsaturated fatty acid with an 18-carbon chain. It has one double bond between the 9th and 10th carbons. (Oleic acid by Openstax; CC BY 4.0)

The position of the hydrogen atoms around the carbon-carbon double bond can be used to further classify the unsaturated fatty acid as either ***cis*** or ***trans***. If hydrogen atoms are present in the same plane at this bond (i.e. on the same side of the double bond), the unsaturated fatty acid is called a ***cis* fatty acid** (Figure **2.5.5**). The *cis* double bond causes a bend or a "kink" in the fatty acid. This bend prevents triglycerides from packing tightly on top of one another and results in a liquid triglyceride at room temperature, otherwise known as an **oil** (Figure **2.5.5**). Olive oil, corn oil, canola oil, and cod liver oil are examples of *cis* unsaturated fats. If the hydrogen atoms at the double bond are on two different planes (i.e. across the double bond), the fatty acid is called a ***trans* fatty acid**. Because of the position of these hydrogen atoms, the *trans* fatty acid is a long, straight molecule. A triglyceride made of trans fatty acids or saturated fatty acids tend to be solid at room temperature because these long, straight fatty acids can be densely packed on top of one another. A solid triglyceride is called a **fat**. Examples of animal fats are stearic acid and palmitic acid (common in meat) and the fat butyric acid found in butter. Mammals store fats in specialized cells called adipocytes, where globules of fat occupy most of the cell's volume.

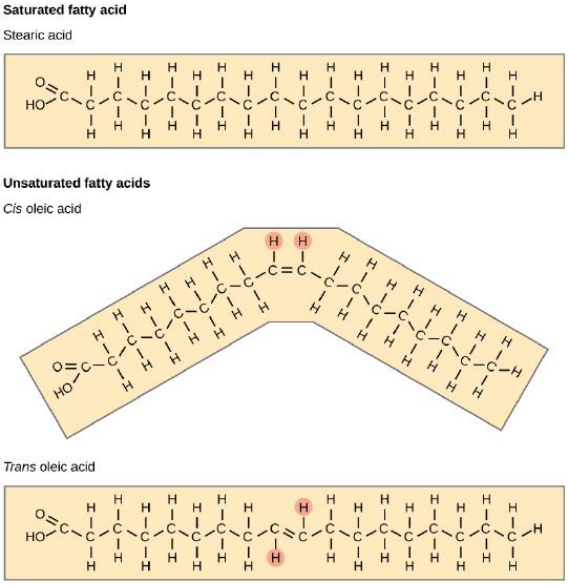


Figure **2.5.5**: Comparison of saturated and unsaturated fatty acids. The saturated fatty acid stearic acid has an 18-carbon chain with a carboxyl group (-COOH) at the left end. All bonds between the carbons are single bonds. Oleic acid is made from stearic acid through the creation of a carbon-carbon double bond between the 9th and 10th carbons. In cis-oleic acid, the hydrogen atoms are on the same side of the double bond causing a kink in the chain. In trans-oleic acid, the hydrogen atoms are on opposite side of the double bond and the fatty acid is linear. (Comparison of saturated and unsaturated fatty acids by Openstax; CC BY 4.0)

In the food industry, oils are **artificially hydrogenated** to increase their shelf-life and give them a semi-solid consistency that desirable for many processed foods. In hydrogenation, hydrogen gas is bubbled through polyunsaturated oils to solidify them. During this hydrogenation process, some double bonds of the *cis* conformation in the hydrocarbon chain may be converted to the *trans* conformation. Margarine, some types of peanut butter, and shortening are examples of artificially hydrogenated *trans* fats. Recent studies have shown that an increase in *trans* fats in the human diet may lead to an increase in levels of low-density lipoproteins (LDL), or "bad" cholesterol, which in turn may lead to plaque deposition in the arteries, resulting in heart disease. Many fast food restaurants have recently banned the use of *trans* fats, and food labels are required to display the trans fat content.

Like carbohydrates, fats (i.e. triglycerides) have received a lot of bad publicity. It is true that eating an excess of fried foods and other "fatty" foods leads to weight gain. However, fats do have important functions. Many vitamins are fat soluble, and fats serve as a long-term storage form of fatty acids: a source of energy. They also provide insulation for the body. Therefore, "healthy" fats in moderate amounts should be consumed on a regular basis.

Phospholipids

Phospholipids are modified triglycerides. Like triglycerides, they are composed of non-polar fatty acid chains attached to a glycerol backbone. However, instead of three fatty acids, the phospholipid has only two fatty acids, with the third carbon of the glycerol bound to a **phosphate group** (Figure **2.5.6**). This phosphate group is modified with a functional group that may be charged or polar in nature. The functional group determines the chemical and physical properties of the phospholipid and can range from

something as simple as a hydroxyl group (e.g. phosphatidic acid) to more complex compounds like carbon rings (e.g. phosphatidylinositol).

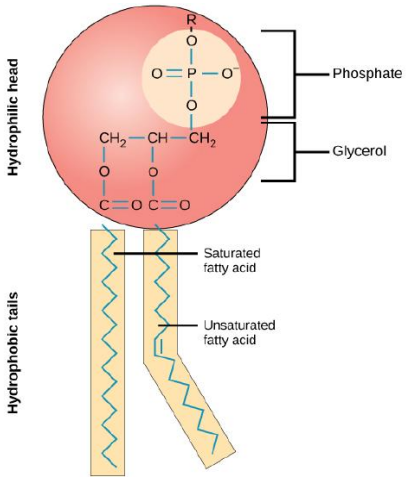


Figure **2.5.6**: The phospholipid. A phospholipid is a molecule with two fatty acids (i.e., the hydrophobic tails) and a modified phosphate group attached to a glycerol backbone. The phosphate group may be modified by the addition of charged or polar functional groups (indicated with an 'R'). The glycerol and phosphate group are called the hydrophilic head group. (Phospholipid structure by Openstax; CC BY 4.0)

The phospholipid is comprised of both hydrophobic and hydrophilic regions. The modified phosphate group bound to the glycerol backbone is often called the **polar head group** of the phospholipid and the two fatty acids are called the **hydrophobic tails**. The hydrophobic and hydrophilic nature of the phospholipid makes it an **amphipathic** molecule. It also means that when added to an aqueous environment, phospholipids will spontaneously arrange themselves into a **phospholipid bilayer,** with the hydrophobic fatty acids tails facing away from water (towards the inside of the bilayer) and the hydrophilic head groups facing out towards the aqueous environment (Figure **2.5.7**). In a cell, the phospholipid bilayer makes up the majority of the plasma membrane and they are responsible for its dynamic nature.

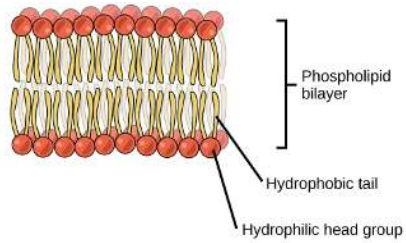


Figure **2.5.7**: The phospholipid bilayer. The phospholipids of the phospholipid bilayer orient themselves so that the hydrophilic head groups face the aqueous solution and the hydrophobic tails face inward. (Phospholipid bilayer by Openstax; CC BY 4.0)

Steroids

A **steroid** is a lipid with a fused ring structure. Although they do not resemble the other lipids, they are grouped with them because they are also hydrophobic and insoluble in water. All steroids have four linked carbon rings and are modified with specific function groups that determine their function (Figure **2.5.8**).

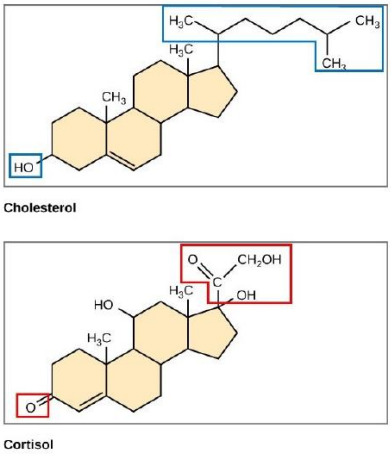


Figure 2.5.8: The steroid. Steroids such as cholesterol (top) and cortisol (bottom) are composed of four fused hydrocarbon rings modified through the addition of specific functional groups (indicated with the boxes). (Steroid structure by Openstax; CC BY 4.0; modified by Patricia Zuk)

The most common steroid is **cholesterol**. As with all steroids, cholesterol is made of four fused carbon rings, giving the molecule rigidity. A hydrocarbon chain fused to one ring makes the molecule mostly non-polar and a hydroxyl group at the other end makes the molecule slightly polar. As such, cholesterol is an amphipathic molecule. Cholesterol is mainly synthesized by animals in the cells of the liver or it can be ingested through food. Although cholesterol is often spoken of in negative terms by lay people, it is necessary for proper functioning of the body. For example, cholesterol is a component of the phospholipid bilayer and is the precursor to many steroid hormones such as testosterone, estradiol, cortisol, and aldosterone. It is also the precursor to Vitamin D and to bile salts, which help in the emulsification and absorption of fats.

Fatty Acids

Essential fatty acids are fatty acids that are not synthesized by an organism but must be obtained through the diet. There are two main types of essential fatty acids: **omega-3 and omega-6**. Omega-3 fatty acids (e.g. alpha-linoleic acid) are a type of polyunsaturated fat that have their first double bond between the third and fourth carbon from the end of the fatty acid chain (Figure 2.5.9). Examples of omega-3 fatty acids are alpha-linoleic acid (ALA) and docosahexaenoic acid (DHA). Omega-6 fatty acids are also a type of polyunsaturated fat. The omega-6 fatty acid has its first double bond at the sixth carbon from the end of the fatty acid chain.

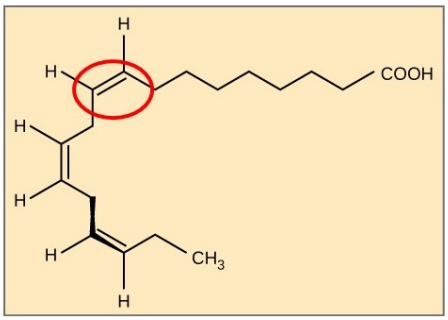


Figure 2.5.9: The omega-3 fatty acid. Omega-3 fatty acids have a double bond located at the third carbon from the end of the hydrocarbon chain (circled). (Omega-3 structure by Openstax; CC BY 4.0; modified by Patricia Zuk)

Salmon, trout, and tuna are good sources of omega-3 fatty acids. Research shows that omega-3 fatty acids are important in brain function and normal growth and development. They may also prevent heart disease, decrease inflammation, and reduce the risk of cancer.

Key Concepts

Lipids are a class of macromolecules that are nonpolar and hydrophobic in nature. Major types include triglycerides, phospholipids, fatty acids, and steroids.

Some major concepts to remember are:

- Fats and oils are a stored form of energy and are also known as triacylglycerols or triglycerides.
- Triglycerides are made up of three fatty acids bound to a glycerol.
- Fatty acids may be unsaturated or saturated, depending on the presence or absence of double bonds in the hydrocarbon chain. If only single bonds are present, they are known as saturated fatty acids. Unsaturated fatty acids may have one or more double bonds in the hydrocarbon chain.
- Phospholipids have a glycerol backbone to which two fatty acid chains and a phosphate-containing group are attached. Phospholipids form the bilayers that make up the plasma membrane.
- Steroids are made of four fused carbon rings modified through the addition of functional groups.
- Cholesterol is the most basic type of steroid
- Modifications to cholesterol result in the production of the steroid hormones (e.g. sex hormones, aldosterone, cortisol)
- Omega-3 and omega-6 fatty acids are known as essential fatty acids

Glossary

Amphipathic - a molecule that has both hydrophilic and hydrophobic regions

Cis - a term used in chemistry and biochemistry to describe the arrangement of atoms or functional groups in a molecule, such as an unsaturated fatty acid

Cis isomer - an isomer that has similar or identical functional groups positioned on the same side of a double bond

Cholesterol - a lipid made of fused carbon rings bound to unique functional groups; a precursor for steroid hormones and bile

Eicosanoids - a group of signaling molecules derived from fatty acids; include the prostaglandins and leukotrienes; involved in inflammation and immunity

Essential fatty acid - a fatty acid that must be obtained through the diet

Fat - a triglyceride that is solid at room temperature

Fatty acid - a hydrocarbon chain with a carboxyl (-COOH) group at one end; can be be saturated or unsaturated

Glycerol - a three-carbon molecule that serves as the backbone of triglycerides and phospholipids

Hydrogenation - a chemical process in which hydrogen is added to unsaturated fats or other organic compounds

Hydrophobic - a property of molecules that repel water; characteristic of the fatty acid tails in lipids

Lipid Bilayer - a double-layered structure formed by phospholipids in aqueous environments, making up the fundamental structure of cell membranes

Lipoprotein - a complex of lipids and proteins that transports fats through the bloodstream, including HDL (high-density lipoprotein) and LDL (low-density lipoprotein)

Oil - a triglyceride that is liquid at room temperature

Omega fatty acid - a type of polyunsaturated fatty acid that play essential roles in human health; categorized based on the position of the first double bond from the methyl (CH_3) end of the hydrocarbon chain

Phospholipid - a modified triglyceride made of two fatty acids "tails" and a polar "head group" attached to a glycerol backbone

Pigment - a lipid that absorbs specific wavelengths of light

Polar head group - the hydrophilic (water-attracting) part of an amphipathic molecule, such as a phospholipid

Polymer - a large molecule made up of repeating units called monomers

Saturated fatty acid - a fatty acid in which all carbons in the hydrocarbon chain are linked to each other through single bonds

Steroid - a lipid made of four fused carbon rings modified with specific functional groups

Trans - a term used in chemistry and biochemistry to describe the arrangement of atoms or functional groups in a molecule, such as an unsaturated fatty acid

Trans isomer - an isomer that has similar or identical functional groups positioned on the opposite side of a double bond

Trans fat - a fat containing artificially modified unsaturated fatty acids with a trans configuration; often found in processed foods and linked to health risks

Triglyceride - a lipid made of three fatty acids attached to a glycerol backbone

Unsaturated fatty acid - a fatty acid in which carbon-carbon double bonds can be found

Wax - a type of lipid that is highly water-resistant and protects various structures in plants, animals and microbes; made of long-chain fatty acids bound to long-chain alcohols rather than glycerol

Attribution

Figures taken from Open Stax Biology 3.3 Lipids

- Figure 2.5.1
- Figure 2.5.3
- Figure 2.5.4
- Figure 2.5.5
- Figure 2.5.6
- Figure 2.5.7
- Figure 2.5.8
- Figure 2.5.9

2.5: Lipids is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.6: Proteins

Biotech Focus

Figure 2.6.1: Rosalyn Yalow in her laboratory. (Rosalyn Yalow; CCO)

In 1977, Rosalyn Sussman Yalow received the Nobel prize in medicine for the development of the **radioimmunoassay (RIA)**. An RIA is a highly sensitive method that uses radioactive-labeled antibodies to measure very low concentration of specific proteins. For example, RIA is used to detect narcotic drugs, viral antigens, hormones, early stage of cancer. RIA can be used for the early diagnosis of diseases such as thyroid disorders, diabetes, and various cancers, enabling timely interventions and treatment plans.

If you would like to read more about Rosalyn Sussman Yalow and her research, you can click on the following link: Rosalyn Yalow and radioimmunoassay.

Introduction

Proteins are one of the most abundant organic molecules in living systems. In the adult human, proteins make up 12 to 18% of the body's mass. Each cell in a living system may contain thousands of proteins, each with a unique function. Their structures, like their functions, vary greatly. They are all, however, polymers of **amino acids**, arranged in a linear sequence that is determined by the genetic code. To learn how the genetic code specifies amino acid sequence, go to Chapter 3.4: Translation of the polypeptide.

Learning Objectives

By the end of this section, you will be able to:

- Describe the functions proteins perform in the cell and in tissues
- Discuss the relationship between amino acids and proteins
- Explain the four levels of protein organization
- Describe the ways in which protein shape and function are linked

Types and Functions of Proteins

There are a wide variety of proteins that serve a diverse set of cellular functions. Some of these are shown in Table 2.6.1.

Table 2.6.1: Protein Types and Functions

Type	Examples	Functions
Catalytic (e.g. enzymes)	Amylase, DNA polymerase, hexokinase	• Help in digestion of food by catabolizing nutrients into monomeric units • Catalyze metabolic reactions • Synthesize molecular compounds • Repair damage • Produce energy
Transport	Hemoglobin, albumin	• Carry substances throughout the organism
Structural	Actin, tubulin, keratin	• Form the structural framework of cells and tissues
Regulatory (e.g. hormones, neurotransmitters, growth factors, receptors)	Insulin, substance P, epidermal growth factor (EGF), EGF receptor	• Coordinate the activity of different organ systems • Detect signals and trigger cellular responses • Form cell signaling pathways
Immunological	Immunoglobulins, interferons	• Protect the organism from foreign pathogens and cancerous cells
Contractile	Actin, myosin	• Mediate muscle contraction
Storage	Legume storage proteins, egg white (albumin)	• Provide nourishment in early development

Enzymes, which are produced by living cells, are **catalysts** in biochemical reactions. A catalyst is a substance that speeds up a chemical reaction without being consumed by that reaction. Because they increase the rate of a reaction, enzymes are considered to be **biologic catalysts**. Each enzyme is specific for the **substrate** (a reactant that binds to an enzyme) that it acts on. Enzymes catabolize a wide variety of chemical reactions, including catabolic (i.e. decomposition), anabolic (i.e. synthesis), substitution, and redox reactions. An example of an enzyme is salivary amylase, which hydrolyzes its substrate amylose, a component of starch.

Hormones are chemical-signaling molecules, usually small proteins or steroids, secreted by endocrine cells, that act to control or regulate specific physiological processes, including growth, development, metabolism, and reproduction. For example, insulin is a protein hormone that helps to regulate the blood glucose level.

Hormones (and other signaling molecules) act by binding to proteins called **receptors**. Receptors are proteins found either on the surface of a cell or inside of a cell. The receptor binds to a specific molecule called a **ligand**. Once bound the receptor triggers a biological response inside of the cell. Examples of ligands include hormones, neurotransmitters, growth factors, and drugs.

Amino Acids are the Building Blocks of Proteins

The building block of a protein is the **amino acid** (Figure **2.6.2**). The name "amino acid" is derived from the fact that they contain an amine group and a carboxyl acid group in their basic structure. Each amino acid consists of a central carbon atom, also known as the **alpha (α) carbon**, bonded to the following:

1. a **carboxyl group (COOH)** that will donate protons when the amino acid is in an aqueous solution
2. an **amino group (NH2)** that will receive proteins from the environment
3. a **hydrogen atom**
4. a **"side chain" or "R group"** that distinguishes one amino acid from another. For each amino acid, the R group (or side chain) is different (Figure).

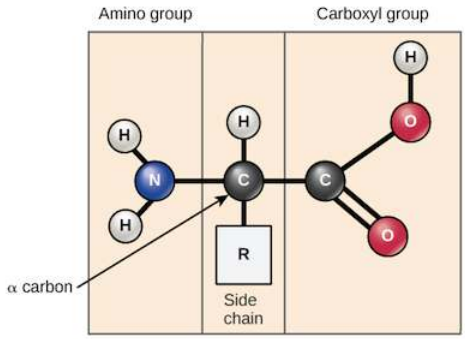


Figure **2.6.2**: The amino acid. An amino acid has an alpha carbon to which an amino group, a carboxyl group, a hydrogen, and a side chain are attached. The side chain varies for different amino acids, and is designated with an "R".

All proteins are made up using the same 20 types of amino acids arranged in a unique sequence according to the genetic code. When in an aqueous environment, the amino acid has a slightly positively charged end (the amine group) and a slightly negatively charged end (the carboxyl group). However, it is the R group that determines the overall physical and chemical characteristic of the amino acid.

Based on the R chain, amino acids are classified into the following groups (Figure **2.6.3**):

- **non-polar (hydrophobic)** - have side chains that are non-polar and uncharged; amino acids such as valine and leucine
- **polar (hydrophilic)** - have side chains that are polar and uncharged; amino acids such as serine, threonine, arginine, glutamine
- **electrically charged acidic** - have side chains that are positively charged at physiological pH; amino acids glutamic acid and aspartic acid
- **electrically charged basic** have side chains that are negatively charged at physiological pH; amino acids histidine, lysine, arginine

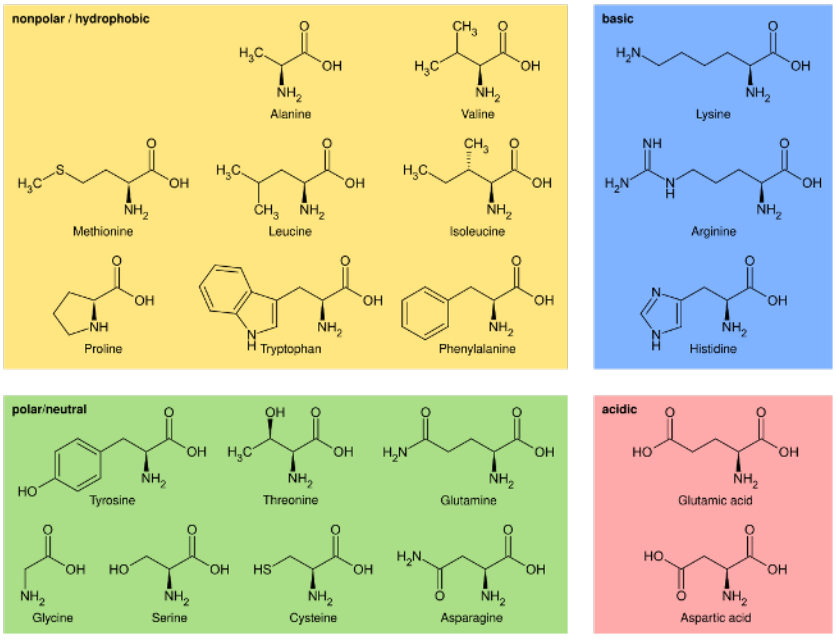


Figure **2.6.3**: The 20 amino acids found in proteins each have different R groups (side chain) that determine whether they are non-polar, polar, acidic, or basic. Nonpolar (or hydrophobic) amino acids include glycine, alanine, phenylalanine, and tyrosine. Polar (or hydrophilic) amino acids are uncharged (i.e., neutral) and include serine, threonine, and cysteine. The three positively-charged amino acids are lysine, arginine, and histidine. The negatively-charged amino acids are aspartic acid (i.e., aspartate) and glutamic acid (i.e., glutamate). (Amino acid types; by isk, CC0 1.0)

Amino acids can represented by a single upper case letter or a three-letter abbreviation. For example, valine is known by the letter V or the three-letter symbol val.

Individual amino acids are linked together through a covalent bond called a **peptide bond.** Formation of the peptide bond results in the production of a water molecule and is a **dehydration synthesis reaction**. To form a peptide bond, the hydroxyl from the carboxyl group of one amino acid and the hydrogen from the amino group of the incoming amino acid combine, forming a molecule of water (Figure **2.6.4**).

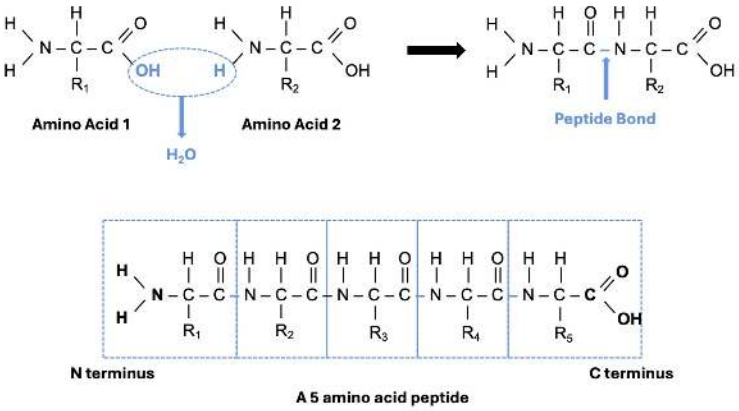


Figure **2.6.4**: Peptide bond formation. Top image: Peptide bond formation is a dehydration synthesis reaction. When the peptide bond forms, the carbon from the carboxyl group of the first amino acid becomes attached to the nitrogen from the amino group of the second amino acid. The OH from the carboxyl group and an H from the amino group form a molecule of water (dotted circle). Bottom image: a chain of 5 amino acids bound by peptide bonds form a short peptide. Since peptide bonds are formed between carboxyl and amino group, the peptide starts with an amino group, called N-terminus, and ends with a carboxyl group called C-terminus. (Peptide bond formation by Kareen Martin; CC BY 4.0)

The joining of two amino acids through a peptide bond produces a **dipeptide**. The joining of three amino acids produces a **tripeptide**. Joining from 4 to 9 amino acids results in a **peptide**. Joining more than 10 amino acids produces a **polypeptide**. A polypeptide can be relatively small (e.g. about 50 amino acids) or could be comprised of thousands of amino acids. . While the terms polypeptide and protein are sometimes used interchangeably, a polypeptide is technically a polymer of amino acids, whereas the term protein is used for a polypeptide or polypeptides that have been folded into a unique three-dimensional structure. In addition, after protein synthesis (i.e. translation), most proteins have been modified through **post-translational modifications** of the polypeptide chain. They may undergo cleavage, phosphorylation, or may require the addition of other chemical groups. Only after these modifications and folding is complete is the protein functional.

Protein Structure

The shape of a protein is critical to its function. For example, an enzyme binds a substrate at a site known as the active site. If the shape of the active site is altered because of changes in protein structure, the enzyme may be unable to bind to the substrate. Protein structure is described using four levels of conformation:

1. **primary** - the amino acid sequence of the polypeptide strand
2. **secondary** - results in regions of alpha-helical coils and beta-pleated sheets
3. **tertiary** - the 3D shape of the protein
4. **quaternary** - produced through the interaction between more than two polypeptide chains

Primary Structure

The **primary structure** of a protein is the specific order of amino acids found in the polypeptide. These amino acids are linked together via peptide bonds. Each polypeptide has a free amino group at one end. This end is called the **N terminus**, or the **amino terminus.** The other end of the polypeptide has a free carboxyl group, which is known as the **C terminus**, or the **carboxy terminus** (Figure **2.6.5**).

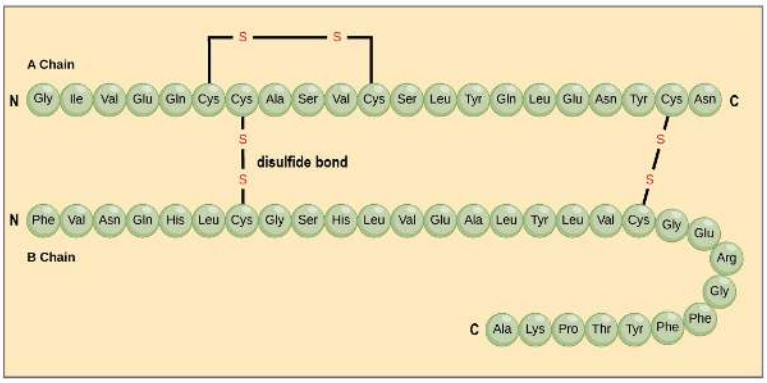


Figure **2.6.5**: The hormone insulin. Insulin is a protein hormone made of two peptide chains (A and B) linked by disulfide bonds (black lines). Each amino acid is indicated by its three-letter abbreviation. The A chain is 21 amino acids long with an N terminal amino acid (N) of glycine (Gly) and a C terminal amino acid (C) of asparagine (Asn). The B chain is 30 amino acids long and has the amino acid phenylalanine (Phe) as the N terminus amino acid and the amino acid alanine (Ala) as the C-terminus amino acid. (Insulin by Patricia Zuk, CC BY 4.0; modified from Primary structure of proteins by Openstax, CC BY-NC 4.0)

The unique sequence found in the primary structure is determined by the gene encoding the protein. A change in the gene's DNA sequence may lead to a change in the amino acid sequence of the protein. A change in just one amino acid in the primary structure can ultimately affect the protein's overall structure and function. Sickle cell anemia (see Figure **2.6.9** below) is a disease caused by a single amino acid change in the primary structure of a hemoglobin polypeptide.

Secondary Structure

Local folding of the polypeptide in certain regions gives rise to the **secondary structure** of the protein. The most common secondary structures in proteins are the **α-helix** and **β-pleated** sheet (Figure **2.6.6**). An α-helix coils like a spring and is produced when a hydrogen bond forms between the oxygen atom in the carbonyl group (C=O) of one amino acid and a hydrogen atom (H) in the amide (N=H) group of another amino acid that is four amino acids farther along the chain. Every helical turn in an α-helix has 3.6 amino acids. The R groups (the variant groups) of the polypeptide protrude out from the α-helix chain. In the β-pleated sheet, sections of the polypeptide lie top of one another together and form "pleats". These pleats are formed by hydrogen bonding between the carbonyl group in one section of the polypeptide and the amide groups of another section lying adjacent to it. The R groups extend above and below the folds of the pleat.

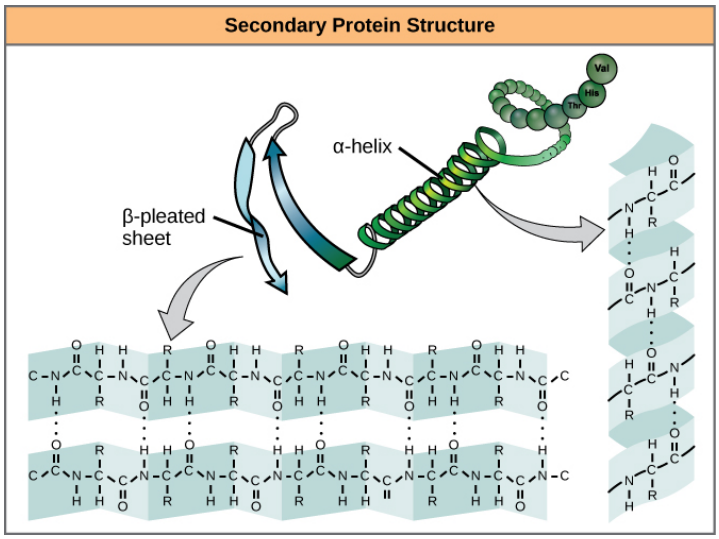


Figure **2.6.6**: Secondary protein structure. The α-helix and ß-pleated sheet make up the secondary structure of a protein. Both form because of hydrogen bonding between carbonyl (C=O) and amide (N=H) groups in the polypeptide backbone. Certain amino acids will form an α-helix, while others will form a ß-pleated sheet. In an α-helix, hydrogen bonding occurs between the carbonyl group of one amino acid and the amide group of the amino acid that occurs four residues later. In a beta-pleated sheet, hydrogen bonding occurs between these two functional groups that are found on different sections of the polypeptide.

Tertiary Structure

Proteins have different shapes; some proteins are globular whereas others are fibrous in nature. The folding of the protein into a specific 3-dimensional shape produces its **tertiary structure**. Tertiary structure is primarily due to *interactions between the R groups of the amino acids* that make up the protein.

The R group interactions that contribute to tertiary structure include (Figure **2.6.7**):

1. **hydrogen bonding**
2. **ionic bonding** - form between basic and acidic R groups.
3. **hydrophobic interactions** - occur between non-polar, hydrophobic R groups; hydrophobic interactions result in the positioning of hydrophobic amino acids in the interior of the protein, leaving hydrophilic amino acids on the outside to interact with surrounding water molecules.
4. **Disulfide bonds** - occur between the sulfur atoms of cysteine amino acids in the presence of oxygen; stabilizes the 3D structure of the protein

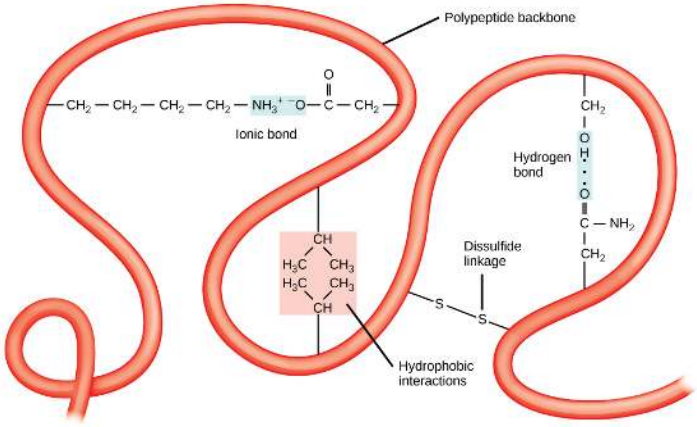


Figure **2.6.7**: Protein tertiary structure. The tertiary structure of a protein is determined by a variety of chemical interactions that occur between the R groups of specific amino acids. These interactions include hydrophobic interactions, ionic bonding, hydrogen bonding and disulfide linkages.

All of these interactions combined together determine the final tertiary structure (i.e. the three-dimensional shape) of the protein. Protein shape is critical to its function. When a protein loses its tertiary structure, it may no longer be functional. Changes in temperature, pH, and exposure to chemicals may lead to permanent changes in the shape of the protein, leading to loss of function. The loss of "natural" shape is known as **denaturation.**

Quaternary Structure

Some proteins are formed from several polypeptides, also known as **subunits**, and the interaction of these subunits is the **quaternary structure** of the protein. Weak interactions between the subunits, such as hydrogen bonds, help to stabilize the overall structure.

The four levels of protein structure (primary, secondary, tertiary, and quaternary) are illustrated below in Figure **2.6.8**.

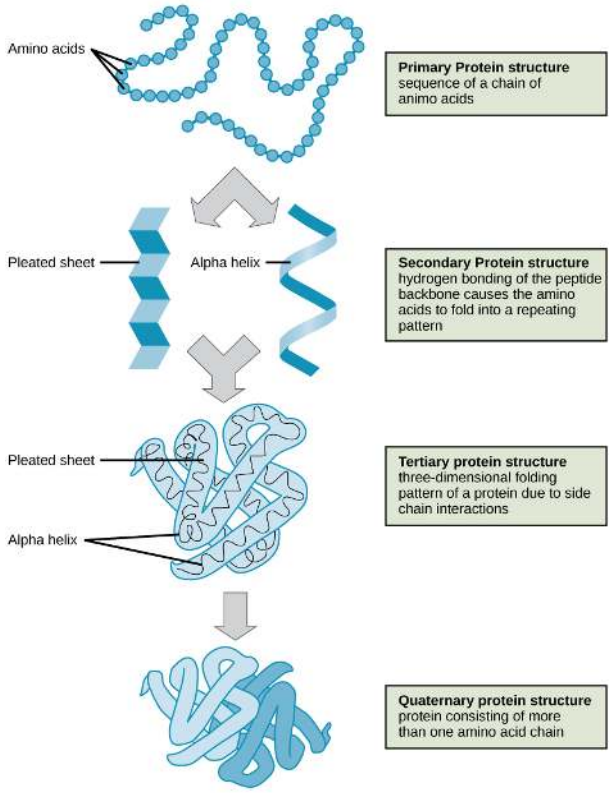


Figure **2.6.8**: The four levels of protein structure. The primary structure is the amino acid sequence. Secondary structure is a regular folding pattern due to hydrogen bonding. Two types of secondary structure are shown: a ß pleated sheet, which is flat with regular ripples, and an α-helix, which coils like a spring. Tertiary structure is the three-dimensional folding pattern of the protein due to interactions between amino acid side chains. Quaternary structure is the interaction of two or more polypeptide chains.

Concept in Action

Video: Protein Structure and Function

Protein Structure and Disease: Sickle Cell Anemia

A functional protein requires that each of its structural levels is correct. Changes to one level of structure can impact another. In sickle cell anemia, the **hemoglobin ß chain** has a single amino acid substitution, causing a change in protein structure and function and leading the disease known as **sickle cell anemia**. **Hemoglobin**, a transport protein that carries oxygen in the blood, is comprised of four polypeptide chains: two α chains and two ß chains. In sickle cell, a genetic mutation changes the hydrophobic amino acid, valine, in the primary structure of the ß chain to the charged and acidic amino acid, glutamic acid (Figure **2.6.9**). This change in the primary structure results in changes to remaining structural levels. The tertiary structure of the ß chain subunits are altered and this affects how these subunit assemble with their α hemoglobin partners. The abnormal quaternary structure results in hemoglobin with a lower oxygen-carrying capacity.

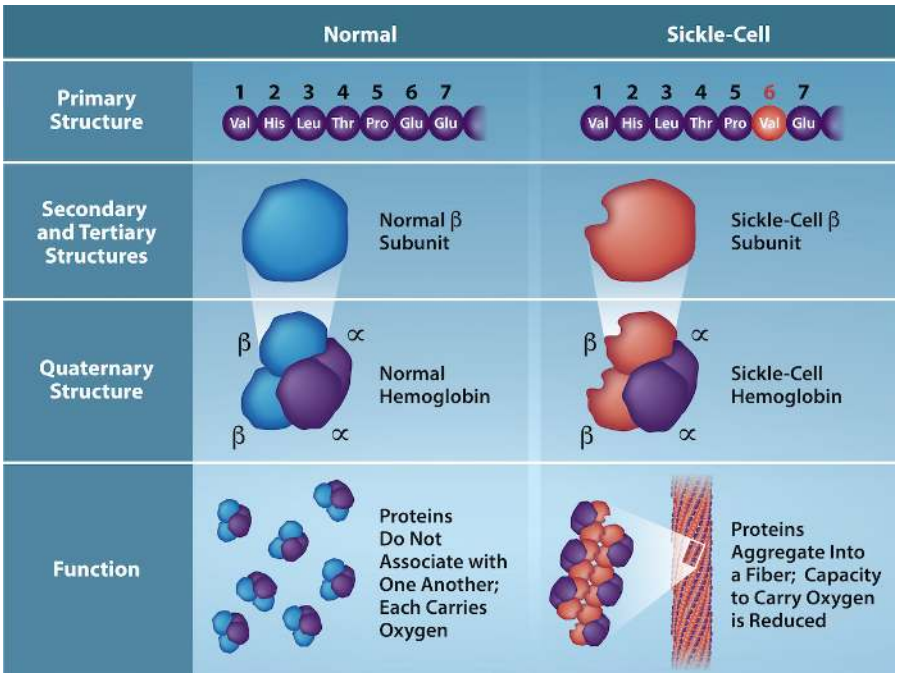


Figure **2.6.9**: Hemoglobin structure and sickle-cell anemia. The ß chain of hemoglobin is 147 residues in length, yet a single amino acid substitution leads to sickle cell anemia. In the normal ß hemoglobin polypeptide, the amino acid at position six is glutamate. In sickle cell, this glutamate is replaced by a valine. This produces abnormal secondary and tertiary structures. When the abnormal ß hemoglobin combines with its α hemoglobin partners to produce the resulting quaternary structure is altered. The abnormal hemoglobin proteins aggregate into fibers that causes the red blood cells to be rigid and have a crescent shape. These red blood cells tend to clog capillaries and decrease the flow of blood through tissues. (Hemoglobin and Sickle Cell by Kareen Martin, CC BY 4.0; adapted from Sickle Cell Anemia by BruceBlaus, CC BY-SA 4.0)

Furthermore, once the oxygen is offloaded to the tissues, the hemoglobin molecules aggregate with one another to form long fibers. This results in the distortion of red blood cells, which become stiffer and assume a crescent or “sickle” shape, clogging arteries

(Figure 2.6.10). This can lead to myriad serious health problems such as breathlessness, dizziness, headaches, and abdominal pain for those affected by this disease.

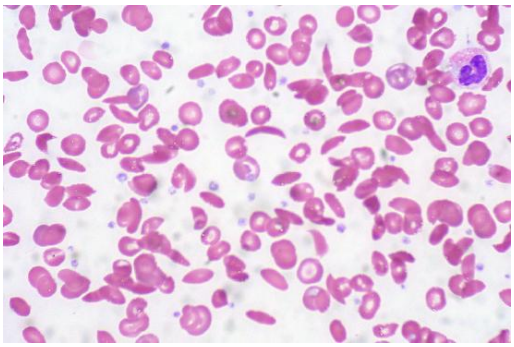


Figure 2.6.10: In this blood smear, visualized at 535x magnification using bright field microscopy, sickle cells are crescent shaped, while normal cells are disc-shaped. (Sickle Cell Anemia - Peripheral blood smear by Ed Uthman, CC BY 2.0)

Denaturation and Protein Folding

Each protein has its own unique shape that is produced and maintained by chemical interactions. If the protein is subject to changes in temperature, pH, or exposure to chemicals, these interactions may be disrupted, causing a change in protein structure and shape. Disruptions in secondary to quaternary structure may result, with the polypeptide maintaining only its primary structure. This loss of "natural structure" is called **denaturation**. Denaturation is often reversible because the primary structure of the polypeptide is conserved. If the denaturing agent is removed, the protein can resume its additional structural levels and function. However, sometimes denaturation is irreversible, leading to a permanent loss of function. One example of irreversible protein denaturation is when an egg is fried. The albumin protein in the liquid egg white is denatured when placed in a hot pan. Not all proteins are denatured at high temperatures; for instance, bacteria that survive in hot springs have proteins that function at temperatures close to boiling. The stomach is also very acidic, has a low pH, and denatures proteins as part of the digestion process; however, the digestive enzymes of the stomach retain their activity under these conditions.

Protein folding is critical to its function. It was originally thought that the proteins themselves were responsible for the folding process. Only recently was it found that often they receive assistance in the folding process from protein helpers known as **chaperones** (or chaperonins) that associate with the target protein during the folding process. They act by preventing aggregation of polypeptides that make up the complete protein structure, and they disassociate from the protein once the target protein is folded.

Key Concepts

Proteins are a class of macromolecules that perform a diverse range of functions for the cell. They help in metabolism by providing structural support and by acting as enzymes, carriers, or hormones.

Some important concepts to remember:

- The building blocks of proteins (monomers) are amino acids.
- Each amino acid has a central carbon that is linked to an amino group, a carboxyl group, a hydrogen atom, and an R group or side chain.
- There are 20 commonly occurring amino acids, each of which differs in the R group.
- Each amino acid is linked to its neighbors by a peptide bond.
- A long chain of amino acids is known as a polypeptide.
- Proteins are organized at four levels: primary, secondary, tertiary, and (optional) quaternary.
    1. The primary structure is the unique sequence of amino acids.
    2. The local folding of the polypeptide to form structures such as the α helix and β-pleated sheet constitutes the secondary structure.
    3. The overall three-dimensional structure is the tertiary structure.
    4. When two or more polypeptides combine to form the complete protein structure, the configuration is known as the quaternary structure of a protein.

- Protein shape and function are intricately linked; any change in shape caused by changes in temperature or pH may lead to protein denaturation and a loss in function.

Glossary

Alpha helix (α helix) - a common secondary structure of proteins; characterized by a coiled, helical shape; stabilized by hydrogen bonds

Amino acid - the building blocks of proteins; consist of a central carbon, an amino group, a carboxyl group, and a side chain (R group)

Beta-pleated sheet (ß-pleated sheet) - a secondary structure in proteins where sections of the polypeptide lie parallel or antiparallel to one another; held together by hydrogen bonds

Catalyst - a compound that speeds up a chemical reaction without being consumed by the reaction

Chaperone proteins - proteins that assist in the proper folding of other proteins, preventing aggregation or mis-folding

Denaturation - the process by which a protein loses its native shape due to external stress, such as heat, pH changes, or chemicals; often results in loss of function

Disulfide Bond - a strong covalent bond formed between sulfur atoms in cysteine residues, stabilizing protein structure

Enzyme - a protein that acts as a biological catalyst to speed up chemical reactions without being consumed.

Essential amino acids - amino acids that must be obtained from the diet because the body cannot synthesize them

Hydrophobic interactions - interactions between R groups of amino acids that place non-polar amino acids in the interior of a protein; one of the interactions that result in a protein's tertiary structure

Peptide bond - a covalent bond linking amino acids together in a polypeptide chain; formed through dehydration synthesis

Polymer - a large molecule made up of repeating units called monomers

Polypeptide - a long chain of amino acids linked by peptide bonds; can be processed to form a protein

Primary structure - the linear sequence of amino acids in a polypeptide chain

Protein - a large, complex molecule made up of several amino acids joined together through peptide bonds

Protein folding - the process by which a polypeptide folds into its functional three-dimensional structure; produces the tertiary structure of a protein

Quaternary structure - the level of protein structure formed by the interaction of multiple polypeptide subunits

Secondary structure - the local folding of a polypeptide into structures such as alpha helices and beta sheets; stabilized by hydrogen bonds

Tertiary structure - the overall three-dimensional shape of a polypeptide, determined by interactions between amino acid side chains

Transport protein - a protein that helps move substances across membranes or within the bloodstream, such as hemoglobin

Attribution

Figures taken from OpenStax 3.4 Proteins

- Figure **2.6.2**
- Figure **2.6.6**
- Figure **2.6.7**
- Figure **2.6.8**

2.6: Proteins is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

2.7: Nucleic Acids

Biotech Focus

Figure **2.7.1**: Mary Maynard Daly. (CCO)

Marie Maynard Daly's early research provided key insights into how DNA is structured and regulated within the cell nucleus. She particularly identified the four nucleic acid bases of DNA (adenine, guanine, thymine and cytosine) leading the way to understanding the structure of DNA. She continued to make significant contributions to science by identifying histones, the proteins around which DNA is wrapped within cells and by researching the relationship between cholesterol and heart disease among others.

If you would like to know more, read: Marie Maynard Daly: A National Historic Chemical Landmark

Introduction

Nucleic acids are the most important macromolecules for the continuity of life. They carry the genetic blueprint of a cell and carry instructions for the functioning of the cell. The two main types of nucleic acids are **deoxyribonucleic acid (DNA)** and **ribonucleic acid (RNA)**.

Learning Objectives

By the end of this section, you will be able to:

- Describe the structure of nucleic acids and define the two types of nucleic acids
- Explain the structure and role of DNA
- Explain the structure and roles of RNA

DNA and RNA are Nucleic Acids

DNA is the genetic material found in all living organisms, ranging from single-celled bacteria to multi-cellular mammals. In eukaryotes, the majority of DNA is found in the nucleus, with smaller amounts found in the organelles, such as the chloroplasts, and mitochondria. In prokaryotes, the DNA is not enclosed in a membranous envelope but is found in a section of the cytoplasm called the **nucleoid**. More about the structure and function of DNA can be found in Chapter 3.1 DNA Structure.

The other type of nucleic acid, **RNA**, plays critical roles in the gene expression and its regulation. In eukaryotic cells, because the DNA molecules never leave the nucleus, an intermediary nucleic acid is needed to communicate with the rest of the cell. This intermediary is RNA. There are several kinds of RNA, with more being discovered. The most studied RNA types are mRNA, tRNA, and rRNA - all of which play key roles in protein synthesis. For more about the the types of RNA and their functions, go to Chapter 3.3 Transcription of RNA.

Nucleotides are the Building Blocks of DNA and RNA

DNA and RNA are polymers known as **polynucleotides.** The building blocks of DNA and RNA are monomers called **nucleotides**.

Each nucleotide is made up of three components (Figure **2.7.2**):

1. **a nitrogenous base**
2. **a pentose (five-carbon) sugar**

3. **a phosphate group**

To build a nucleotide, the pentose sugar is linked to a specific nitrogenous base at its first carbon and to one or more phosphate groups at its fifth carbon. The base and sugar linked together are called a **nucleoside**. Adding the phosphate group(s) creates a nucleotide.

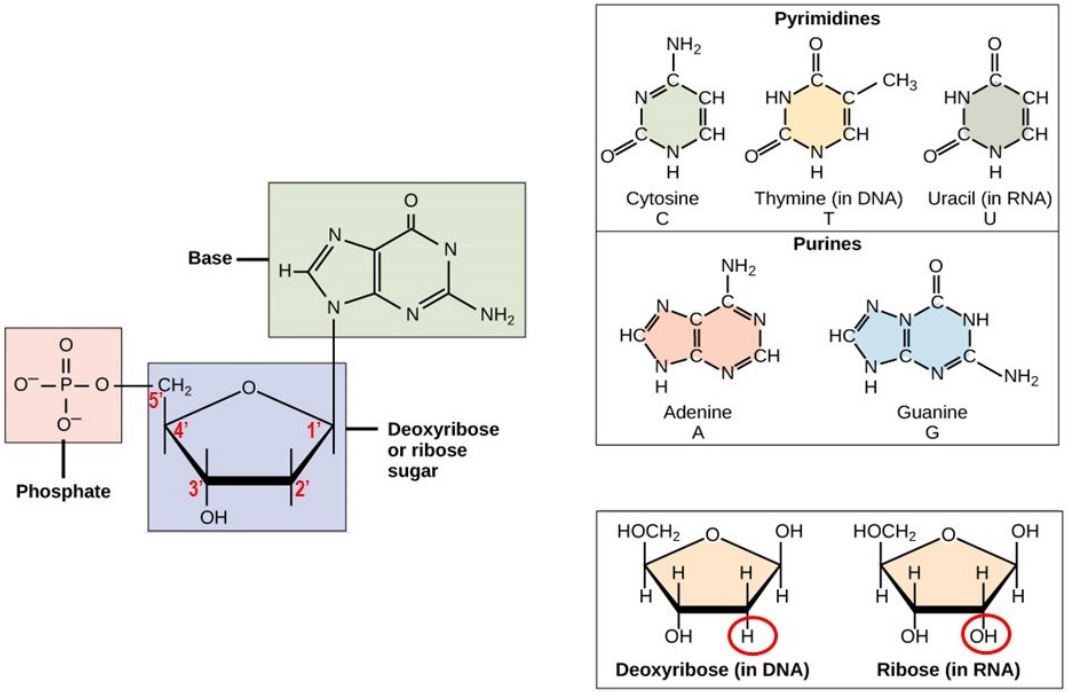


Figure **2.7.2**: Nucleotide structure. Left image: a nucleotide is made up a nitrogenous base (green box), a pentose sugar (blue box), and one or more phosphate groups (pink box). Carbon residues in the pentose are numbered clockwise from the oxygen as 1 "prime" (1′) through 5 "prime" (5′) . The base attaches to the 1′ carbon of the pentose sugar, and the phosphate is attached to the 5′ carbon. Two types of pentose sugars are found in nucleotides, deoxyribose (DNA) and ribose (RNA). Top right image: the nitrogenous bases are classified as either purines and pyrimidines. The purine bases, adenine and guanine and the pyrimidine base, cytosine, are found in DNA and RNA. The pyrimidine base thymine is found only in DNA while the pyrimidine, uracil, is only found in RNA. Bottom right image: deoxyribose is similar in structure to ribose, but it has an H instead of an OH at the 2′ position. (Nucleotide Structure by Patricia Zuk, CC BY 4.0; modified from Molecular structure of a nucleotide by Openstax, CC BY-NC 4.0)

The nitrogenous bases are organic molecules containing carbon and nitrogen. Because of the presence of an amino group with the potential of binding an extra hydrogen, the nitrogenous base is capable of decreasing the hydrogen ion concentration in its environment, thus acting as a base. The nitrogenous bases of nucleic acids are **adenine (A), guanine (G) cytosine (C), thymine (T), and uracil**. These nitrogenous bases are classified as either **purines** or **pyrimidines**. The primary structure of a purine (i.e. adenine and guanine) is two carbon-nitrogen rings, while the pyrimidine (cytosine, thymine, uracil) has a single carbon-nitrogen ring (Figure **2.7.2**). The nitrogenous base is specific to the nucleic acid. DNA contains A, T, G, and C, whereas RNA contains A, U, G, and C.

The pentose sugar in DNA is called **deoxyribose**, and in RNA, the sugar is **ribose**. The carbon atoms of the sugar are numbered clockwise from the oxygen within the ring. They are numbered from 1′ to 5′ (e.g. 1′ is read as “one prime”). The use of the term "prime" to number the carbons in the pentose sugar is meant to distinguish between these carbons and those found in the rings of the nitrogenous base. While the overall structure of deoxyribose and ribose are the same, there is an important difference - *deoxyribose lacks an oxygen at the 2' carbon.*

DNA and RNA nucleotides are named based on the nitrogenous base, the type of sugar and the number of phosphate groups. For example, *adenosine triphosphate (ATP)* is an RNA nucleotide with an adenine base and three phosphate groups attached to the ribose sugar. The DNA nucleotide counterpart would be *deoxynucleotide triphosphate (dATP)*. If these nucleotides have only one

phosphate group bound to the pentose sugar, they would be named *adenosine monophosphate (AMP)* and deoxynucleotide monophosphate (dAMP), respectively. Table **2.7.1** below summarizes the nomenclature for DNA and RNA nucleotides.

Table **2.7.1**: Nucleotide Nomenclature

Nucleoside Name	Number of phosphates	Abbreviation	Name
adenosine/deoxyadenosine	1 2 3	AMP/dAMP ADP/dADP ATP/dATP	adenosine monophosphate/deoxyadenosine monophosphate adenosine diphosphate/deoxyadenosine diphosphate adenosine triphosphate/deoxyadenosine diphosphate
guanosine/deoxyguanosine	1 2 3	GMP/dGMP GDP/dGDP GTP/dGTP	guanosine monophosphate/deoxyguanosine monophosphate guanosine diphosphate/deoxyguanosine diphosphate guanosine triphosphate/deoxyguanosine triphosphate
cytidine/deoxycytidine	1 2 3	CMP/dCMP CDP/dCDP CTP/dCTP	cytidine monophosphate/deoxycytidine monophosphate cytidine diphosphate/deoxycytidine diphosphate cytidine triphosphate/deoxycytidine triphosphate
deoxythymidine (found in DNA only)	1 2 3	dTMP dTDP dTTP	deoxythymidine monophosphate deoxythymidine diphosphate deoxythymidine triphosphate
uridine (found in RNA only)	1 2 3	UMP UDP UTP	uridine monophosphate uridine diphosphate uridine triphosphate

Phosphodiester Bonds Join Nucleotides

Nucleotides join to one another through **phosphodiester bonds** (Figure **2.7.3**). In phosphodiester bond formation, the hydroxyl on the 3' carbon of one nucleotide forms a bond with the phosphate group closest to the pentose sugar in another nucleotide. When the diphosphate or triphosphate forms of the nucleotides are used in this reaction, water is not released and it is not considered a dehydration synthesis reaction. However, if a monophosphate nucleotide is used, water is removed through dehydration synthesis. When forming DNA and RNA, the phosphodiester bond forms in a specific direction. Specifically, the bond forms between the phosphate group(s) of an incoming nucleotide and the 3' hydroxyl of the existing polynucleotide chain. Because of this, one end of a nucleic acid has an exposed phosphate group and is named the "5' end" while the other end has a 3' hydroxyl and is called the "3' end". The phosphodiester bond is said to form *"in the 5' to 3' direction"*.

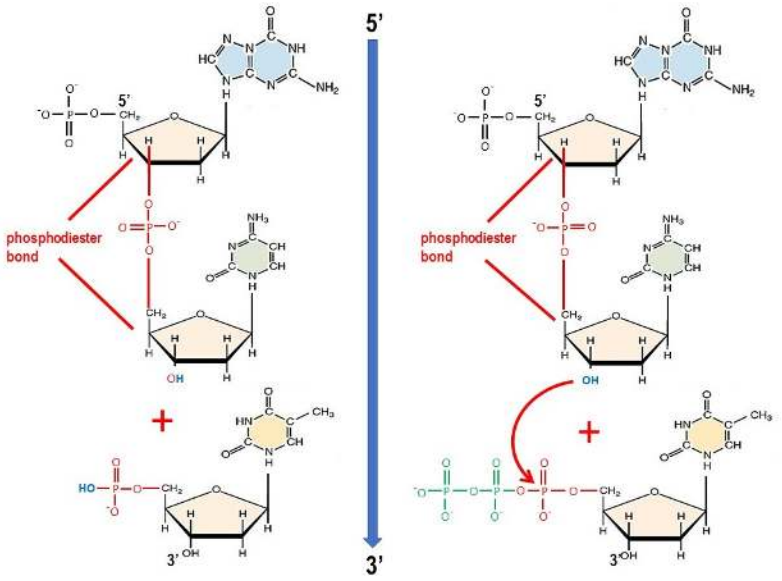


Figure 2.7.3: The phosphodiester bond forms in a specific direction. Left image: the addition of the monophosphate form of the nucleotide (bottom) to a nucleotide chain (top) via a phosphodiester bond. Bond formation involves the removal of a water molecule (atoms shown in blue). Right image: the addition of the triphosphate form (bottom) to the nucleotide chain (top). The phosphodiester bond forms as a result of a "nucleophilic attack" by the 3' OH group (blue) on the first phosphate group of the incoming nucleotide. This attack removes the remaining two phosphate groups (green). The atoms involved in the phosphodiester bond are shown in red. The direction of phosphodiester bond formation from 5' to 3' is indicated with an arrow. (Phosphodiester bond by Patricia Zuk, CC BY 4.0)

DNA is Double-Helix Structure

DNA exists as a **double-helix** (Figure 2.7.4), consisting of two long strands of DNA polynucleotides that twist like a "spiral staircase". In the DNA helix, the sugars and phosphates are found on the outside of the helix, not unlike the rails of the spiral staircase. The nitrogenous bases project to the interior of the helix, like the steps. The two polynucleotide strands of the helix run in opposite directions, or **anti-parallel** to one another, meaning that the 5′ end of one strand will be next to the 3′ end of the other strand. The bases from each nucleotide chain pair up with one another and are bound to each other by hydrogen bonds. This base pairing is restricted in the DNA helix and is known as **complementary base pairing**. In this pairing scheme, *purines pair with pyrimidines*. Specifically, adenine (A, the purine) pairs with thymine (T, the pyrimidine) through the formation of two hydrogen bonds and guanine (G, the purine) pairs with cytosine (C, the pyrimidine) through the formation of three hydrogen bonds. What results is a DNA helix in which the two DNA strands are complementary to each other. This complementary base pairing has implications for cellular processes like DNA replication. During DNA replication, each of the DNA strands are "copied" to produce two new DNA helices. However, the term copy is misleading, as a identical copy is not made rather a complementary copy is. For example, if the sequence of one strand is AATTGGCC, the complementary strand would have the sequence TTAACCGG. For more information about DNA replication, read Chapter 3.2 Replication of DNA.

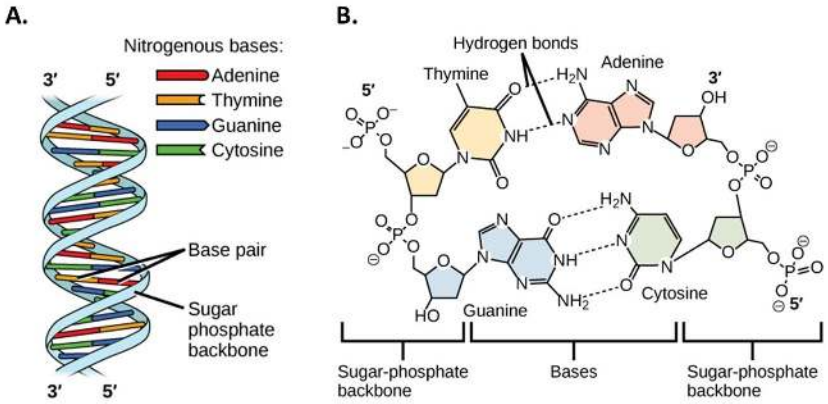


Figure **2.7.4**: The DNA Helix. (A) Two phosphate-sugar backbones (curved blue lines) run on the outside of the DNA helix with the bases (adenine, thymine, guanine, cytosine) projecting to the inside. The two DNA strands run "anti-parallel" to one another. (B) Each base from one strand interacts with a "complementary" base from the opposing strand via hydrogen bonding (dotted black lines); adenine pairs with thymine and cytosine pairs with guanine. (DNA Helix by Jerome Walker and Denis Myts, Concepts of Biology; CC BY-SA 4.0)

Comparison between DNA and RNA

Like DNA, RNA is a polynucleotide chain made up of nucleotides joined by phosphodiester bonds that form in the 5' to 3' direction. However, RNA does differ from DNA in several aspects. Table **2.7.2** below outlines these differences.

Table **2.7.2**: Differences between DNA and RNA

	DNA	RNA
Function	Stores and carries genetic information	Transfers genetic information; involved in protein synthesis
Types	One type	Several types
Location	Mostly in the nucleus; some in organelles	Nucleus and cytoplasm
Structure	Double-stranded; forms a helix	Usually single-stranded
Pentose Sugar	Deoxyribose (lacks an oxygen at the 2' carbon)	Ribose (has an oxygen at the 2' carbon
Pyrimidine bases	Cytosine, thymine	Cytosine, uracil
Purine bases	Adenine, guanine	Adenine, guanine

Key Concepts

DNA and RNA comprise the two types of nucleic acids found in cells. DNA provides the code for a cell's activities, while RNA is involved in translating that code into proteins that carry out these activities.

Some important concepts to remember:

- Both DNA and RNA are made from nucleotides
- Nucleotides contain a five-carbon sugar backbone, one to three phosphate groups, and a nitrogenous base.
- Nucleotides are joined to one another by a phosphodiester bond.
- Phosphodiester bonds form in the 5' to 3' direction.
- The bases of nucleic acids are adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U).
- The bases of DNA are A, G, C, and T; the bases of RNA are A, G, C, U.
- Bases are classified as purines (A, G) or pyrimidines (C, T, U)
- The nitrogen bases A pairs with its complementary base T (or U in RNA); the base C pairs with its complement G
- DNA is found as a double-stranded helix, made of two "anti-parallel" DNA strands

- Most RNA is single-stranded

Glossary

Adenine (A) - a nitrogenous base found in DNA and RNA; pairs with thymine (T) in DNA and uracil (U) in RNA.

Anti-parallel - the orientation of the two strands of DNA in opposite directions, one running 5' to 3' and the other 3' to 5'.

Complementary base pairing - the specific hydrogen bonding between complementary nitrogenous bases in nucleic acids (A-T/U, C-G).

Cytosine (C) - a nitrogenous base found in DNA and RNA; pairs with guanine (G).

Deoxyribonucleic Acid (DNA) - a double-stranded nucleic acid that carries genetic information and directs cellular functions.

Deoxyribose - the five-carbon sugar found in DNA; lacks an oxygen atom at the 2' carbon.

Double helix - the spiral-shaped structure of DNA, consisting of two complementary strands.

Guanine (G) - a nitrogenous base found in DNA and RNA; pairs with cytosine (C).

Hydrogen bond - a weak bond found between the positively-charged hydrogen of one molecule and a negatively-charge atom of another molecule (usually O or N); holds complementary nitrogenous bases together in DNA and RNA.

Nitrogenous base - an organic molecule found bound to the pentose sugar of a nucleic acid.

Nucleic Acid - a macromolecule composed of nucleotides, essential for storing and transmitting genetic information (DNA and RNA).

Nucleotide - the basic building block of nucleic acids, consisting of a nitrogenous base, a sugar, and a phosphate group.

Pentose sugar - a 5-carbon sugar.

Phosphate group - a functional group that links nucleotides together in the sugar-phosphate backbone of nucleic acids.

Polymer - a large molecule made up of repeating units called monomers

Purines - a category of nitrogenous bases with a double-ring structure; adenine (A) and guanine (G).

Pyrimidines - a category of nitrogenous bases with a single-ring structure; cytosine (C), thymine (T), and uracil (U).

Ribonucleic Acid (RNA) - a single-stranded nucleic acid involved in protein synthesis and gene expression.

Ribose - the five-carbon sugar found in RNA; has an oxygen atom at the 2' carbon.

Sugar-Phosphate backbone - the repeating structure of sugar and phosphate groups that forms the framework of DNA and RNA strands.

Thymine (T) - a nitrogenous base found in DNA; pairs with adenine (A); replaced by uracil (U) in RNA.

Uracil (U) - a nitrogenous base found only in RNA; pairs with adenine (A) instead of thymine.

2.7: Nucleic Acids is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

3: Molecular Biology Fundamentals

3.1: DNA Structure

3.2: Replication of DNA

3.3: Transcription of RNA

3.4: Translation of the polypeptide

3.5: Mutations and DNA Repair

3: Molecular Biology Fundamentals is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

3.1: DNA Structure

Biotech Focus

Viewing the three dimensional structures of molecules, like DNA, remained a challenge in the early part of the twentieth century. Following the discovery of X-rays in 1895, researchers soon proposed that the atomic and molecular structure of a compound, such as a crystal, could be determined by beaming X-rays at them and observing their diffraction patterns. From this work, the field of **X-ray crystallography** was born. X-ray crystallography has been fundamental in numerous scientific breakthroughs, including determining the size of atoms, the lengths and types of chemical bonds, and revealing the structure and function of biological molecules, such as proteins and nucleic acids. Dorothy Crowfoot Hodgkin used X-ray crystallography to determine the structures of cholesterol (1937), penicillin (1946), and vitamin B12 (1956), for which she was awarded the Nobel Prize in Chemistry in 1964. Rosalind Franklin, the British X-ray crystallographer, was one of the first to produce high quality X-ray diffraction images of DNA. From her work, we now know the intricacies of the DNA helix. Today, over 130,000 X-ray crystal structures of proteins, nucleic acids, and other biological molecules have been determined.

Introduction

The first recorded discovery of DNA came in 1869, when an unknown substance found in the pus of wounded soldiers was described by the Swiss physician Friedrich Miescher. As it was found in nuclei of cells, he named this substance "nuclein". In 1878, Albrecht Kossel isolated the nucleic acid component of nuclein. In 1909, Phoebus Levene identified the base, sugar and phosphate components of a nucleotide and would later suggest that DNA consists of a string of four nucleotides (A, C, T, G) linked together through the phosphate groups. In 1910, Albrecht Kossel received the Nobel Prize in Physiology or Medicine for his discovery of the four bases of DNA, as well as of uracil in RNA. In 1928, Frederick Griffith showed that a molecule obtained from pathogenic *Streptococcus pneumonia* strains could be introduced into healthy *S. pneumonia* cells, "transforming" them into pathogenic cells. While we now know that Griffith introduced DNA into these bacteria, he did not know the chemical identity of this transforming molecule. It wasn't until the 1930s, that O. Avery, C. MacLeod, and M. McCarty, using improved purification techniques, showed that the transforming molecule was, in fact, DNA. In spite of these results, DNA was not readily accepted as the genetic material. Confirmation of this would come from the experiments of Alfred Hershey and Martha Chase, who determined that DNA could be passed from bacteriophage to *E.coli* cells. For his contributions to determining DNA as the “stuff of genes”, Hershey shared the 1969 Nobel Prize in Physiology or Medicine with Max Delbruck and Salvador E. Luria.

Learning Objectives

Understanding how DNA is used in biotechnology requires that you understand the structure of this molecule. In this this section, you will be able to:

- Discuss who "discovered the DNA helix"
- Explain the structure of a nucleotide
- Describe how DNA is structured
- Describe the forms of DNA is found in cells: chromatin, chromosomes and plasmids
- Compare and contrast prokaryotic and eukaryotic DNA
- Explain DNA packaging in eukaryotes

The History of the DNA Helix

The history of determining the structure of the DNA helix is rich and long. In 1937, William Astbury used **X-ray crystallography**, a method for investigating molecular structure by observing the patterns formed by X-rays shot through a crystal of a substance, to show that DNA had a regular structure. In 1952, Rosalind Franklin, a researcher in the lab of Maurice Wilkins at King's College, together with her research assistant, Raymond Gosling, produced one of the most well-known X-ray diffraction images of the DNA helix, known as "Photo 51" (Figure **3.1.1**). This image helped solidify the theory that DNA was a double helix. At the same time, James Watson and Francis Crick, working together at the University of Cambridge, had started to build a model of the DNA helix. Using data from other research groups, including that of Rosalind Franklin and Erwin Chargaff, they eventually pieced together the

puzzle of the DNA helix and published their seminal work in the journal Nature in 1953. In 1962, James Watson, Francis Crick, and Maurice Wilkins were awarded the Nobel Prize in Medicine for their work on DNA structure. Because Nobel Prizes are not awarded posthumously, Rosalind Franklin did not receive this award, having died of ovarian cancer in 1958. Several controversies still exist surrounding the discovery of the DNA helix, including the story that James Watson was shown Photo 51 without Rosalind Franklin's permission. Crick and Watson also received a copy of a report containing several of Franklin's crystallographic calculations. How much of this data contributed to their final DNA model will never be known. However, to simply attribute the discovery of the DNA helix to just James Watson and Francis Crick would discount the critical contributions of researchers like William Astbury, Erwin Chargaff, Raymond Gosling, and Rosalind Franklin. Today our knowledge of DNA structure is because of all of them.

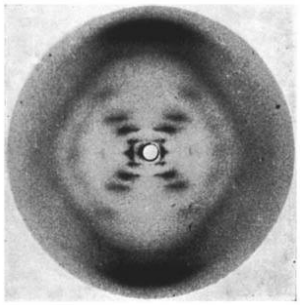


Figure **3.1.1**: Photo 51, an X-ray diffraction image of the DNA helix. (Photo 51 by Raymond Gosling, public domain)

Interestingly, in an ironic twist, the Russian biologist Nikolai Koltsov proposed, in 1927, the presence of a "giant hereditary molecule" in cells made up of "two mirror strands that would replicate in a semi-conservative fashion using each strand as a template". A pretty fantastic theory since it was proposed long before Watson, Crick, Gosling, and Franklin worked out the structure of the DNA double-helix.

DNA is Made of Nucleotides and Forms a Double Helix

The DNA molecule is a nucleic acid polymer made of subunits called nucleotides. A **nucleotide** (Figure **3.1.2**) consists of three chemical groups:

- a **pentose sugar**
- one to three **phosphate groups**
- a **nitrogenous base**

The sugar component of a nucleotide is a pentose sugar made of five carbons. The carbons are numbered clockwise from the oxygen as 1', 2', 3', 4', and 5' (e.g. 1' is read as “one prime”). In the case of DNA, this pentose sugar is called **deoxyribose** as it lacks an oxygen at the 2' carbon. The nitrogenous bases of DNA are **adenine, guanine, cytosine,** and **thymine** (Figure **3.1.2**). Adenine and guanine are known as the **purines**, whereas cytosine and thymine are classified as **pyrimidines**. An introduction to nucleic acids can be found in Chapter 2.7: Nucleotides.

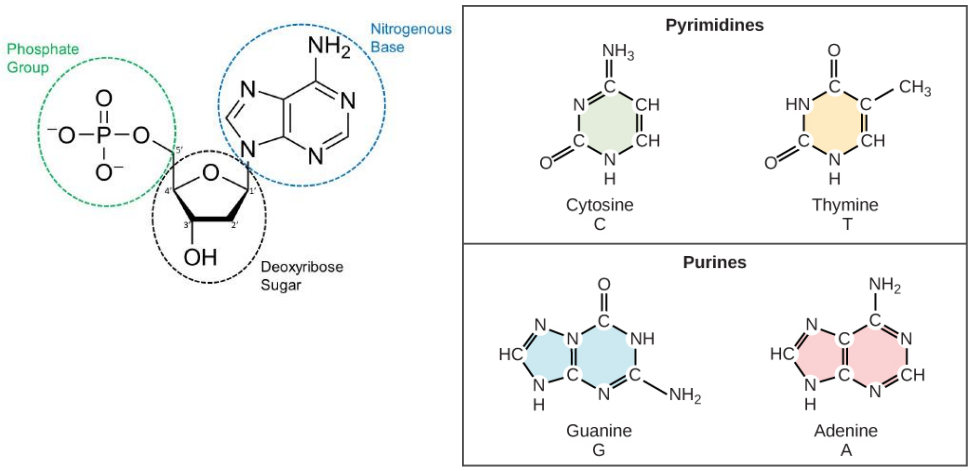


Figure **3.1.2**: Nucleotide structure. Left image: The nucleotide consists of three chemical groups: the pentose sugar (black circle), the nitrogenous base attached to the 1' carbon of the sugar (blue circle), and the phosphate group attached to the 5' carbon (green circle); Right image: DNA bases are carbon-based ring structures that are classified as either purines (guanine, adenine) or as pyrimidines (cytosine, thymine). (Nucleotide Structure by Patricia Zuk, CC 4.0; adapted from DAMP chemical structure by Hbf878, CC01.0 and OpenStax, CC BY-SA 4.0)

Making a DNA molecule requires linking nucleotides so that they form a long polynucleotide chain. Joining nucleotides is done by forming a **phosphodiester bond** between the phosphate group found attached to the 5' carbon of one nucleotide and the hydroxyl group attached to the 3' carbon of the next nucleotide. The resulting single-strand of DNA has its sugar–phosphate groups lined up as a “backbone” with the nucleotide bases sticking out from this backbone. However, DNA is not a single-strand but two strands that form a coiled **double helix (**Figure **3.1.3**). The two DNA strands of the helix have the opposite orientation, or run "anti-parallel" to each other. Hydrogen bonds form between complementary bases (i.e., adenine with thymine; cytosine with guanine), linking the two strands together along their length. Refer back to Chapter 2.7: Nucleotides to read more about the structure of the DNA helix.

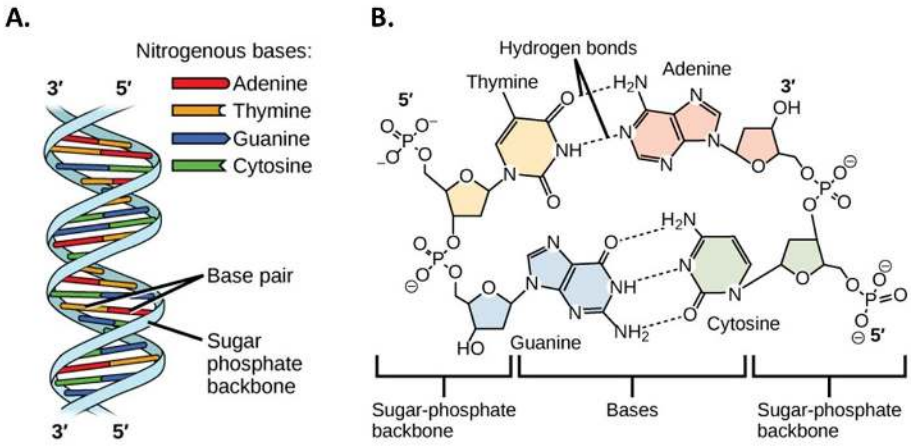


Figure **3.1.3**: The DNA Helix. (A) The DNA molecule is made of two single polynucleotide strands joined together to form a helix with the sugar-phosphate backbone (blue curved lines) found on the periphery and the complementary bases (adenine and thymine; guanine and cytosine) connected to each other in the center of the helix. The two DNA strands forming the helix run anti-parallel to each other; (B) Complementary nitrogenous bases connect to each other via hydrogen bonds. (DNA Helix by Jerome Walker and Denis Myts, Concepts of Biology, CC BY-SA 4.0)

DNA Comes in Many Forms: Chromatin, Chromosomes, and Plasmids

Stretched end-to-end, a DNA molecule in a single human cell would come to a length of about 2 meters. The DNA of *E. coli* is about 1000 times as long as the bacterial cell itself. As a result, DNA must be packaged in a very ordered way to fit and function within a cell. The strategy used to package DNA is dependent upon whether the cell is prokaryotic or eukaryotic.

Prokaryotic DNA

Prokaryotic cells, because of the absence of a nucleus, package the majority of their DNA into a region of the cytoplasm known as the **nucleoid**. In most prokaryotic cells, the DNA found within the nucleoid is a large circular piece of double-stranded DNA called a **bacterial chromosome** or **genophore** (Figure **3.1.4**). However, other bacteria may have linear chromosomes found within this region.

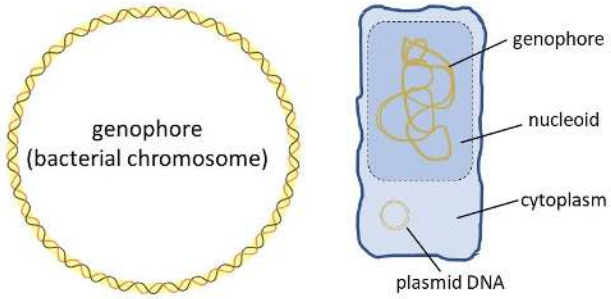


Figure **3.1.4**: The nucleoid and genophore. Left image - the bacterial chromosome is a large, circular piece of double-stranded DNA called the genophore. Right image - the genophore is found within the nucleoid region of the cytoplasm. Outside of the nucleoid, bacteria can have smaller pieces of circular DNA, called plasmids. (The Genophore by Patricia Zuk, CC BY 4.0; adapted from Bacterial Transformation by Rosanna Hartline, CC BY-SA 4.0)

To ensure the bacterial chromosome can fit into the nucleoid, it is packaged through **supercoiling.** Supercoiling happens in both prokaryotic and eukaryotic cells when the DNA helix is subject to strain by being either underwound or overwound (Figure **3.1.5**). A good analogy for supercoiling is the twists found in a telephone cord. Most DNA found in bacterial cells is underwound, or negatively supercoiled, because it makes the separation of the two DNA strands easier during DNA replication and transcription. Several structural proteins and enzymes within the nucleoid help to maintain the supercoiled nature of the bacterial chromosome. One of these enzymes is known as a **topoisomerase**.

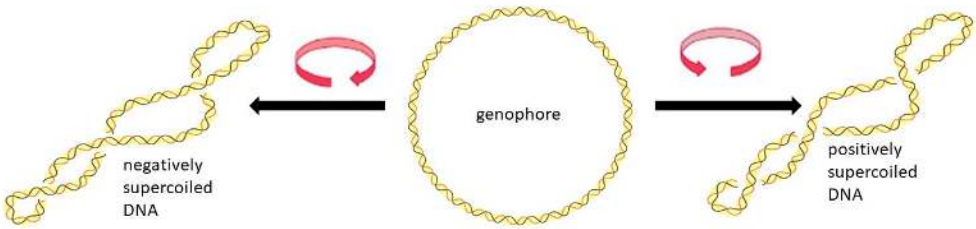


Figure **3.1.5**: Supercoiled DNA. The bacterial chromosome, or genophore (center image), found within the nucleoid region, can be underwound or negatively supercoiled (left image) or can be overwound or positively supercoiled (right image). (Supercoiled DNA by Patricia Zuk, CC BY 4.0; adapted from DNA Topoisomers by Fdardel, CC BY-SA 4.0)

The bacterial chromosome contains the genes responsible for the day-to-day functions of the cell. However, in addition to the chromosome, bacteria also have smaller pieces of circular DNA known as plasmids. A **plasmid** is a small, extrachromosomal DNA molecule that is found outside of the nucleoid and can replicate independently of the bacterial chromosome. Naturally occurring plasmids contain genes for specialized functions, such as antibiotic resistance, virulence, nitrogen fixation, or conjugation. They are significantly smaller than the bacterial chromosome, typically several thousand base pairs, although plasmids as large as several hundred thousand base pairs have been found.

In addition, plasmids can be artificially constructed and introduced into bacterial cells. These types of plasmids (Figure **3.1.6**) serve as tools in genetics and biotechnology labs, where they are used to clone and amplify genes for commercial applications. Plasmids that are sold commercially contain several regions in common with naturally occurring ones, such as antibiotic resistance genes. In addition, they will have a region for the insertion of genes, referred to as a **multiple cloning site** or **MCS**. This MCS is flanked by a **promoter sequence** and a **polyadenylation signal** so that the inserted gene will be properly transcribed into mRNA by the bacterial cell. More about these flanking regions of DNA can be found in Chapter 3.3 Transcription of RNA.

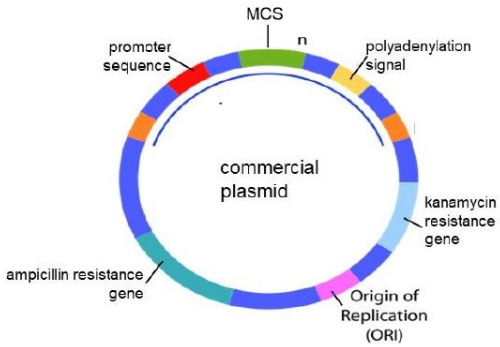


Figure **3.1.6**: Plasmid DNA. The elements of a typical commercial plasmid includes sequences such as resistance genes for antibiotics like ampicillin or kanamycin, a multi-cloning site (MCS), a promoter sequence, and an origin of replication. (Ti plasmid by Mouagip, Public Domain)

Eukaryotic DNA

If the DNA of all 46 human chromosomes were stitched together into a single molecule, it would be over 2 meters long. This is a problem that all eukaryotic cell have - the DNA is too long to fit into a nucleus. As such, eukaryotic cells must employ a unique type of packaging strategy to fit their DNA inside the nucleus. Eukaryotes begin to package their DNA by converting it into a form called **chromatin**. Chromatin is a complex of DNA and proteins. Eukaryotic cells have two types of chromatin: **euchromatin** and **heterochromatin**. In euchromatin, the DNA is found associated with proteins known as **histones**. A histone is often referred to as a **octamer** - a complex of 8 small, positively charged protein subunits (H2A, H2B, H3, and H4) found in the histone as pairs (Figure **3.1.7**). To form euchromatin, the DNA helix wraps around a histone octamer almost two times, creating a structure called a **nucleosome**. The DNA is then secured to the histone octamer by the histone protein, H1. The interaction between the DNA and the histone to form a nucleosome is simply the result of the attraction between negatively charged DNA and positively charged histone proteins. The nucleosome is linked to the next one by a short strand of "linker" DNA that is free of histones and is essentially the DNA helix. The length of linker DNA varies from cell type to cell type but is consistent within the cell, producing nucleosomes at regular intervals. Because of its appearance under the electron microscope, euchromatin is frequently referred to as "beads on a string", with the "bead" as the nucleosome and the "string" the linker DNA (Figure **3.1.7**). Once formed, certain sections of euchromatin condense further when neighboring nucleosomes stack compactly onto each other to form a 30-nm–wide fiber called **heterochromatin**. Heterochromatin condensing is thought to be the result of interactions between the H1 proteins.

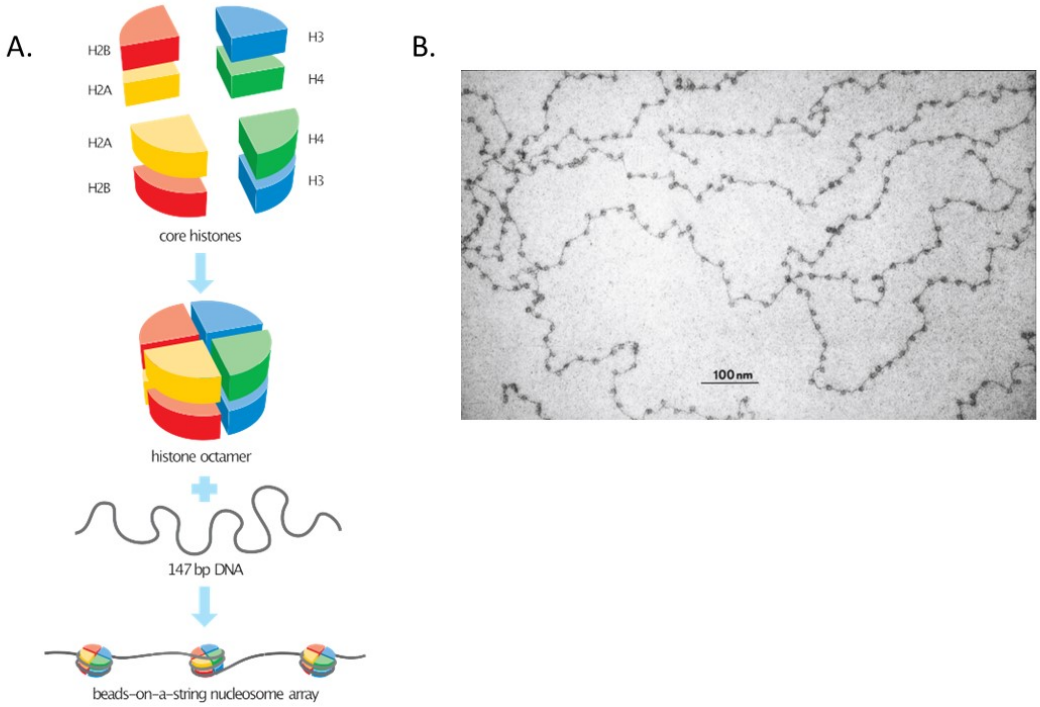


Figure **3.1.7**: (A) The histone octamer is made of two subunits each of four core histone proteins (H2A, H2B, H3, and H4). About 147 bp of DNA helix is wrapped around the histone octamer to form the nucleosome. The nucleosomes are arrayed in a loosely packed "beads-on-a-string" form called euchromatin. (Chromatin Structure by David O Morgan, CC0). (B) An electron microscope image of euchromatin showing several nucleosomes. (Nucleosomes by ORNL History, CC BY 2.0)

Eukaryotic cells, in the interphase stage of the cell cycle, will be a mixture of heterochromatin and euchromatin. However, as the cell progresses towards division (i.e. mitosis or meiosis), the DNA is packaged beyond the heterochromatin level to form a **chromosome**. A chromosome ("chromo" = color; "soma" = body) is a highly organized thread-like structure composed of chromatin and associated proteins. The chromosome represents the highest level of DNA organization found within a eukaryotic cell. In dividing cells, condensing into a chromosome begins after the DNA has been replicated and concludes during metaphase (Figure **3.1.8**). Euchromatin is condensed into heterochromatin. The heterochromatin forms loops that are 300 nm wide. These loops are condensed further to form the 700 nm wide **chromatid**. During metaphase, the chromosome is made of two chromatids, known as **sister chromatids**, joined at a region of heterochromatin called a **centromere**. Once cell division is complete, the DNA will de-condense back to its euchromatin and heterochromatin forms.

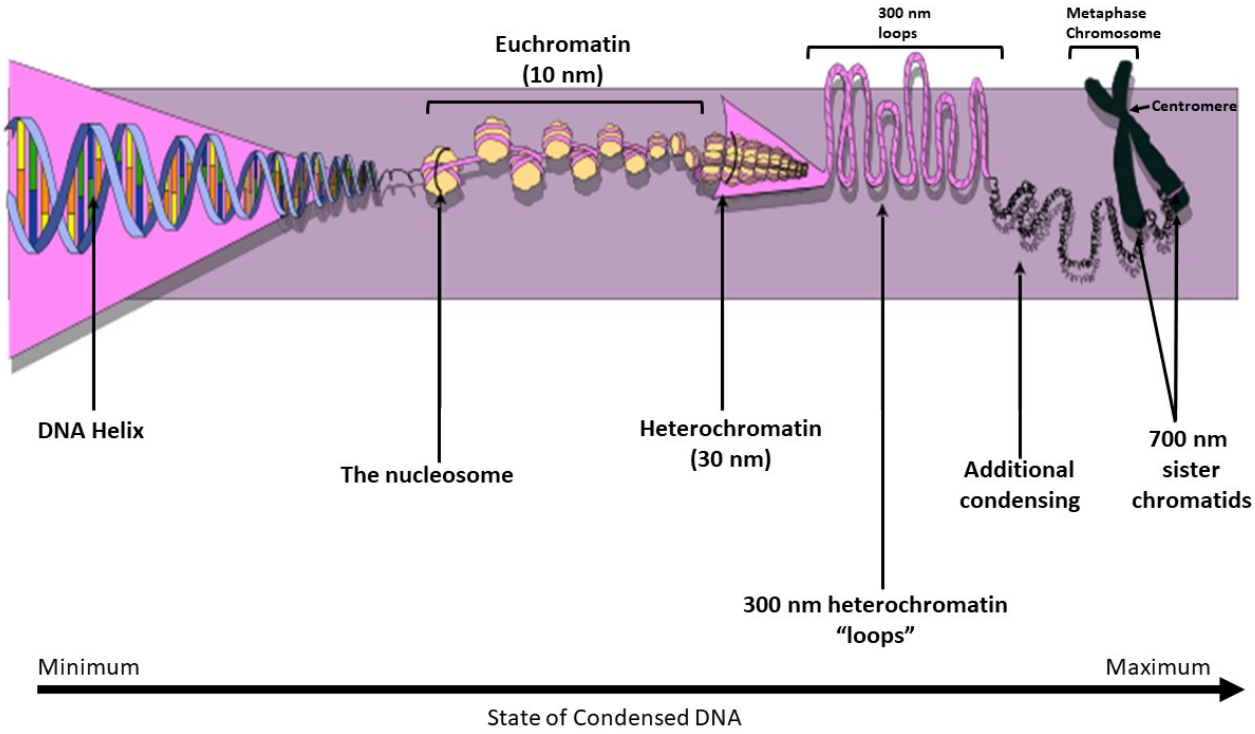
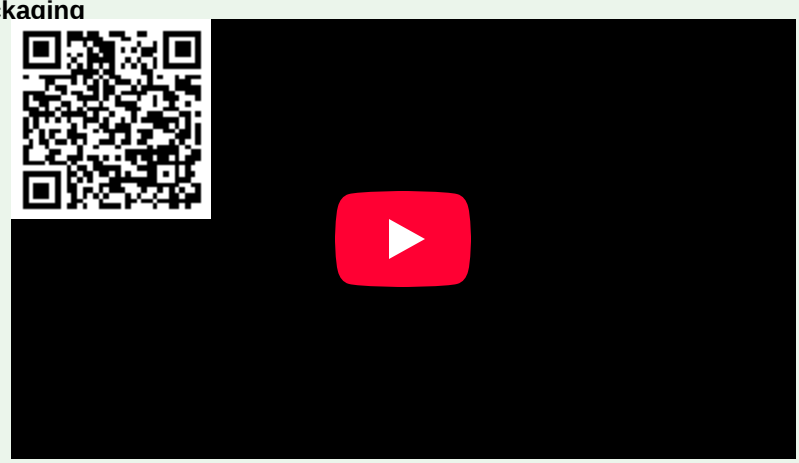


Figure **3.1.8**: DNA packing. DNA is packaged to form chromatin and then a chromosome. The DNA helix is wrapped around the histone to form the nucleosome. An array of nucleosomes is known as euchromatin. The euchromatin is condensed into a 30 nm heterochromatin fiber. Heterochromatin is looped and condensed further to form the 700 nm chromatid found in the duplicated chromosome. (DNA packing by Patricia Zuk, CC 4.0; adapted from DNA packaging by Մարիամ, CC BY-SA 4.0)

Concept in Action

Animation: DNA Packaging



The form of chromatin in the eukaryotic cell has a direct correlation to the function of its DNA. The majority of chromatin in the nucleus is euchromatin and represents the form of DNA where transcription occurs. Stretches of euchromatin are rich in genes and are transcriptionally active in the cell. DNA in the form of heterochromatin has fewer genes and is thought to be inaccessible to the transcription machinery. As such, it represents a more inactive or "silent" form of DNA. For more information about transcription, refer to Chapter 3.3 Transcription of RNA. All eukaryotic cells will have stretches of heterochromatin that do not de-condense to euchromatin but persist as "permanent" heterochromatin. Examples of these regions are the **centromere** and **telomeres** of the chromosome.

Key Concepts

The DNA helix is made of two anti-parallel polynucleotide strands that resemble a "spiral staircase". The sugar-phosphate groups of the nucleotides join to form an outer "backbone" that coils like the rails, with the bases projecting to the inside of the helix, like the steps of the staircase.

The major concepts to remember are:

- the two polynucleotide chain of the DNA helix run in the opposite direction to one another (i.e. are anti-parallel)
- the bases of the DNA helix pair to one another in a complementary fashion - C with G and A with T
- DNA can be found in a prokaryotic cell as a circular chromosome, called a genophore, and as small pieces of circular DNA, known as plasmids
- DNA in eukaryotic cells is found as chromatin or chromosomes
- Eukaryotic cells have two forms of chromatin: euchromatin and heterochromatin
- Euchromatin is made of a DNA helix wrapped around histone protein complexes
- Heterochromatin is formed by condensing euchromatin further into larger coils
- Heterochromatin is condensed further to form the chromatids of a chromosome

Glossary

Adenine (A): a nitrogenous base that pairs with thymine (T) in DNA through two hydrogen bonds.

Base pair: a pair of complementary nitrogenous bases in DNA, consisting of adenine-thymine (A-T) and guanine-cytosine (G-C).

Chromatin: a form of organized DNA found within the nucleus of a cell; found as two types: euchromatin and heterochromatin

Chromosome: the most highly condensed and organized form of DNA; a thread-like structure composed of DNA and associated proteins

Complementary base pairing: the specific pairing of nitrogenous bases in DNA (A pairs with T, G pairs with C) through hydrogen bonding

Cytosine (C): a nitrogenous base that pairs with guanine (G) in DNA through three hydrogen bonds

Deoxyribonucleic Acid (DNA): a molecule that carries genetic instructions for the growth, development, function, and reproduction of living organisms

Deoxyribose: a five-carbon sugar found in DNA nucleotides

Double Helix: a twisted ladder-like structure of DNA, consisting of two strands running in opposite directions

Euchromatin: a type of chromatin; composed of the DNA helix wound around proteins called histones

Gene: a segment of DNA that contains the instructions for synthesizing a specific protein or RNA molecule

Genophore: the chromosome equivalent found in prokaryotes and viruses

Guanine (G): a nitrogenous base that pairs with cytosine (C) in DNA through three hydrogen bonds

Heterochromatin: a type of chromatin; composed of euchromatin wound into a large solenoid structure through its associated with nuclear proteins

Histone: a complex of assembled histone proteins that provides structural support for euchromatin; classified into 5 types: 1, 2A, 2B, 3, 4, and 5

Nucleosome: the section of euchromatin that is composed of a DNA helix wrapped around a histone

Nucleotide: the basic building block of DNA, consisting of one to three phosphate groups, a deoxyribose sugar, and a nitrogenous base

Phosphodiester bond: the bond found between nucleotides in DNA and RNA

Purine: the organic bases adenine and guanine found in nucleic acids

Pyrimidine: the organic bases cytosine, uracil and thymine found in nucleic acids

Supercoiling: the twisting of the DNA helix

Thymine (T): a nitrogenous base that pairs with adenine (A) in DNA

Topoisomerase: the enzyme complex responsible for the elimination of supercoiling

Transcription: the process of copying a gene's DNA sequence into messenger RNA (mRNA)

3.1: DNA Structure is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

3.2: Replication of DNA

Biotech Focus

In 1983, while working at the biotechnology company Cetus Corporation, Dr. Kary Mullis created a laboratory process for DNA amplification that mimicked the *in vivo* process of DNA replication. This process, named **Polymerase Chain Reaction or PCR**, used repeated cycles of heating and cooling to exponentially replicate a targeted DNA segment. Mullis set-up three water baths: a high temperature bath for DNA denaturation (95°C), a lower temperature bath for primer annealing (e.g. 50°C to 60°C), and a third water bath for the extension phase of the polymerase (72°C). The DNA was incubated in each water bath for a defined time period and then repeated over and over again. Initially, the process used a DNA polymerase from *E. coli*, which was not stable enough at the high temperatures required for DNA denaturation and had to be replenished during each cycle. The breakthrough came with the discovery of **Taq polymerase,** an enzyme isolated from the thermophilic bacterium *Thermus aquaticus*, which could withstand the high denaturing temperatures. PCR quickly gained recognition for its versatility and has became a foundational tool in genetics, diagnostics, forensics, and research. Mullis' work earned him the Nobel Prize in Chemistry in 1993.

Introduction to DNA Replication

During cell division, each daughter cell receives an identical copy of the DNA. For this to happen, the DNA must be copied through a process called **DNA replication**. The replication of DNA occurs during the synthesis phase, or S phase, of the cell cycle, before the cell enters mitosis or meiosis. The structure of the double helix provides a hint as to how DNA is copied. During DNA replication, each of the two "parental" DNA strands that make up the double helix will separate and serve as a template from which the new "daughter" DNA strands are copied. The newly made daughter DNA strand is complementary to the parental DNA strand. After replication, each new DNA helix will consist of one parental DNA strand and one new daughter DNA strand. This is known as **semi-conservative replication** because each of the parental DNA strands remains (i.e. is conserved) within the new helix (Figure **3.2.1**).

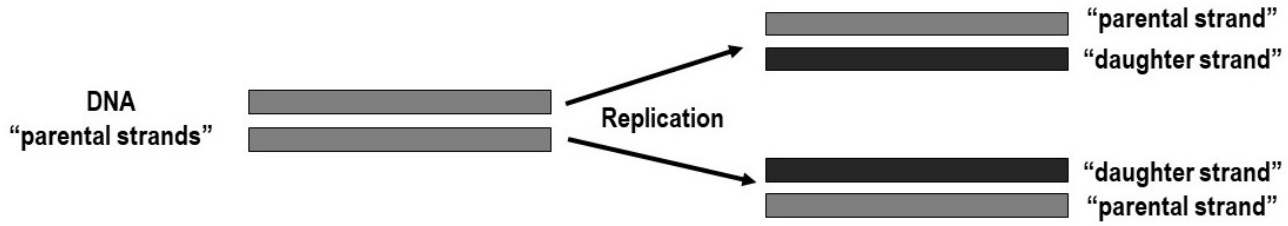


Figure **3.2.1**: The semi-conservative replication of DNA. During DNA replication the two original "parental" strands of DNA (gray rectangles) serve as templates for the synthesis of new "daughter" strands (black rectangles). After DNA replication, each DNA helix is made of a parental strand and a daughter strand. (Semi-conservative Replication by Patricia Zuk, CC BY 4.0)

Learning Objectives

DNA is replicated in cells and in the laboratory using a series of enzyme-catalyzed steps. At the end of this section, you will be able to:

- List the major steps of DNA replication
- Explain the process of DNA replication in prokaryotes
- Define the role for each component of DNA replication
- Compare and contrast DNA replication in prokaryotes and eukaryotes
- Explain the steps of the Polymerase Chain Reaction (PCR) workflow
- List and explain the components of a PCR reaction
- Explain the events of one PCR cycle
- Explain how real-time PCR differs from semi-quantitative PCR

The Mechanism of DNA Replication

The process of DNA replication requires many components and is comprised of many stages. However, the process can be simplified into the following steps:

1. The DNA helix is unwound at the origin of replication
2. RNA primers bind to specific sequences of DNA
3. DNA polymerases bind the primers and "read" the parental strands in order to create the new daughter strands. One new strand is made continuously, while the other strand is made in pieces
4. Primers are removed, new DNA nucleotides are put in place of the primers and missing phosphodiester bonds are re-formed

Both the secondary and tertiary structures of DNA make it impossible to replicate. To eliminate the tertiary DNA structure of supercoiling, a **topoisomerase** enzyme complex breaks a phosphodiester bond in one of the two DNA strands, eliminating the supercoiling. The secondary structure of the helix is then eliminated by "unwinding" the helix into its two parental template strands. This step is accomplished by an enzyme called a **DNA helicase (or helicase)** (Figure 3.2.2). The helicase recognizes a specific sequence of DNA known as an **origin of replication**. Two helicase complexes bind at this sequence and move along the DNA in opposite directions. As they move, they unwind or "unzip" the double-stranded DNA into single-strands. As the DNA helix unwinds, Y-shaped structures called **replication forks** are formed. Two replication forks are formed at each origin of replication as two helicase complexes move away from one another. The forks will grow in opposite directions as replication proceeds. Bacterial chromosomes, with their smaller sizes, typically have one origin of replication. The larger eukaryotic chromosomes will have several origins along their lengths so that replication can occur simultaneously from several places along each chromosome.

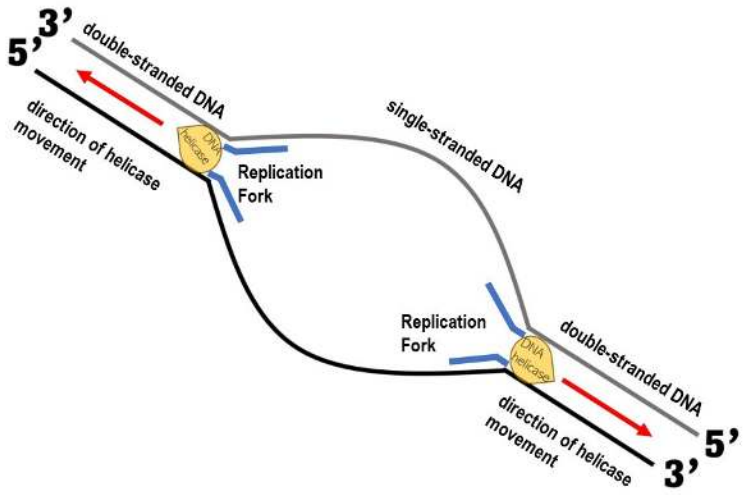


Figure 3.2.2: The DNA Helicase. A DNA helicase (yellow circles) binds to double-stranded DNA and unwinds it. Two DNA helicases move in opposite directions (red arrows), working together to produce single-stranded DNA ready for replication. The region of DNA unwinding is called a replication fork (blue lines). (The DNA Helicase by Patricia Zuk, CC BY 4.0; adapted from DNA Helicase by RosieScienceGal, CC BY-SA 4.0)

The unwinding of the DNA helix produces the two parental strands that serve as templates for daughter strand synthesis. The parental strands are prevented from reforming the DNA helix through the binding of several small **single-stranded binding proteins (SSBs)** to each parental strand. As the helicase unwinds the helix, the parental DNA is "copied" by a **DNA polymerase**, a large enzyme complex made of several subunits, each with specific functions in replication. The DNA polymerase is limited, however, in that it cannot bind single-stranded DNA. To fix this, an enzyme complex called a **primase** creates a short **RNA primer** that is complementary to the parental DNA template. The DNA polymerase clamps itself onto the parental DNA strand at the 3' end of the primer and glides along this strand towards its 5' end. As it moves to the 5' end, it uses the parental strand as a template, adding and linking complementary DNA nucleotides to create the DNA daughter strand. As DNA replication proceeds, RNA primers are removed and the gaps filled with new DNA nucleotides by another type of DNA polymerase. Finally, missing phosphodiester bonds between nucleotides in the daughter strands are formed by a **DNA ligase** enzyme complex, creating a continuous piece of daughter DNA.

DNA replication (Figure **3.2.3**) is a complex process that presents numerous challenges to the cell. Several of these are solved by enzyme complexes like the helicase (which unwinds the DNA helix) and the primase (which creates the primer). But the biggest challenges are: 1) the fact that both parental strands must be replicated at the same time and 2) the requirement that daughter DNA must be made in the 5' to 3' direction. In other words, incoming DNA nucleotides can only be added to a daughter DNA strand at its 3' end. The first challenge is solved easily. Two DNA polymerases, linked to one another, replicate both parental templates at the same time. The second challenge is more difficult. The requirement that daughter DNA "grows" in the 5' to 3' direction is not a problem for the DNA polymerase replicating the parental strand that is in the 3' to 5' orientation. The daughter DNA strand made from this template will be synthesized continuously in a process known as **leading strand synthesis**. The daughter strand made through this process is often called the **leading strand**.

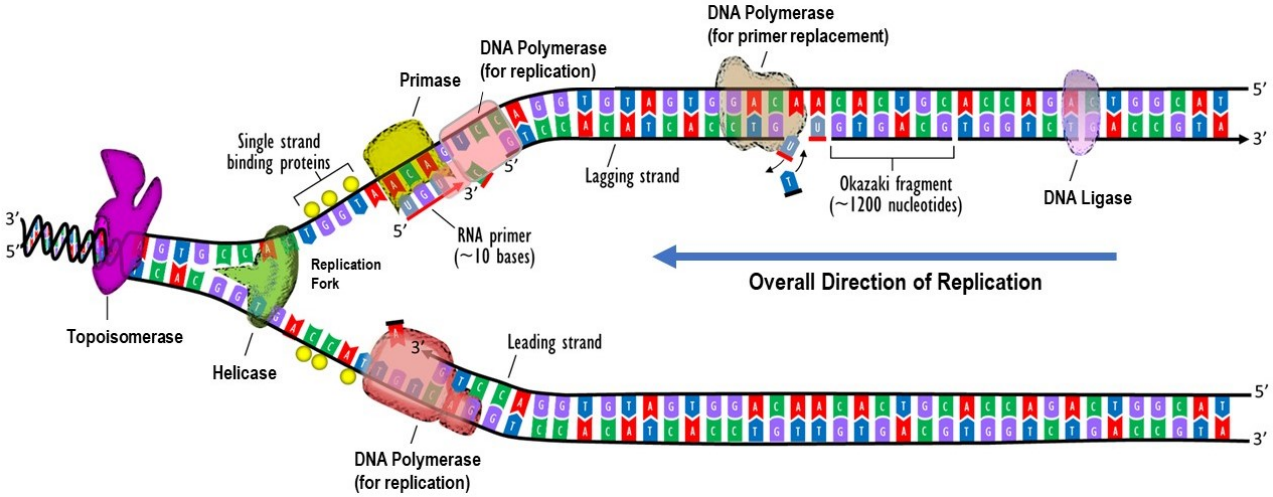


Figure **3.2.3**: DNA replication. The replication of DNA involves multiple enzyme complexes, including a Topoisomerase (dark purple enzyme) to remove supercoiling, a Helicase (green enzyme) to unwind the DNA helix into single template strands, a Primase (yellow enzyme) to create RNA primers, and DNA polymerases for replication (orange enzyme) and for RNA primer removal (tan enzyme) and replacement with DNA. The single strands of DNA are prevented from reforming the helix by the binding of several small single-stranded binding proteins (yellow circles). The leading daughter strand (Leading strand) is synthesized continuously. The lagging daughter strand (Lagging strand) is made in pieces known as Okazaki fragments of about 1200 nucleotides. The DNA Ligase (light purple) joins the Okazaki fragments together to complete synthesis of the lagging strand. (DNA Replication by Patricia Zuk, CC BY 4.0; adapted from DNA Replication in Prokaryotes by Eunice Laurent, CC BY-SA 4.0)

However, replication of the other parental template strand that is in the 5' to 3' orientation cannot be done continuously. Instead, this template must be replicated in short pieces called **Okazaki fragments**, in a process called **lagging strand synthesis**. In lagging strand synthesis, a short stretch of parental DNA, beginning with an RNA primer, is "looped" so that it *has its orientation reversed* (i.e. from 5' to 3' to 3' to 5'). This reversal of orientation to 3' to 5' means that the DNA polymerase can synthesize daughter DNA in the 5' to 3' direction and it creates an Okazaki fragment. The DNA polymerase then takes a new section of parental DNA and reverses and replicates it. As these Okazaki fragments are made, a **DNA ligase** will link them together by forming new phosphodiester bonds, thus creating a continuous piece of DNA that is the lagging strand.

Concept in action

Animation: DNA Replication

DNA Replication in Prokaryotes vs. Eukaryotes

DNA replication was first studied in prokaryotes, primarily because of the small size of the genome. As an example, *E. coli* has 4.6 million base pairs in its single, circular chromosome, and all of it gets replicated in approximately 42 minutes, starting from a single origin of replication and proceeding around the chromosome in both directions. This means that approximately 1000 nucleotides are added per second. The process is much more rapid than in eukaryotes, whose rate of replication ranges from 500 to 5000 nucleotides added per minute at each replication fork.

While there are many similarities in DNA replication between prokaryotes and eukaryotes, there are several important differences (Table **3.2.1**. The major difference lies in the composition and function of the DNA polymerases. Specifically, three polymerases (DNA polymerase I, II, and III) are involved in prokaryotic DNA replication, whereas eukaryotes utilize five polymerases (alpha, beta, delta, gamma, and epsilon).

Table **3.2.1**: **Comparison of DNA replication in prokaryotes and eukaryotes.**

Category	Prokaryotes	Eukaryotes
Replication site	nucleoid	nucleus
DNA organization	single, circular chromosome	multiple, linear chromosomes
DNA condensing	supercoiling	chromatin condensed into chromosomes
Origin of replication	one	multiple
Direction of replication	bidirectional	bidirectional
Replication rate	1000 - 2000 nucleotides per second	100 nucleotides per second
Removal of supercoiling	DNA topoisomerase II (DNA gyrase)	DNA topoisomerase II (DNA gyrase)
Unwinding of DNA helix	Helicase	Helicase
RNA primer production	Primase	DNA polymerase a
Synthesis of DNA	DNA polymerase III	DNA polymerase d (lagging strand) DNA polymerase e (leading strand)
Removal of RNA primers	DNA polymerase I	RNAse H???
Replacement of primers with DNA	DNA polymerase I	DNA polymerase e
Joining of DNA fragments	DNA Ligase	DNA Ligase
DNA repair	DNA polymerase II	DNA polymerases, b and d

Polymerase Chain Reaction (PCR): DNA Replication in the Laboratory

Polymerase chain reaction (PCR) is a laboratory protocol that amplifies a small amount of DNA into significant amounts that can be manipulated in a lab. PCR has become an incredibly powerful tool in the lab, widely used in forensics, research and clinical labs. Included in the myriad of uses for PCR is its use in amplifying enough DNA for downstream analysis (e.g. sequencing, restriction digest, DNA fingerprinting) and cloning.

The workflow for PCR is relatively straightforward (Figure **3.2.4**). PCR reactions are set-up and placed into a "PCR machine" or **thermocycler**. The thermocycler is programmed to run a repetitive series of **PCR cycles**. Upon the conclusion of the PCR program, the PCR reactions are analyzed through agarose gel electrophoresis, to confirm the presence of the correct PCR product, in addition to other downstream applications, such as sequencing.

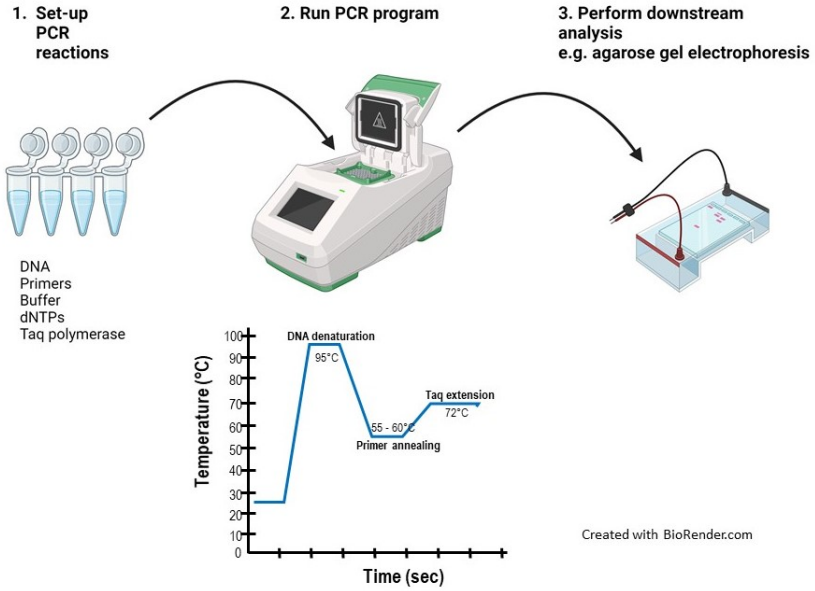


Figure **3.2.4**: The PCR workflow. 1) Set up PCR reactions: The PCR reaction is set up in PCR tubes to contain the DNA to be amplified (DNA), together with sequence-specific primers (Primers), a polymerase-specific buffer (Buffer), DNA nucleotides (dNTPs), and aTaq polymerase. 2) Run PCR Program: The PCR tubes are placed into a PCR machine programmed to repeatedly cycle through three specific temperatures (lower figure): DNA denaturation at 95°C, Primer annealing at 55°C to 60°C , and Taq extension at 72°C. 3). Perform downstream analysis: Following PCR, the reactions are analyzed through downstream applications, such as agarose gel electrophoresis. (The PCR Workflow by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

The PCR reaction

In amplifying DNA, PCR mimics DNA replication of the cell. Indeed, the components found in a PCR reaction are the same components, or very similar, to those found in cells undergoing DNA replication (Figure **3.2.5**).

The components of a PCR reaction are:

1. **The DNA polymerase:** At the core of a PCR reaction is a DNA polymerase capable of replicating a DNA template. The most common DNA polymerase used in PCR reactions is called **Taq polymerase** – a thermostable bacterial polymerase isolated from *Thermus aquaticus*, a thermostable DNA polymerase isolated from the bacteria Thermus aquaticus in 1988. Taq polymerase maintains activity across a wide range of temperatures that will be encountered during a PCR cycle.
2. **The template DNA:** The DNA added to the PCR reaction will contain the target region of DNA to be amplified.
3. **Two DNA primers:** As with *in vivo* DNA replication, replication by Taq polymerase requires a primer to initiate DNA synthesis. PCR uses two primers, known as the **forward primer** and the **reverse primer**. The forward and reverse primers will flank the DNA region to be amplified and are made of short sequences of DNA complementary to the DNA template. The forward primer sequence is designed to be complementary to the anti-sense template and the reverse primer is designed to be complementary to the sense DNA strand. PCR primers are often referred to as **oligonucleotides** or **"oligos"** because of their short length. Rather than being made of RNA, the two primers are made of DNA. However, they will act as the *in vivo* RNA

primer does in replication by base pairing to the template DNA and providing a 3' end from which the Taq polymerase can extend.

4. **The four DNA Nucleotides:** As with *in vivo* DNA replication, the replicated DNA strands will be synthesized using the four DNA nucleotide bases, dATP, dGTP, dCTP, and dGTP.
5. **A reaction buffer:** To optimize Taq polymerase activity, the PCR reaction will be set-up using a reaction buffer that is a mixture of monovalent and divalent cations (e.g. Mg2+) buffered to a specific pH.

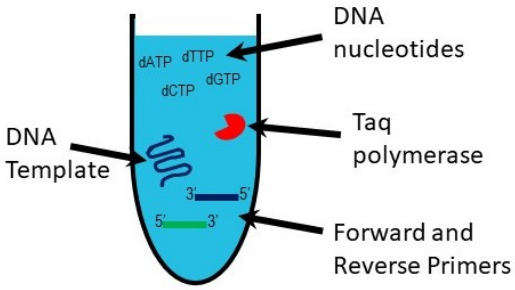


Figure 3.2.5: The PCR reaction. The components of a typical PCR reaction are similar to the components of DNA replication. (The PCR Reaction by Patricia Zuk, CC BY 4.0)

The components listed above are missing some critical components of *in vivo* DNA replication. First and foremost, the helicase of DNA replication is not used in a PCR reaction. The unwinding of the template DNA helix is achieved by incubating the PCR reaction at a very high temperature (i.e. 95°C). This high temperature unwinds or **denatures** the double-stranded DNA into two single strands. There is no need for a topoisomerase because the template DNA used in PCR reactions is typically small enough that supercoiling is not found. Finally, the "reannealing" of the two parental DNA strands back into a helix is prevented by the rapid binding of the forward and reverse primers to their complementary regions. In this way, single-stranded binding proteins (SSBs) are not required.

The PCR "cycle" is made of three temperatures

A PCR reaction is run using a repetitive series of temperatures. Each series is known as a "cycle" and consists of three temperatures (Figure 3.2.6). These temperatures are the:

1. **Denaturing temperature:** A very high temperature (~95°C) that breaks the hydrogen bonds between complementary bases and causes the two parental DNA strands to separate. The denaturing step of PCR is typically very short (~30 to 60 seconds).
2. **Annealing temperature:** A lower temperature designed to promote the base-pairing between the forward and reverse primers to their complementary sequences in the template strands. Because the PCR primers are added to the reaction in excess amounts, when the reaction is cooled, they will anneal to their complementary DNA regions before the two parental strands can re-anneal with each other. Annealing temperatures typically range from 50°C to 60°C and depend on the primer sequences. Annealing times in a PCR reaction usually range from 1 to 2 minutes to ensure sufficient primer annealing.
3. **Extension temperature:** The temperature that allows the Taq polymerase to bind and extend off the annealed primers. For Taq polymerase this temperature is 72°C. Extension steps are usually between 30 and 60 seconds.

A typical PCR program will repeat this three-temp cycle 30 to 40 times. Each cycle increases the amount of target DNA amplified. Only the region of template DNA found between the two annealed primers is replicated At the end of the program, millions of replicated pieces of DNA outlined by the two primers will be created.

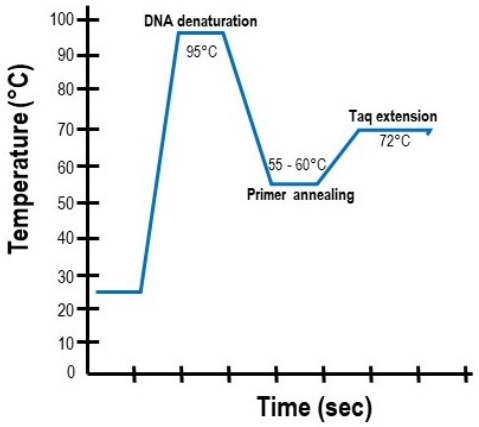


Figure **3.2.6**: The PCR cycle. A PCR cycle is made of three temperatures: denaturing, annealing, and extension. The DNA denaturing temperature of 95°C produces single stranded DNA templates. Cooling to the annealing temperature allows for the binding of the primer to its complementary sequence on the template. The extension temperature of 72°C allows extension off the primer leading to template strand replication.
(The PCR Cycle by Patricia Zuk, CC BY 4.0)

The Taq polymerase amplifies the DNA found between the two PCR primers

The strength of PCR lies in its ability to amplify a targeted region of DNA inside a much larger piece. For example, a scientist wanting to study a single human gene (or a specific region inside of it) only needs to amplify that gene (or region) from the enormous human genome of approximately three billion base pairs. “Outlining” the target DNA to be amplified is done using the forward and reverse primers. Therefore, researchers will design these primers to flank the region of DNA they wish to amplify. The animation below illustrates how PCR will amplify the regions between the two primers.

Concept in Action

Animation: PCR amplification of a target region

Real-time PCR: Quantitative PCR

The PCR described above is known as "semi-quantitative" because it can give a general idea of DNA quantity found in the PCR product. However, this type of PCR can be adapted into a truly quantitative protocol known as **real-time PCR** or **qPCR**. In real-time PCR, a fluorescently-labeled **probe** is used to quantitate the degree of cDNA amplification in the PCR reaction. Two major approaches are used in real-time PCR: **Taqman and SYBR green**.

The "Taqman protocol" was developed by Applied Biosystems (now part of Thermo Fisher). In this protocol, a **20X Gene Expression Mix**, containing custom forward and reverse primers, in addition to a fluorescently-labeled "Taqman probe", is used to amplify a sequence of DNA through a standard PCR reaction. The Taqman probe binds the DNA template somewhere between the forward and reverse primers. The DNA sequence of the probe is bound to a fluorophore and a quencher that prevents fluorescence.

Commonly used fluorescent probes include fluorescein (FAM), VIC, HEX, and JOE. When the probe is bound to the DNA template, the quencher prevents fluorescence. However, as the Taq polymerase amplifies the region between the forward and reverse primers, it displaces the fluorescent probe, which is followed by probe cleavage. As a result, the fluorescent probe is separated from its quencher and is excited. Fluorescent emission is then detected by the thermocycler (Figure **3.2.7**). The expression data for each PCR reaction is provided as a value known as a **Ct (threshold cycle) value** - the cycle number at which a detectable fluorescent signal was measured. These Ct values can be analyzed and either converted to absolute values or compared to one another so that changes in gene expression can be determined.

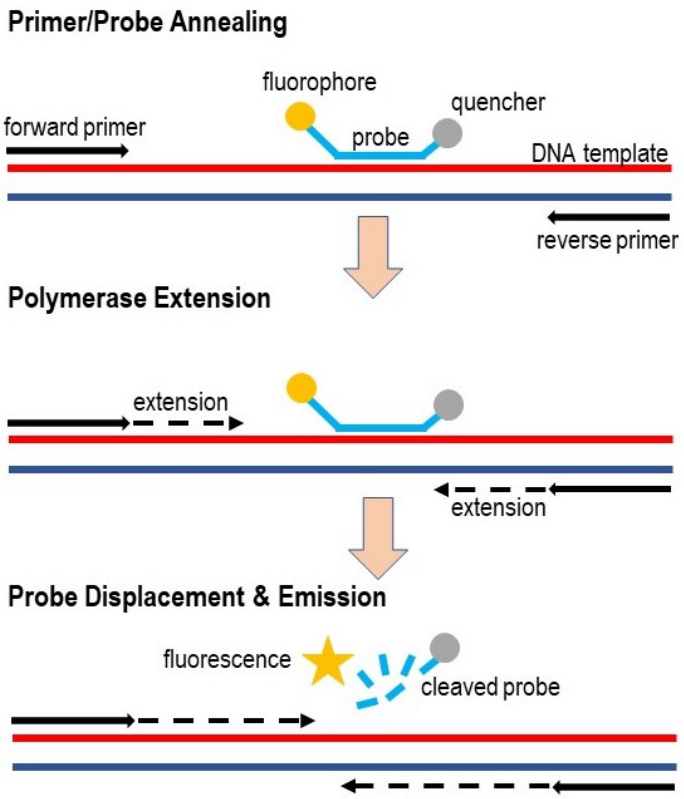


Figure **3.2.7**: Real-time PCR quantitation using primers and a Taqman probe. During each cycle of PCR amplification, the forward and reverse primers anneal to their complementary regions on the DNA strand, in addition to a fluorescently-labeled Taqman probe that is comprised of a fluorophore and a quencher. Extension from the primer by the Taq polymerase results in probe displacement from the DNA template. Displacement results in probe cleavage and "release" of the fluorophore from the quencher. The resulting fluorescence is quantified. (Real-time PCR by Taqman by Patricia Zuk, CC BY 4.0)

The "SYBR green protocol" depends on the intercalating dye, **SYBR Green I**. When unbound in the PCR reaction, the SYBR Green dye has negligible fluorescence. However, when the Taq polymerase synthesizes it complementary DNA strand, SYBR Green I is incorporated into the PCR product and emits a strong fluorescent signal. At the start of the next PCR cycle, the fluorescent signal is decreased when the double-stranded DNA (dsDNA) template is denatured but increases again as the PCR product is made. Therefore, the fluorescent signal of the sample is collected at the end of the elongation step of each PCR cycle to determine the relative change in amplified products (Figure **3.2.8**). Since SYBR Green I is a non-specific dsDNA binding dye, it cannot distinguish between amplified PCR products and non-specific piece of dsDNA. Therefore, corrective methods must be used in data analysis.

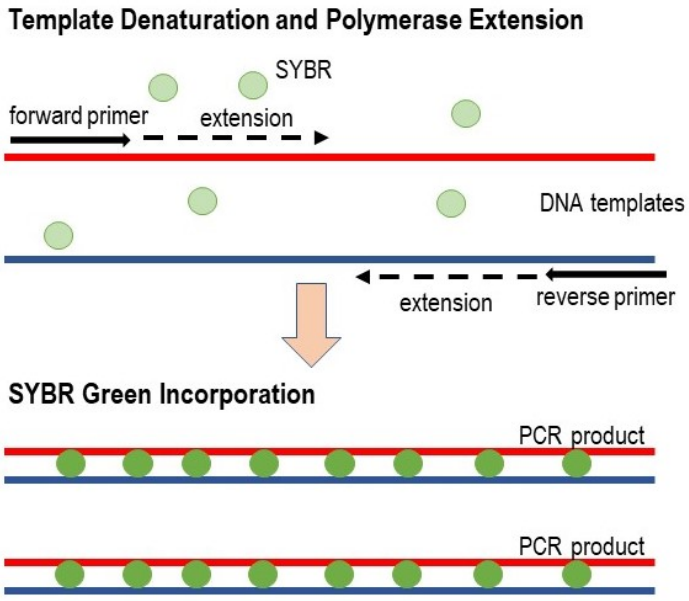


Figure 3.2.8: SYBR green real-time PCR. SYBR green cannot bind single-stranded template DNA and shows negligible fluorescence when dsDNA is denatured. Following polymerase extension from the primers, the SYBR green dye becomes incorporated into the double-stranded PCR product. The fluorescence is then measured. (SYBR Green Real-Time PCR by Patricia Zuk, CC BY 4.0)

Lab Protocols

- Lab Technique: Polymerase Chain Reaction (PCR)
- Lab Technique: Quantitative PCR
- Lab Technique: Agarose Gel Electrophoresis

Key Concepts

DNA replication duplicates the two strands of the DNA helix. Some major concepts to remember are:

- each of the two "parental" DNA strands serve as templates for "daughter" DNA strand synthesis
- the two"parental" DNA strands of the DNA helix are separated by a Helicase enzyme prior to replication
- single-stranded binding proteins hold the two parental DNA strands apart
- DNA replication is performed by a DNA polymerase that reads a parental template strand to create a daughter strand made of complementary DNA nucleotides
- DNA polymerase requires a short primer of RNA to begin replication of a daughter strand. This primer is made by a Primase enzyme
- DNA polymerase makes the daughter DNA strand in the 5' to 3' direction. This means that one daughter strand (i.e. the leading strand) can be synthesized continuously. The other daughter strand (i.e. the lagging strand) is made in short pieces called Okazaki fragments
- the enzyme DNA ligase joins all DNA fragments to finish DNA replication
- after replication, each new DNA helix consists of one parental strand and one new daughter strand (i.e. semi-conservation replication)

Glossary

DNA Ligase: An enzyme that joins Okazaki fragments together by forming phosphodiester bonds.

DNA Polymerase: An enzyme that adds nucleotides to a growing DNA strand during replication.

DNA Replication: The biological process by which a cell makes an identical copy of its DNA before cell division.

Daughter strand: The complementary DNA strand made by DNA polymerase during DNA replication.

Helicase: An enzyme that unwinds the DNA double helix by breaking hydrogen bonds between base pairs.

Leading Strand: The DNA strand that is synthesized continuously in the 5’ to 3’ direction during replication.

Lagging Strand: The DNA strand that is synthesized discontinuously in short segments known as Okazaki fragments.

Okazaki Fragments: Short DNA fragments synthesized on the lagging strand during replication; joined together by DNA ligase.

Origin of Replication: A specific sequence in the genome where DNA replication begins; the site of binding of the helicase and other replication proteins.

Parental DNA strand: One of the two original strands found in the DNA helix; used as a template for synthesis of the leading and lagging strands.

Primase: An enzyme that synthesizes a short RNA primer to provide a starting point for DNA polymerase.

Replication Fork: The Y-shaped region where the DNA double helix is unwound and new strands are synthesized**.**

RNA Primer: A short RNA sequence synthesized by primase that initiates DNA synthesis.

Topoisomerase: An enzyme that prevents DNA supercoiling and relieves tension ahead of the replication fork.

Single-Strand Binding Proteins (SSBs): Proteins that stabilize the separated DNA strands during replication to prevent them from reannealing.

Semi-Conservative Replication: A method of DNA replication in which each new DNA molecule consists of one original (parental) strand and one newly synthesized strand.

3.2: Replication of DNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

3.3: Transcription of RNA

Biotech Focus

The process of transcribing DNA into RNA is a complex system involving numerous enzyme complexes all working together in a highly coordinated fashion. In the 1990s, a research group led by **Roger Kornberg** at Stanford University successfully developed an *in vitro* transcription system from baker's yeast that faithfully replicated the *in vivo* process found in cells. The group then used this system to isolate several dozen proteins required for transcription, including the Mediator protein complex required for efficient transcription in eukaryotes. Thanks to this groundbreaking research, we now know that the protein components of transcription are remarkably conserved across the spectrum of eukaryotes, from yeast to human cells. Based on this work, Roger Kornberg won the Nobel Prize in Chemistry in 2006.

Read Roger Kornberg's recollections of his research that culminated in this Nobel Prize.

Introduction

The goal of **transcription** is to make an RNA copy of a gene's DNA sequence. Transcription is the first step in **gene expression**, in which information from a gene is used to produce a functional product, such as RNA or a protein. This flow of genetic information is also known the **central dogma of molecular biology** (Figure **3.3.1**), which states that a gene becomes transcribed and then can be translated into a protein.

For a protein-coding gene, the RNA copy, made through transcription, carries the information needed to build the protein. Transcription is relatively straightforward. RNA nucleotides, complementary to the DNA template, are linked together to form an RNA copy known as an **RNA transcript**. The translation of this transcript into a polypeptide sequence is more complex and will require the reading of RNA nucleotides in a specific manner. This process will be covered in Chapter 3.4: Translation.

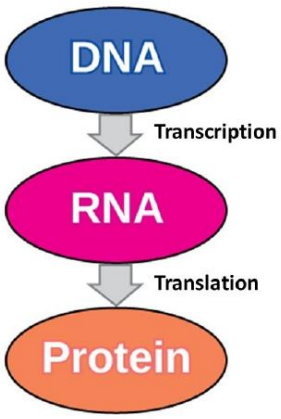


Figure **3.3.1**: The Central Dogma of Molecular Biology. DNA is transcribed into RNA which is then translated into protein. (Central Dogma, from LibreText, CC BY-SA 4.0)

Learning Objectives

RNA is a nucleic acid that converts the genetic code found in DNA into the proteins that perform cellular functions.At the end of this section, you will be able to:

- List and explain types of RNA found in cells
- Describe the modifications needed to create mRNA, rRNA, and tRNA
- Explain the structure of a protein-coding gene and its regulatory sequences (i.e., the transcription unit)
- List the types of RNA polymerases involved in transcription
- List and explain the stages of transcription

- Explain the steps involved in mRNA processing
- Explain the spliceosome and its role in spicing
- Explain alternative splicing and its importance

Types of RNA

RNA, like DNA, is a nucleic acid made up of nucleotides. However, there some important differences between RNA and DNA. RNA nucleotides have a pentose sugar, named **ribose,** that has a hydroxyl group (OH), rather than a hydrogen, at the 2' carbon. The bases of RNA are adenine, guanine, cytosine, and **uracil.** Finally, RNA is a single polynucleotide chain. For more information about nucleic acids, refer to Chapter 2.7 Nucleic Acids.

There are several types of RNA found in cells. These RNA types can be broken down into two categories: **coding and non-coding**. There is only one type of RNA that is classified as coding. This RNA is known as **messenger RNA or mRNA** and it is produced following the transcription of protein-coding genes in the nucleus.

There are numerous types of non-coding RNA molecules, including:

- **ribosomal RNA or rRNA**
- **transfer RNA of tRNA**
- **microRNA or miRNA**
- **small nuclear RNA or snRNA**

Non-coding RNA molecules, like coding RNA, are transcribed. However, they are not translated into proteins. More about protein translation can be found in Chapter 3.4: Translation.

mRNA

Messenger RNA, or mRNA, is produced upon transcription of protein-coding genes. This transcription takes place in the **nucleoplasm**, the fluid of the nucleus. Following transcription, this RNA transcript, which is often called **pre-mRNA**, is exported out into the cytoplasm to be modified into mRNA.

Pre-mRNA processing into mRNA involves three events (Figure **3.3.2**):

- addition of a modified guanine nucleotide, known as the **5'-methylated cap**, to the 5' end of the pre-mRNA
- addition of a long stretch of adenine nucleotides, known as the **poly-A tail**, to the 3' end of the pre-mRNA
- splicing to remove all **introns** from the pre-mRNA

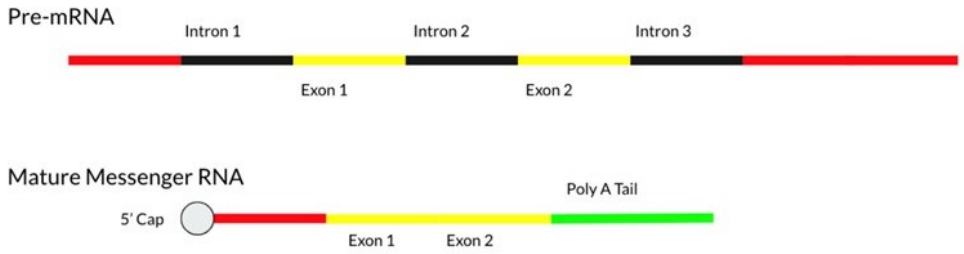


Figure **3.3.2**: mRNA processing. Mature mRNA is processed from the pre-mRNA molecule (i.e. RNA transcript) through the addition of a 5'-methylated cap to the 5' end and a polyA tail added to the 3' end. Introns are removed through splicing. (mRNA Maturation by Editorzcj, CC BY-SA 4.0)

rRNA

Ribosomal RNA, or rRNA, makes up the majority of RNA found in cells. rRNA is produced upon transcription of a class of genes called ribosomal genes. This transcription takes place in the **nucleolus** of the nucleus. Following transcription, the rRNA is folded into a series of **stems and loops**, resulting in a characteristic secondary structure (Figure **3.3.3**). This mature rRNA will become part of the ribosome, the "machine" for translation of proteins. rRNA molecules are named based on their size. rRNA size is quantified using a technique called **sedimentation**. In sedimentation, molecules are added to columns of sugar and then centrifuged. The rate of sedimentation through the column is denoted with a capital "S". Larger rRNA molecules will have a faster sedimentation rate and a larger "S" value in comparison to smaller rRNA molecules. The types of rRNA molecules made are dependent upon species.

For example, humans have the following rRNA molecules: 5S, 5.8S, 18S, and 28S. In contrast, *E.coli* cells produce 5S, 16S, and 23S rRNA molecules. More about rRNA can be found in Chapter 3.4: Translation.

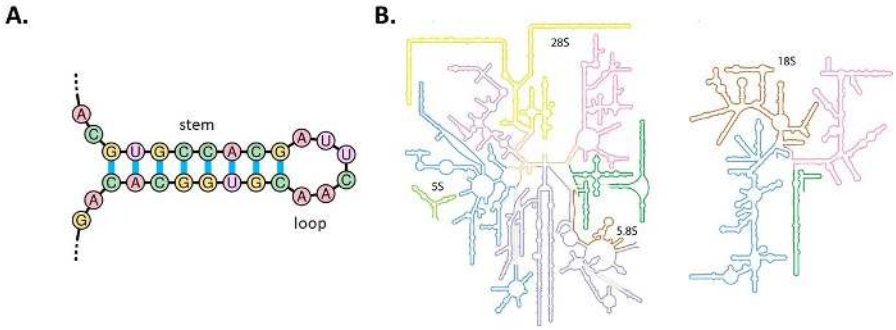


Figure **3.3.3**: rRNA molecules (A) The stem-loop configuration of rRNA is produced through complementary base pairing to create a stem, with the non-complementary bases producing a loop. (Stem-loop by Sakurambo, CC BY 2.5)
(B) The 4 types of rRNA molecules found in human cells are 18S and a complex made of 28S, 5.8S, and 5S. (adapted from Petrov et al, CC BY-SA 4.0)

tRNA

Transfer RNA, or tRNA, is transcribed in the nucleoplasm of the nucleus. tRNA is a short, single-stranded RNA molecule approximately 80 nucleotides in length that is then folded into a characteristic secondary structure of stems and loops not unlike rRNA. This secondary structure is then folded further to create a unique tertiary structure (Figure **3.3.4**). More about tRNA can be found in Chapter 3.4: Translation.

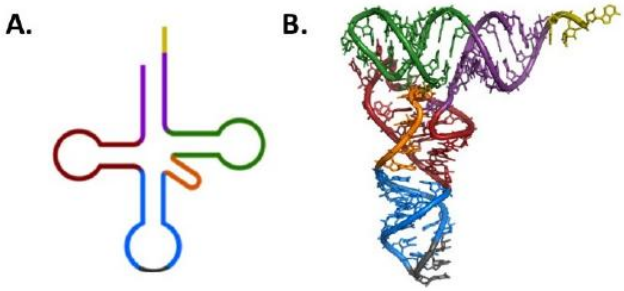


Figure **3.3.4**: The tRNA molecule. (A) The secondary structure of the tRNA molecule shows numerous stem and loop regions. (B) The tertiary structure shows the proposed three-dimensional structure of a tRNA molecule. (tRNA-Phe by Yikrazuul, CC BY-SA 3.0)

Genes are Transcribed

Not every stretch of DNA in our genome is transcribed into RNA. A significant portion of our genome is considered to be "junk" DNA, sequences to which a function has not yet been assigned. Those stretches of DNA that are transcribed are known as genes. A **gene** is defined as a sequence of DNA that is transcribed into functional RNA. Protein-coding genes are transcribed into coding RNA (i.e. mRNA). Non-protein coding genes are transcribed into a specific type of non-coding RNA.

The eukaryotic gene is part of a "transcription unit", which is comprised of the stretch of DNA that is transcribed, in addition to several regulatory sequences that will control transcription (Figure **3.3.5**).

For a protein-coding gene, the gene itself is made of three regions:

- the **coding sequence (CDS)**, which is the majority of the transcribed DNA. The coding sequence is often called an **Open Reading Frame (ORF)**. The CDS/ORF is made of **exons**, which are DNA sequences that will be translated into amino acids, and **introns**, which are intervening stretches of DNA that are not translated
- the **5' untranslated region (5'UTR)**, a regulatory DNA sequence before (upstream from) the coding sequence
- the **3' untranslated region (3'UTR)**, a regulatory DNA sequence after (downstream from) the coding sequence

Both prokaryotic and eukaryotic protein-coding genes will have UTRs flanking the coding sequence. However, these UTRs are significantly shorter in prokaryotes.

Associated with the gene are regulatory sequences known as the **promotor** and the **enhancer.** The promoter is a DNA sequence located immediately upstream of the gene that "promotes" transcription. The majority of prokaryotic and eukaryotic genes will have some type of promoter. In both prokaryotes and eukaryotes, the promoter region of most genes typically contains a sequence of DNA known as a **core promoter**. The DNA sequence of this core promoter is very similar in many prokaryotic and eukaryotic genes (i.e., is conserved). The most well-known eukaryotic core promoter has the sequence "TATAAA" and is known as the **TATA box.** In prokaryotic cells, this core promoter sequence is "TATAAT" and is called the Pribnow box, after its discoverer. In eukaryotes, additional sequences can be found next to the core promoter. These sequences are called **proximal promoter elements (PPEs)** and collectively they are known as the **proximal promoter**. The sequences making up the proximal promoter will vary from gene to gene. If present, they function as regulators of transcription.

In addition to a promoter region, eukaryotic genes are also associated with a stretch of DNA known as the enhancer. As its name implies, the enhancer "enhances" the efficiency of transcription. The enhancer is usually found at a significant distance upstream of the promoter and gene, although some enhancers can be found downstream of the gene. The enhancer is a sequence that is unique to eukaryotic cells and is not found in prokaryotes.

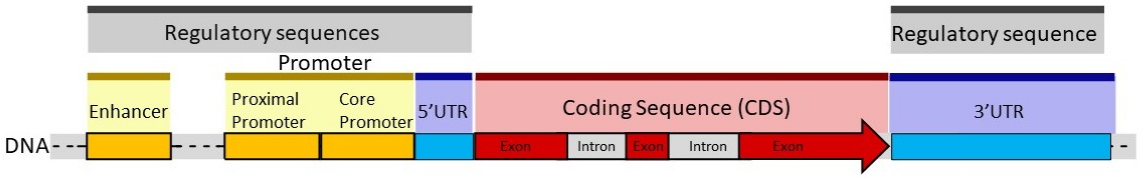


Figure **3.3.5**: The transcription unit of a eukaryotic gene. The transcription unit includes a coding sequence/CDS (shown in red) and regulatory sequences known as an enhancer and a promoter (shown in yellow). The promoter region of a transcription unit often contains distinct sequences known as the core promoter and the proximal promoter. The coding sequence contains sequences that will be expressed as amino acids in the polypeptide (exons) and regions that will be spliced out (introns). The coding sequence is also flanked on either side by untranslated regions, or UTRs (shown in blue). (The Transcription Unit by Patricia Zuk, CC BY 4.0; adapted from Gene Structure by Thomas Shafee, CC BY-SA 4.0)

Transcription by the RNA polymerase

The enzyme complex responsible for transcription is the **RNA polymerase**. In prokaryotes, there is a single RNA polymerase that transcribes all genes. In eukaryotes, there are three RNA polymerases: RNA polymerase I, RNA polymerase II, and RNA polymerase III. Each of these are responsible for the transcription of specific RNA molecules in the eukaryotic cell. Table **3.3.1** summarizes these polymerases.

Table **3.3.1**: **Comparison of RNA polymerases**

RNA Polymerase	Transcription Location	RNAs transcribed
RNA polymerase I	nucleolus	5.8S, 18S, and 28S rRNA
RNA polymerase II	nucleoplasm	mRNA
RNA polymerase III	nucleoplasm	5S rRNA, tRNA

Both prokaryotic and eukaryotic RNA polymerases are made of several subunits, with each subunit performing a specific task in transcription. For example, specific subunits in the polymerase act to recognize the promoter sequence, open the DNA helix (i.e., act like a helicase), or hold the two DNA strands apart (i.e. act like SSBs). Additional subunits bind to the DNA template and synthesize the RNA transcript by linking RNA nucleotides together.

Transcription: from DNA to RNA

In both prokaryotes and eukaryotes, transcription occurs in three stages: **initiation, elongation, and termination**.

Initiation

Transcription begins with the binding of a large complex of proteins, called the **Transcription Initiation Complex,** to the promoter of the transcription unit (Figure **3.3.6**). Contained with this complex is the RNA polymerase. The RNA polymerase recognizes the

sequence of the core promoter and binds to the DNA at that region. This binding places the RNA polymerase in close proximity to the first nucleotide to be transcribed, otherwise known as the **transcription start site (TSS).** In eukaryotes, the TSS is found in the 5' UTR. Polymerase binding in eukaryotes is made more efficient by the proximal promoter elements found next to the core promoter. In eukaryotes, the binding of the RNA polymerase to the promoter region is further enhanced by the presence of DNA-binding proteins called **transcription factors**. Transcription factors called **general transcription factors** will help position the RNA polymerase at the core promoter, while more specialized transcription factors, called **specific transcription factors**, will bind to the proximal promoter and help regulate RNA polymerase activity. Additional transcription factors, known as **activators**, bind the gene's enhancer, significantly enhancing the initiation of transcription by the RNA polymerase. In order to do this, a "DNA bending protein" bends the DNA so that the enhancer sequence with its activators is brought into proximity of the promoter. Additional proteins, forming a **mediator complex,** facilitates the interaction between the activators at the enhancer and the transcription factors at the promoter so that the initiation of transcription occurs efficiently and rapidly. Together with the RNA polymerase, the transcription factors, activators, and the mediator complex make up a significant portion of the Transcription Initiation Complex. Once this complex is finished forming, the RNA polymerase acts to partially unwind the DNA helix at the TSS creating a "transcription bubble". Transcription then enters the elongation stage.

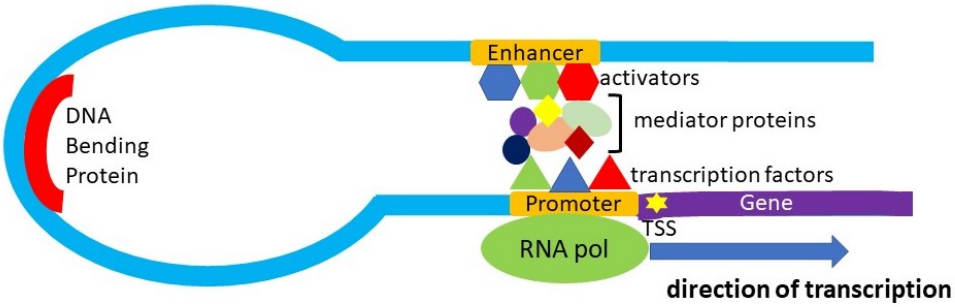


Figure **3.3.6**: The transcription initiation complex. The start of transcription in eukaryotes requires a transcription initiation complex containing several proteins, including transcription factors, activators, and a mediator protein complex that facilitates the interactions between these proteins. A DNA bending protein bends the DNA, bringing the enhancer region close to the promoter region. The transcription initiation complex forms to increase the efficiency of RNA polymerase (RNA pol) binding to the promoter and the initiation of transcription at the transcription start site (TSS). (The Transcription Initiation Complex by Patricia Zuk, CC BY 4.0; adapted from Regulation of transcription by Bernstein0275, CC BY-SA 4.0)

Elongation

Elongation in both prokaryotes and eukaryotes occurs when the RNA polymerase reads the DNA sequence and links the incoming RNA nucleotides together to produce the RNA transcript (Figure **3.3.7**). The RNA polymerase reads the DNA strand with the 3' to 5' orientation so that it can synthesize the RNA transcript in the 5' to 3' direction. This DNA strand is referred to as the **template strand**. The strand in the 5' to 3' direction is not read by the RNA polymerase and is called the non-template strand. During elongation, the RNA polymerase acts as a helicase to unwind the DNA helix, increasing the size of the transcription bubble. It holds the two DNA strands away from each other, acting as single-stranded binding proteins (SSBs). The RNA polymerase also does not require primers to bind to the DNA template strand. As such, a primase is not required for transcription. The RNA transcript that is made during elongation is complementary to the template strand, with the nucleotide uracil (U) used in place of thymine (T). This means if the template sequence is TAAGTG, the RNA transcript sequence would be UUCAC.

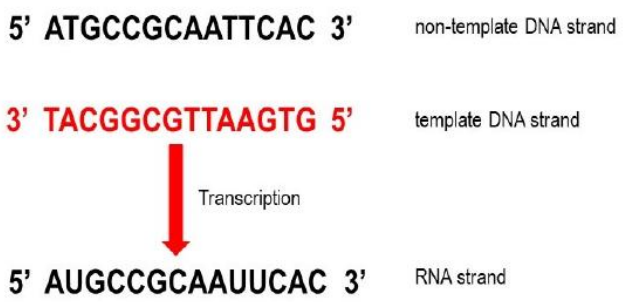


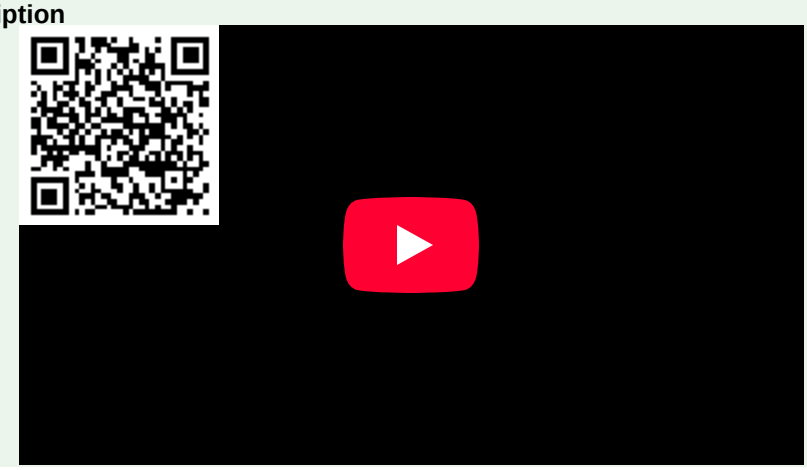
Figure **3.3.7**: Transcription elongation. During transcription elongation, the DNA strand with the 3' to 5' orientation is used as the template for transcription (template DNA strand, red letters). The DNA strand with the 5' to 3' orientation (non-template strand, black letters) is not involved in transcription. In this figure, the template DNA sequence of TACGGCGTAAGTG is transcribed into an RNA strand with the sequence AUGCCGCAAUUCAC. (Transcription Elongation by Patricia Zuk, CC BY 4.0)

Termination

Once the gene is transcribed, the RNA polymerase needs to dissociate from the template strand and release the newly made RNA transcript. This is happens at a **termination sequence** located at the 3' end of the gene. The termination sequence can vary depending on the gene and on whether that gene is prokaryotic or eukaryotic. In eukaryotes, the termination sequence is found within the 3'UTR. The sequence is usually comprised of repeated nucleotide sequences that result in the RNA polymerase stalling, detaching from the template, and freeing the RNA transcript.

Concept in Action

Animation: Transcription



mRNA Processing in Eukaryotes

Newly transcribed RNA molecules made from eukaryotic protein-coding genes undergo specific processing steps in order to turn them into mature mRNA molecules. As listed in the section on mRNA above, these modifications are: the addition of a "cap" to the 5' end of the RNA, the addition of a "poly-A tail" to the 3' end of the RNA, and the removal of introns within the coding sequence of the gene. These three modifications must be completed before the resulting mRNA can be transferred from the nucleus to the cytoplasm so that it can translated into a protein. These modifications also increase the stability of the mRNA once in the cytoplasm. As a result, eukaryotic mRNAs last for several hours, whereas the typical prokaryotic mRNA, which lack most of these modifications, lasts no more than five seconds before being degraded.

Addition of the "Cap"

As the RNA transcript is being transcribed, a modified guanine nucleotide called 7-methylguanosine, is added as a “cap” to the 5' end of the growing RNA transcript through an unusual 5'-to-5' triphosphate linkage. This nucleotide is referred to as the **5' methylated cap** owing to the presence of a methyl group attached to the guanine base of the nucleotide (Figure **3.3.8**). The 5'

methylated cap plays a role in the export of the mRNA from the nucleus and protects the 5' end of the mRNA from degradation by RNAses, RNA-degrading enzymes. As will be discussed in Chapter 3.4: Translation, the cap also helps to regulate the start of translation.

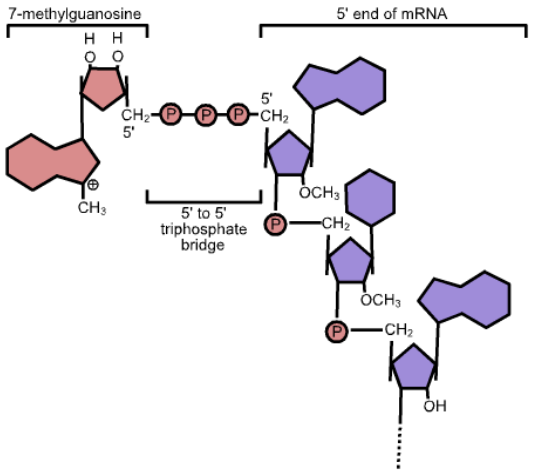


Figure **3.3.8**: The 5' methylated cap of mRNA. mRNA is capped with a unique nucleotide called 7-methylguanosine (pink) that is attached to the 5' end of the mRNA (blue) through a unique 5' to 5' triphosphate bridge. (5' cap structure by Zephyris, CC BY-SA 3.0)

Addition of the "Tail"

Following the termination of transcription, a long chain of adenine nucleotides, called the **poly-A tail**, is added to the 3' end of the RNA transcript through a process called **polyadenylation**. A sequence in the 3'UTR region, known as the **polyadenylation signal** (AAUAAA) determines the location of this poly-A tail. During polyadenylation, the majority of the 3'UTR is cleaved off and a specialized RNA polymerase, called polyA polymerase, catalyzes the addition of about 250 adenine nucleotides. Like the cap, the poly-A tail plays a role in nuclear export, protects the RNA transcript from RNAse activity (at the 3' end) and plays a role in regulating translation. Interestingly, the addition of a poly-A tail to prokaryotic transcripts enhances mRNA degradation.

Splicing

The coding sequence of a eukaryotic protein-coding gene will contain regions that will be translated into the amino acids of a polypeptide chain. These regions are called **exons** because they are expressed. In addition, there are regions that will not be translated as amino acids. These intervening sequences are called **introns.** Introns must be removed or "spliced out" of the RNA transcript and the exons ligated or joined together during processing. The process of removing introns and reconnecting exons is called **splicing.** Splicing take place in the nucleus by a complex of RNA and proteins, called a **spliceosome.** The spliceosome is made up of five **snRP subunits**, with each snRP comprised of a stretch of **snRNA (small, nuclear RNA)** and supportive proteins (Figure **3.3.9**).

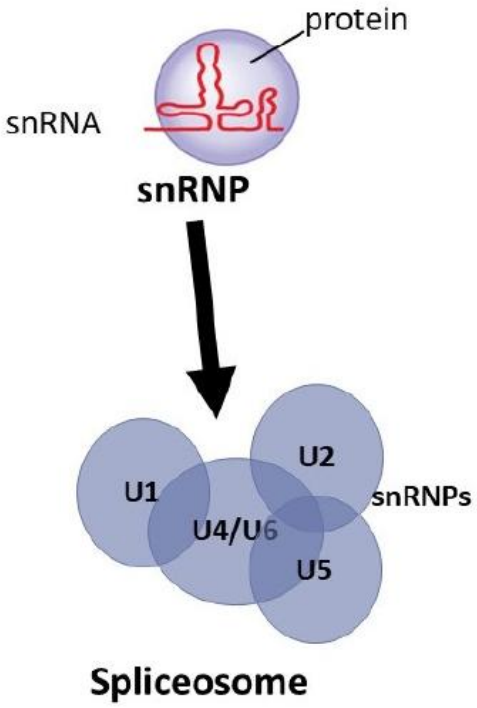


Figure **3.3.9**: The spliceosome. Splicing is performed in eukaryotes by a spliceosome. The spliceosome is made of subunits called small nuclear ribonucleoproteins (snRPs). Each snRP is made of protein and a piece of small nuclear RNA (snRNA). Five snRPs (U1, U2, U4, U5, U6) assemble to form the functional spliceosome. (The Spliceosome by Patricia Zuk, CC BY 4.0)

The individual snRPs (U1, U2, U4, U5, and U6) assemble in a specific order on the coding sequence of the RNA transcript. They assemble at sequences flanking the intron called **splice sites.** The sequence at the start of an intron is called the **donor site** and usually has the nucleotides "GU" somewhere within the sequence. The sequence at the end of the intron is called the **acceptor site** and contains the nucleotides "AG" within its sequence. These two sequences are recognized by specific parts of the spliceosome. Once the spliceosome forms, it cuts out the intron and ligates the flanking exons (Figure **3.3.10**). Following splicing, translation of the coding sequence produces the polypeptide that will become the functional protein.

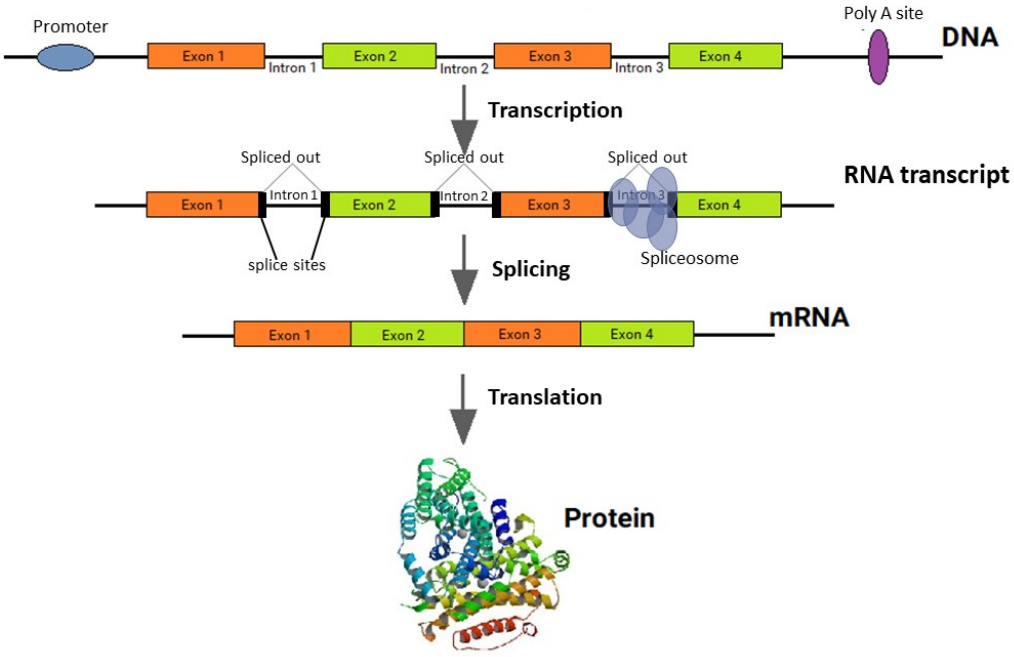


Figure **3.3.10**: Intron Splicing in eukaryotes. DNA is transcribed into an RNA transcript containing introns and exons. The spliceosome (blue circles) assembles at an intron using splice site sequences found flanking the intron (shown in black). Each intron is spliced out and exons are ligated together by the spliceosome. Translation of the spliced mRNA produces a polypeptide which will become a functional protein. (Intron Splicing by Patricia Zuk, CC BY 4.0; adapted from Transcript and Splicing by Ganeshmanohar, CC BY-SA 4.0)

The splicing activity of the spliceosome is actually performed by the *snRNA of the spliceosome and not the proteins*. An RNA with enzymatic activity is classified as a **ribozyme**. Therefore, the spliceosome is a ribozyme.

Concept in Action

Animation: Splicing

While all introns must be spliced from eukaryotic mRNA, exons also can be spliced. The removal of exons by the spliceosome creates novel mRNA transcripts and new "versions" of polypeptides. In this way, multiple polypeptide isoforms can be made from one coding sequence, depending on the exons spliced out. The process of exon removal to create different polypeptide isoforms is known as **alternative splicing** (Figure **3.3.11**). Alternative splicing increases genetic diversity as more than one polypeptide can be made from a single mRNA.

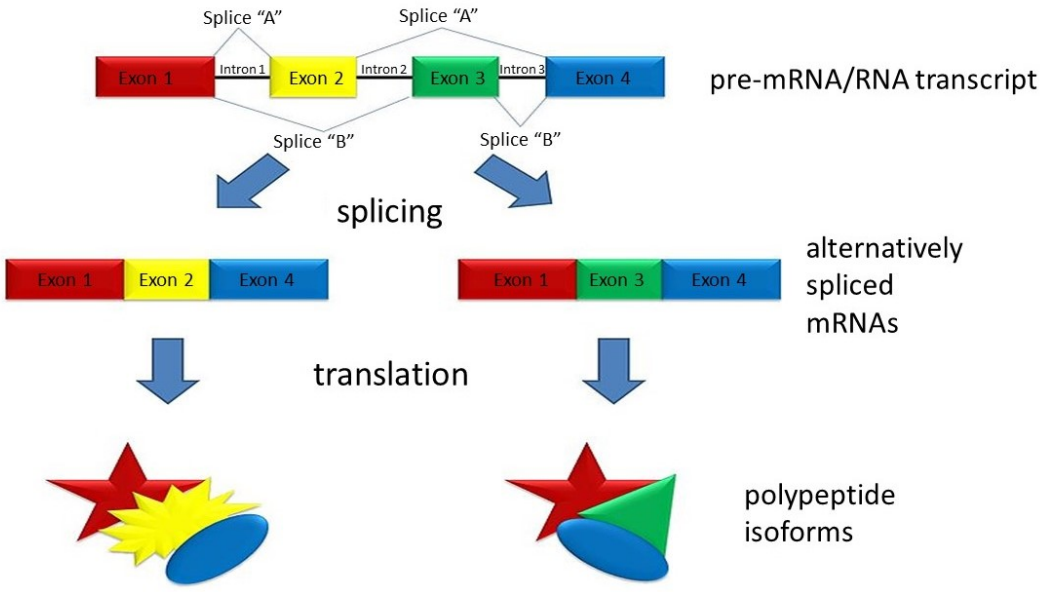


Figure **3.3.11**: Alternative splicing of RNA in eukaryotes.The removal of different exons can produce distinct, alternatively spliced mRNA molecules that, when translated, produce different protein isoforms. In splice plan "A", exon 3 is spliced out of the RNA transcription along with all introns; exons 1, 2, and 4 are retained. In splice plan "B", exon 2 is spliced out of the RNA transcription along with all introns; exons 1, 3, and 4 are retained. As each exon produces a region of the polypeptide, different polypeptide isoforms can be produced. (Alternative Splicing by Patricia Zuk, CC BY 4.0; adapted from Splicing Overview by Agathman, CC BY-SA 3.0)

Splicing must be carefully performed by the spliceosome. If the splicing process errs by even a single nucleotide, the nucleotide sequence of the rejoined exons would be shifted, and the resulting protein would be nonfunctional following translation.

Key Concepts

Transcription is the first step in gene expression in which the DNA coding sequence is copied to make an RNA molecule. However, distinctions do exist. Protein-coding genes in both prokaryotes and eukaryotes are transcribed into mRNA molecules. These mRNA molecules will form the starting point for the last step in gene expression: translation. Transcription is performed by an RNA polymerase, which links nucleotides to form an RNA transcript.

The major concepts to remember are:

- Prokaryotes and eukaryotes share many similarities when undergoing transcription
- Only the DNA strand with the 3' to 5' orientation serves as a template for transcription
- Transcription has three stages: initiation, elongation, and termination
- Transcription of DNA into RNA is performed by an RNA polymerase
- Protein-coding genes are transcribed into mRNA molecules
- In eukaryotes, mRNA is made through the addition of a 5' cap, and a 3' poly-A tail to the RNA transcript. All introns are cut out of the mRNA coding sequence through splicing.
- In eukaryotes, most protein-coding genes require the formation of a transcription initiation complex to increase the efficiency of transcription

Glossary

5' methylated cap: a modified guanine (G) nucleotide found at the 5' end of the mRNA strand; prevents mRNA degredation

3' poly-A tail: a long chain of adenine (A) nucleotides attached to the 3' end of the mRNA strand

Activators: DNA binding proteins that bind to the enhancer regions of a DNA strand

Alternative splicing: the process that allows a single gene to code for multiple proteins by varying the combination of exons included in the final mRNA

Coding sequence: the section of the DNA/RNA sequence that is translated into a polypeptide strand; composed of exons and introns; has the same sequence as the transcribed RNA (with thymine replaced with uracil)

Enhancer: a sequence of DNA that binds activators and enhances the process of transcription

Exon: a section of the coding sequence that is translated into amino acids

Intron: a section of the coding sequence that is not translated

Mediator protein: a large complex of proteins that mediates the interaction between activator proteins and transcription factors during transcription initiation

Messenger RNA (mRNA): a sequence of RNA produced through the process of transcription; specifies the sequence of amino acids following translation; comprised of a coding sequence and flanked by a 5' methylated cap and a 3' poly-A tail

Pre-mRNA: unmodified RNA produced through transcription; must be modified through the addition of a 5' cap, a 3' poly-A tail, and splicing; also called an RNA transcript

Polyadenylation: the addition of a long chain of adenine nucleotides (called the poly-A tail) to the 3' end of mRNA; enhances stability of the mRNA and translation

Promoter sequence: a sequence of DNA where the RNA polymerase binds; determines the site of transcription initiation

Ribosomal RNA (rRNA): a type of RNA that is a structural component of ribosomes; aids in protein synthesis

RNA polymerase: an enzyme that reads the DNA template strand and synthesizes an complementary RNA strand using RNA nucleotides

RNA processing: the modifications made to pre-mRNA, including capping, splicing, and polyadenylation, to produce mature mRNA

RNA transcription: the process by which a DNA sequence is copied into a complementary RNA strand

Spliceosome: a large complex of snRPs that splices out introns (and exons) from the coding sequence of mRNA

Small nuclear RNA (snRNA): a small sequence of RNA found as part of a small nuclear ribonucleoprotein (snRNP)

Small nuclear ribonucleoprotein (snRNP): a combination of an snRNA molecule and a protein; also known as a ribonucleoprotein (RNP)

Splicing: the process of removing introns (and exons) from the pre-mRNA transcript; also called RNA splicing

TATA Box: a specific DNA sequence found in the promoter region that helps position RNA polymerase for transcription initiation

Template strand: the DNA strand copied into a complementary RNA strand through the process of transcription; has the 3' to 5' orientation; also called the anti-sense strand

Termination Sequence: a specific DNA sequence that signals the end of transcription

Transcription factors: DNA binding proteins that bind specific sequences of DNA and assist in the initiation and regulation of transcription

Transfer RNA (tRNA): an RNA molecule that helps decode mRNA sequences into proteins by carrying specific amino acids to the ribosome

Untranslated region (UTR): a stretch of RNA that is not translated; contains important regulatory sequences for transcription and translation; found in front of the coding sequence (5' UTR) and behind the coding sequence (3' UTR); found in both DNA and RNA

3.3: Transcription of RNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

3.4: Translation of the polypeptide

Biotech Focus

One of the first experiments to try and determine the nature of the genetic code was performed by Francis Crick, Sydney Brenner, Leslie Barnett, and RJ Watts-Tobin. In this experiment, a mutagen known as **proflavin** was used to induce mutations in the DNA of **T4 bacteriophage**. The researchers knew that proflavin inserts itself between DNA bases, typically resulting in insertion or deletion of a single base pair. Additional treatment of bacteriophage with more proflavin introduces additional insertions or deletions. The researchers treated bacteriophage with proflavin and then used these mutated bacteriophage to infect *E.coli* bacteria. The infected *E.coli* were then grown on plates of agar. They proposed that proflavin-treated bacteriophage (i.e. mutated) would be unable to kill their bacterial hosts. As a result, the bacteria would grow as a sheet on the plate, known as a "lawn" of bacteria. In contrast, *E.coli* infected with non-treated bacteriophage (i.e. non-mutated) would be killed, producing a clear spot, or a "plaque", on the agar plate. Crick and his colleagues discovered that bacteriophage treated once with proflavin to introduce single insertions or deletions could not kill their *E.coli* hosts. With each round of proflavin exposure, additional single insertions or deletions were introduced and depending on the number of proflavin treatments, plaques would be seen. In this way, the Crick and his colleagues were able to determine that the codon consisted of three DNA nucleotides.

Introduction

Once DNA was accepted as the genetic material, the question became how could such a simple molecule with only four bases account for the inheritance of so many different physical traits? The recognition that enzyme activity could be inherited in the same way as morphological characteristics led to the "*one-gene-one enzyme"* hypothesis that earned G. W. Beadle, E. L. Tatum and J. Lederberg the 1958 Nobel Prize for Physiology and Medicine. When enzymes were later shown to be proteins, the hypothesis became "*one-gene-one protein"*. When proteins were shown to be composed of one or more polypeptides, the final hypothesis became ***"one-gene-one-polypeptide".*** This hypothesis stands today. In both prokaryotic and eukaryotic cells, protein-coding genes are expressed (i.e. transcribed and translated) as polypeptides. These polypeptides are then processed so that a functional protein can be produced.

Learning Objectives

Translation decodes the genetic code and produces proteins. At the end of this section, you will be able to:

- Describe the structure of the ribosome
- Explain how tRNA molecules are attached to amino acids
- Explain the genetic code used in translation
- Describe the initiation, elongation, and termination steps of translation
- Define the reading frame of translation and its importance in translation
- List and explain the four levels of protein processing

The Ribosome

The “machine” of protein translation is the **ribosome**. A ribosome is a complex macromolecule composed of rRNA and ribosomal proteins. Ribosomes exist in the cytoplasm of prokaryotes and can be found in the cytoplasm and attached to the rough endoplasmic reticulum of eukaryotes. In *E. coli*, there are between 10,000 and 70,000 ribosomes present in each cell at any given time. Both prokaryotic and eukaryotic ribosomes are made of two subunits: **the small subunit** and the **large subunit.** Each subunit is created by combining specific rRNA molecules with polypeptides. In prokaryotes, this assembly takes place in the cytoplasm, with the assembly occurring in the nucleolus in eukaryotes. Table **3.4.1** summarizes the ribosomes of prokaryotes and eukaryotes.

Table **3.4.1**: Comparison of Prokaryotic and Eukaryotic Ribosomes

Cell type	Subunit	rRNA	Number of proteins	Location of Synthesis
Prokaryote	Small	16S	21	Cytoplasm
	Large	5S, 23S	34	Cytoplasm
Eukaryote	Small	18S	33	Nucleolus
	Large	5S, 5.8S, 28S	48	Nucleolus

The small subunit of the ribosome has a site that recognizes and binds the mRNA, while the large subunit has three binding sites for tRNA molecules: the **A site (A = aminoacyl)**, the **P site (P = peptidyl)** and the **E site (E = "exit")** (Figure 3.4.1). The tRNA molecule at the P site is attached to the growing peptide chain. The tRNA at the A site is attached to the new, "incoming" amino acid to be added to the peptide chain. The tRNA at the E site has no amino acid and "exits" the ribosome from this site. During translation, these subunits will assemble around the mRNA.

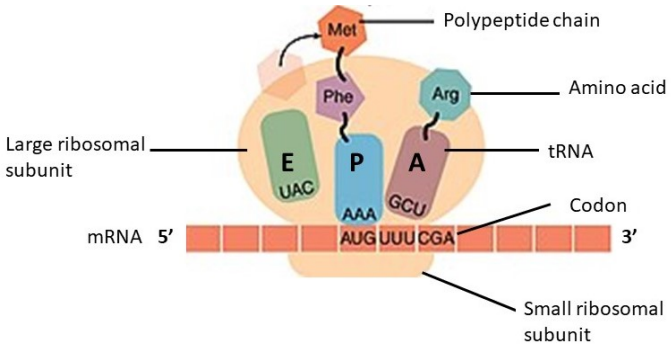


Figure 3.4.1: The ribosome. The assembled ribosome is made of a large and small ribosomal subunit attached to the mRNA molecule. The large ribosomal subunit is shown with three tRNA molecules positioned at the E (left binding site), P (center binding site), and A (right binding site) sites. (The Ribosome by Patricia Zuk, CC BY 4.0; adapted from Translation by OpenStax, CC BY-SA 4.0)

tRNAs

Transfer RNAs (tRNAs) are structural RNA molecules, roughly 70 to 80 nucleotides in length. They are transcribed from genes in the nucleoplasm by RNA polymerase III. Once transcribed, they assume a distinctive secondary and tertiary conformation (Figure 3.4.2). The secondary conformation can be visualized as a series of stems and loops. One loop, the **anti-codon loop**, has a three-nucleotide sequence that is complementary to the codon of the mRNA. At the opposite end of the molecule is the **amino acid attachment site**, the site where the amino acid is covalently bound. This site has a conserved sequence of "CCA". These two loops are also found at the opposite ends of the tRNA molecule when in its tertiary conformation.

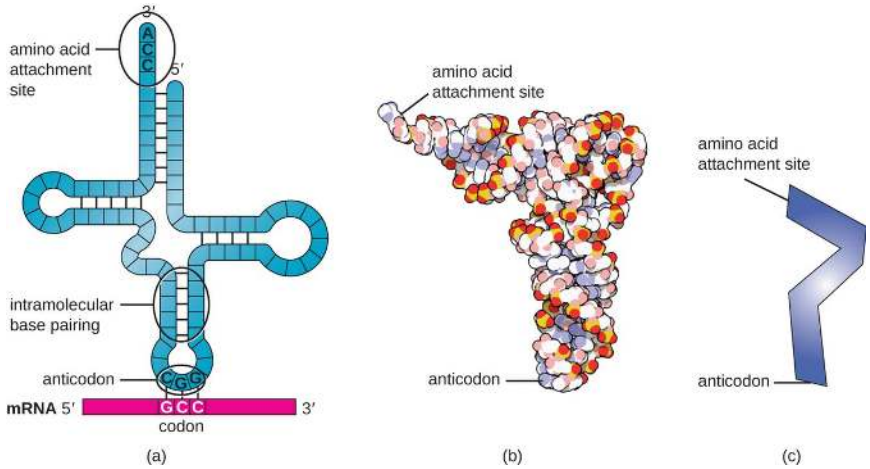


Figure **3.4.2**: The tRNA molecule. (a) The secondary structure of a tRNA molecule with its anti-codon loop bound to the mRNA codon (bottom) and its amino acid attachment site (top). (b) Tertiary conformation of the tRNA with these two sites identified. (c) Simplified representation of the tRNA. (attribution provided below)

The attachment of the amino acid to the amino acid site occurs in the cytoplasm by an enzyme complex called an **amino acyl-tRNA synthetase** (Figure **3.4.3**). Once attached, the tRNA is said to be "charged". This charging is powered by ATP hydrolysis and is highly specific, requiring that the shape of the enzyme's active site corresponds to the shape of the amino acid and tRNA molecule. Eukaryotic cells have at least 20 synthetases, each responsible for the attachment of a specific amino acid to the correct tRNA molecule. Depending on species and cell type, 40 to 60 types of charged tRNAs are used in eukaryotic translation.

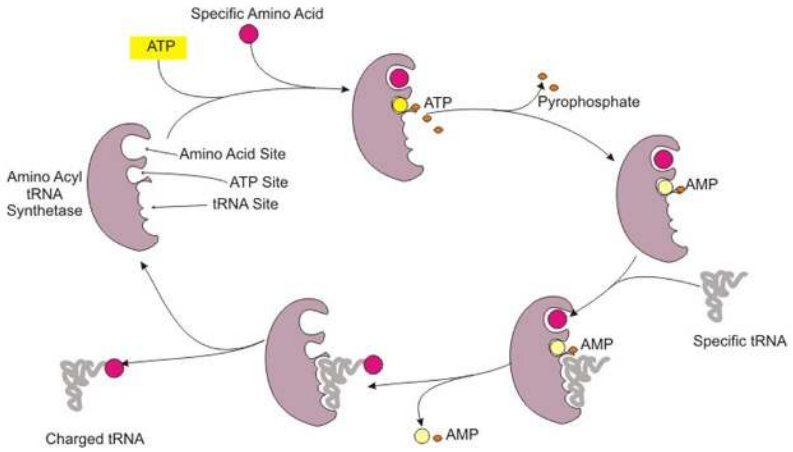


Figure **3.4.3**: tRNA "charging". Loading of a tRNA molecule with an amino acid, also known as tRNA "charging", is done using an enzyme called aminoacyl-tRNA synthetase. ATP and the specific amino acid (red circle) bind to their sites on the synthetase, followed by hydrolysis of the ATP to AMP. The corresponding tRNA (gray line) binds the synthetase. The release of the AMP is followed by linking of the amino acid to the tRNA. The "charged" tRNA is released from the synthetase and can be used in translation. (Charge tRNA by Boumphreyfr, CC BY-SA 3.0)

The Genetic Code of Translation

To translate the "message" of the mRNA into a polypeptide strand, the mRNA is read three nucleotides at a time by the ribosome. This three-nucleotide group is called a **codon**. Specific codons correspond to specific amino acids. This relationship between codon sequence and amino acid is known as the **genetic code** (Figure **3.4.4**). Of the 64 possible mRNA codons, 61 of them code for specific amino acids and three specify the termination of translation as **stop codons**. The genetic code is **universal**, meaning that the amino acids coded for are the same in most known organisms. In addition, the genetic code is **degenerate**, meaning a given amino acid can be encoded by more than one codon. For instance, the amino acid leucine is encoded by the codons CUA, CUU, CUC, and CUG. The exception to the degenerate code is the amino acid methionine, coded for by AUG. This is the only amino acid to have a single codon sequence. Similarly, stop codons are encoded by a single codon sequence.

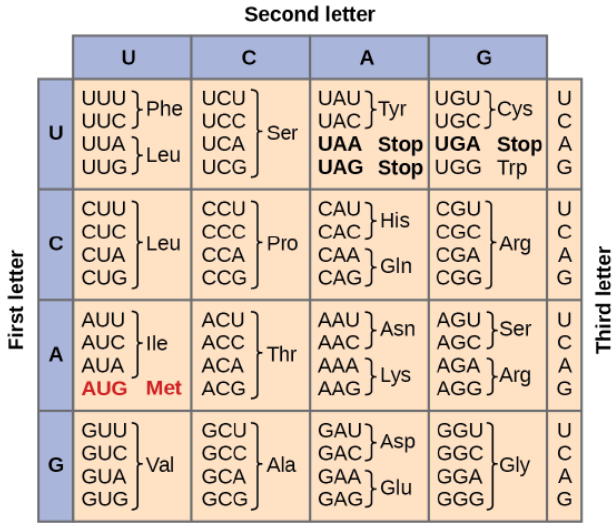


Figure **3.4.4**: The genetic code for translation. Specific sequences of 3 nucleotide letters, known as codons, code for specific amino acids or stop codons. (The Codon Table, from OpenStax Biology 2e, CC BY 4.0)

Translation

As with transcription, translation can be broken down into three stages: **initiation, elongation, and termination.** The actual process of translation in prokaryotic and eukaryotic cells is very similar. However, there are some differences in the initiation stage. In addition, because of the lack of a nucleus in prokaryotes, translation can take place at the same time as transcription.

Initiation of Translation

Translation in both prokaryotes and eukaryotes begins with the formation of an **translation initiation complex**, comprised of the small and large ribosomal subunits, the mRNA template, and a special **initiator tRNA (**Figure **3.4.5**). In prokaryotes, the initiator tRNA is called **tRNA^{fMet}**. In eukaryotes it is known as **tRNA-Met$_i$**. The assembly of the translation initiation complex requires the assistance of **initiation factors (IFs)** in prokaryotes and **eukaryotic initiation factors (eIFs)** in eukarryotes. To assemble the complex, the 5' end of the mRNA is first recognized by the small subunit. In eukaryotes, the small subunit recognizes the 5' methylated cap. The subunit binds the mRNA and "scans" toward the 3' end of the mRNA looking for the first AUG codon, or the "start codon" . This codon is located in the 5' UTR and is flanked on either side by specific nucleotides. Together, the AUG with its flanking sequences is known as the **Kozak sequence,** named for its discoverer, Marilyn Kozak. Because prokaryotic mRNA molecules do not have a 5' methylated cap, the small subunit recognizes a sequence of mRNA called the **Shine-Dalgarno sequence** (AGGAGG; named for John Shine and Lynn Dalgarno). The prokaryotic small subunit then stops at the AUG located just downstream sequence. Once the small subunit has stopped, the tRNA^{fMet} positions itself at the start codon. In eukaryotes, the tRNA-Met$_i$ accompanies the small subunit to the start codon. The large subunit is the last component to join the translation initiation complex, binding to the small ribosomal subunit so that the initiator tRNA is positioned at the P site.

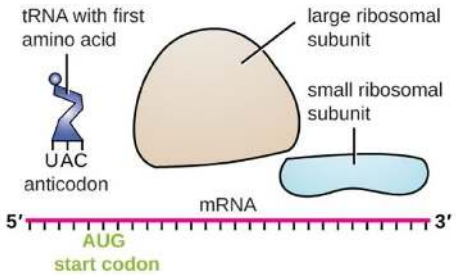


Figure **3.4.5**: The translation initiation complex. The components of the translation initiation complex in prokaryotes and eukaryotes include the two ribosomal subunits, the mRNA and the initiator tRNA bound to the first amino acid (i.e., methionine). (attribution provided below)

Elongation of Translation

Once the translation initiation complex forms around the mRNA, a new tRNA molecule, bearing the amino acid that corresponds to the next codon, binds to the ribosome at the A site. This binding represents the start of the **elongation phase**. The rRNA molecules of the ribosome, acting as a ribozyme, catalyze the formation of a peptide bond between the methionine and this new amino acid. The methionine is transferred to the tRNA at the A site, making dipeptide. The ribosome will then shift to the 3' end of the mRNA. A new tRNA/amino acid complex will enter the A site. The tRNA with the two amino acids will shift to the P site and the "empty" tRNA will enter the E site and exit the ribosome. The ribosome will move the dipeptide over to the new amino acid forming a peptide bond and a tripeptide. The ribosome will shift again and the process will repeat itself over and over again. As the ribosome moves to the 3' end of the mRNA, the peptide chain will elongate one amino acid at a time, producing a polypeptide.

Termination of Translation

Eventually, the ribosome's A site will be positioned over one of the three possible stop codons. At this point, a **release factor** will enter the A site, catalyzing the dissociation of the ribosomal subunits from the mRNA and the release of the polypeptide into the cytoplasm. Because the release factor is not bound to an amino acid, *the stop codon does not code for an amino acid*. As long as the mRNA molecule remains intact, the ribosomal subunits can reform around the mRNA to start the process of translation again.

The following summarizes the major events of translation (Figure **3.4.6**):

- **Initiation:** A translation initiation complex forms, involving the small ribosomal subunit, the initiator tRNA (with methionine), and the large ribosomal subunit. The small ribosomal subunit binds the mRNA and positions itself at the first AUG (i.e., the start codon), setting the “reading frame” of translation. The initiator tRNA and large subunit bind to complete the complex.
- **Elongation:** A tRNA with a new amino acid binds to the A site of the ribosome. The ribosome catalyzes the formation of a peptide bond between the peptide chain bound to the tRNA at the P site and the new amino acid bound to the tRNA at the A site. The ribosome shifts to the 3’ end of the mRNA so that the tRNA-peptide chain at the A site moves into the P site and the empty tRNA shifts into the E site to exit the ribosome.
- **Termination:** When the A site of the ribosome reaches a stop codon, a release factor binds to the A site, releasing the ribosome and peptide strand. The peptide strand is now considered to be a completed polypeptide

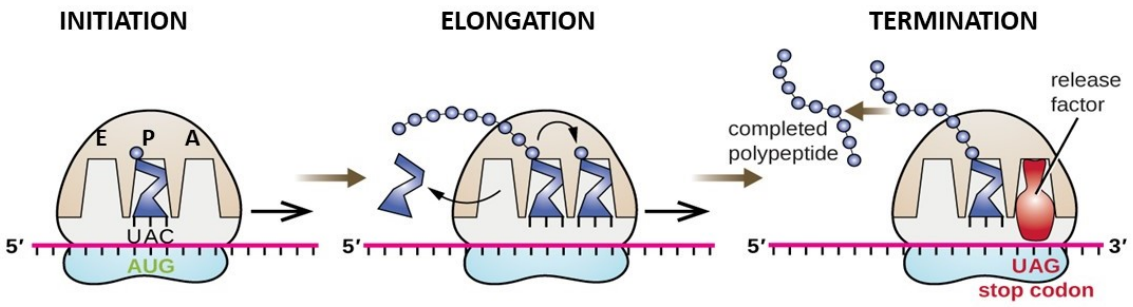


Figure **3.4.6**: Translation summary. Initiation: Translation begins when the small and large ribosomal subunits assemble around the mRNA and the first tRNA, bearing a methionine, binds to the AUG start codon that is positioned at the P site. Elongation: Translation continues as new tRNA molecules bind to the mRNA at the A site. The peptide chain at the P site is transferred from its tRNA to the tRNA located at the A site and forms a bond with the new amino acid, growing the peptide chain by one amino acid. Termination: Translation stops when the A site of the ribosome reaches a stop codon and a release factor, releasing the ribosome and peptide strand as a completed polypeptide. (attribution provided below)

Following translation, the polypeptide chain undergoes specific processing steps that drives its folding into a functional protein (see Protein Processing below). In addition, the polypeptide will undergo specific chemical modifications like the attachment of phosphate groups (i.e. phosphorylation) or methyl groups (i.e., methylation) to specific amino acids in the polypeptide sequence. The chemical modifications play important roles in the folding of the polypeptide and its resulting function.

Concept in Action

Animation: Translation

The Translation Reading Frame

Synthesis of polypeptides in both prokaryotic and eukaryotic cells begins with the amino acid methionine. In the majority of eukaryotic and prokaryotic mRNA sequences, this amino acid is specified by the first AUG codon encountered by the ribosome. Because it is the start of the polypeptide, this codon is called the **start codon**. Three codons, UAA, UAG, UGA, do not specify amino acids, but signal the end of translation. As such, they are called **stop codons**. The sequence of codons found in between the start codon and the stop codon is called the **reading frame**. The reading frame determines the sequence of amino acids in the polypeptide. "Mistakes" found in the mRNA sequence that eliminate or add nucleotides to the reading frame can "shift" the reading frame and alter the sequence of amino acids. These mistakes will be discussed next in Chapter 3.5: Mutations and DNA Repair.

Protein Processing

The translation of mRNA into a polypeptide strand is not the end of protein synthesis. Rather, it is only the beginning. Once translated, the polypeptide strand will undergo additional modifications so that a functional protein is made. These modifications produce distinct levels of **protein conformation or structure**, that directly affect function (Figure **3.4.7**). To read more about these levels of structure, go to Chapter 2.6 Proteins.

Primary structure/conformation: The amino acid sequence of the polypeptide produced through translation. The amino acid sequence has a direct effect on the additional conformation levels described below.

Secondary structure/conformation: The folding of the amino acid sequence into alpha helices and beta-pleated sheets. The secondary level is a result of hydrogen bonding between amino groups and carboxyl groups in neighboring regions of the polypeptide chain.

Tertiary structure/conformation: The unique three dimensional shape of the protein. Protein folding is a spontaneous process driven by interactions between the R groups of amino acids. These interactions include: hydrophobic (van der Waals) interactions, ionic bonding and hydrogen bonds. The final shape that results is the most energetically favorable one and is stabilized by the formation of disulfide bridges that form between the R groups of cysteine amino acids. Protein folding in eukaryotes is monitored by a class of proteins called chaperones.

Quaternary structure/conformation: The shape of a protein complex comprised on more than one protein subunit. Each subunit has its own unique tertiary conformation and interacts with one another through hydrogen bonds and hydrophobic/van der Waals forces. There are many types of proteins complexes, with globular (spherical) and fibrous being the most common. The majority of prokaryotic and eukaryotic proteins are made of multiple protein subunits.

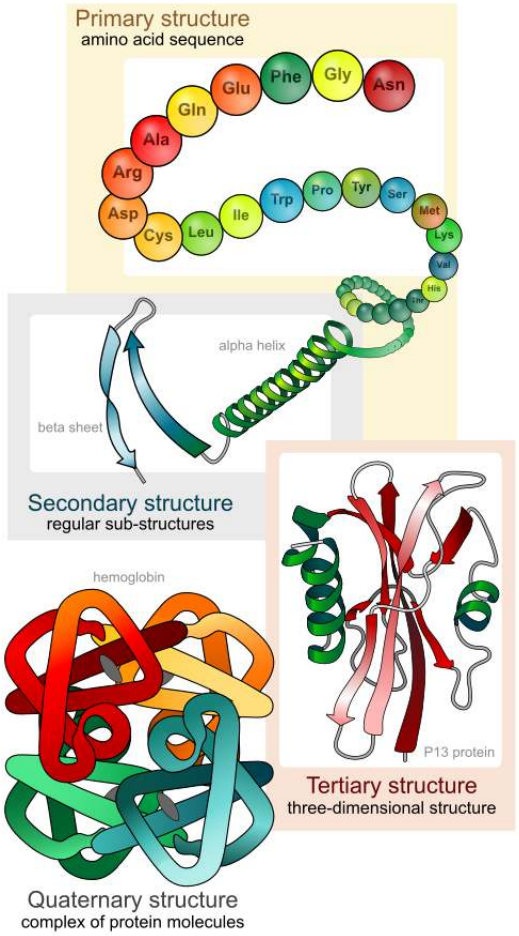


Figure **3.4.7**: The four levels of protein conformation. The primary structure of the protein (i.e. the amino acid sequence) is folded into regions of alpha helices and beta-pleated sheets to form the secondary structure of the protein. The folding of the secondary level into a unique three-dimensional shape is the protein's tertiary structure. Complex proteins assembled from more than protein subunit is the protein's quaternary structure. (Main Protein Structure by LadyofHats; public domain)

Key Concepts

Translation is the process that converts the "message" held in the mRNA into the polypeptide made of amino acids. Through specific post-translational modifications the polypeptide becomes a functional protein

Some major concepts to remember are:

- The genetic information in the mRNA "message" is translated into a sequence of amino acids through the process of translation
- Translation is performed by ribosomes that read the mRNA sequence in triplets called codons. Amino acids are brought to the ribosome by tRNA molecules.
- Each codon specifies one amino acid. Most amino acids are encoded for by more than one codon
- The first AUG codon of the mRNA sequence is the start codon and it codes for methionine
- Three codons, UAA, UAG, UGA, are stop codons and do not code for amino acids
- The sequence between the start and stop codon is the reading frame
- Translation is comprised of three stages: initiation, elongation and termination
- Translation initiation involves the assembly of the two ribosomal subunits on the mRNA sequence and the binding of the initiator tRNA to the start codon
- During elongation, the ribosome translates the reading frame into a polypeptide made of amino acids
- In termination, a release factor binds the stop codon and the ribosome dissociates from the mRNA and releases its polypeptide

- Once translated, the polypeptide undergoes additional modification that will produce the secondary, tertiary, and possibly a quaternary level of structure or conformation

Glossary

Anticodon: a sequence of three nucleotides found in tRNA that is complementary to a codon in mRNA; ensures the correct amino acid is added to the growing polypeptide chain

Aminoacyl-tRNA synthetase: an enzyme that attaches the correct amino acid to its corresponding tRNA

Codon: a sequence of three DNA or RNA nucleotides; corresponds to a specific amino acid or a stop signal during translation

Elongation: the process in which amino acids are sequentially added to the growing polypeptide chain

Initiation factor (IF): a protein that helps in the assembly of the ribosome on the mRNA and is required to start the process of translation; known as eukaryotic initiation factors (eIFs) in eukaryotic translation

Initiation: the first stage of translation, where the ribosome assembles around the mRNA and the start codon is recognized

Initiator tRNA: the first tRNA to enter the ribosome; carries methionine; binds the first AUG codon in the mRNA

Kozak sequence: a sequence of mRNA recognized by the small subunit of the eukaryotic ribosome

Messenger RNA (mRNA): a type of RNA that carries genetic instructions from DNA to the ribosome for protein synthesis

Release factor: a protein that binds within the ribosome to stop translation and release the ribosome from the mRNA

Ribosomal RNA (rRNA): a piece of RNA that is a structural and functional component of a ribosome

Ribosome: a large complex comprised of several rRNA molecules and multiple proteins; made in the nucleolus as a small subunit and a large subunit; the engine of translation

Shine-Dalgarno sequence: a sequence of mRNA recognized by the small subunit of the prokaryotic ribosome

Start codon: the first AUG codon of the coding sequence; codes for methionine

Stop codon: one of three codons (UAA, UAG, UGA) that stops translation; does not code for an amino acid

Termination: the final stage of translation, where a stop codon is recognized, and the completed polypeptide is released from the ribosome

Transfer RNA (tRNA): a small stretch of RNA that functions as an amino acid donor during translation

Translation: the process by which ribosomes synthesize proteins using messenger RNA (mRNA) as a template

Attributions

Figures taken from or adapted from OpenStax Microbiology, CC BY 4.0

- Figure **3.4.2**
- Figure **3.4.5**
- Figure **3.4.6**

3.4: Translation of the polypeptide is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

3.5: Mutations and DNA Repair

Biotech Focus

In 500 BCE, the Greek physician Hippocrates identified crab-like tumors that he called *karkinos*. From this word, we eventually derived the term **cancer**. Through our study of cancer, researchers began to study **mutagens** and their production of DNA mutations. The association between physical mutagens, like X-rays and ultraviolet (UV) light, and cancer was confirmed in the early part of the 20th century. However, a direct link to DNA mutations and chemical mutagens came later, when, in the 1940s, Charlotte Auerbach and JM Robson found that mustard gas caused mutations in the genomes of fruit flies. Today, through the use of the **Ames test**, a widely employed assay that exposes bacteria to a chemical followed by DNA sequencing, researchers can determine if a chemical will cause DNA mutations and be classified as a mutagen. The Ames test serves as a quick and convenient assay to determine the carcinogenic potential of a compound.

Introduction

The information encoded in the DNA sequence of a protein-coding gene determines the sequence of amino acids that will be found in the polypeptide. Because the amino acid sequence affects the folding of the polypeptide and the function of the protein, the DNA sequence of a protein-coding gene must be free of errors. The correct DNA sequence also determines the secondary structure and function of rRNA and tRNA molecules. Therefore, the correct DNA sequence is essential to the viability and function of an organism. A change in the normal DNA sequence is a **mutation**. Mutations in DNA can be caused by errors in replication, mitosis, meiosis or by exposing DNA to a **mutagen,** an agent that causes a heritable and permanent change in DNA sequence. This agent can be chemical or physical.

Learning Objectives

Errors in DNA sequences are called mutations. At the end of this section, you will be able to:

- Explain how mutations are classified
- Compare small scale vs. large scale mutations
- Explain the types of point mutations (frame-shift, missense, nonsense, and silent)
- Explain the difference between the two substitution mutations (transversion and transition)
- Explain frame-shift mutations (insertions and deletions) and their importance
- List and explain the types of chromosomal mutations
- Explain the major DNA repair mechanisms used in cells

Mutagen Classification

DNA mutations can be classified into two categories: **small-scale and large-scale.** Small-scale mutations affect only one or two nucleotides in a gene, whereas large-scale mutations can affect large stretches of DNA, or even an entire chromosome.

Small-scale mutations include:

- **substitution or point mutations**
- **insertions**
- **deletions**

Large-scale mutations include:

- **duplications**
- **deletions**
- **insertions**
- **inversions**
- **translocations**

Small-Scale Mutations

- **Substitution mutation:** a mutation that exchanges a single nucleotide for another (Figure **3.5.1**). An exchange of a purine for a purine (A for G; G for A) or a pyrimidine for a pyrimidine (C for T; T for C) is known as a **transition mutation**. The exchange of a purine for a pyrimidine (C/T for A/G; A/G for C/T) is called a **transversion mutation**. Transition mutations are more common. Substitution mutations can occur as errors made during DNA replication or can also be caused by chemical mutagens. A subsequent round of DNA replication will result in the mutation becoming a change of a base pair. A substitution mutation that occurs in the coding sequence of a protein-coding gene is often called a **point mutation**.

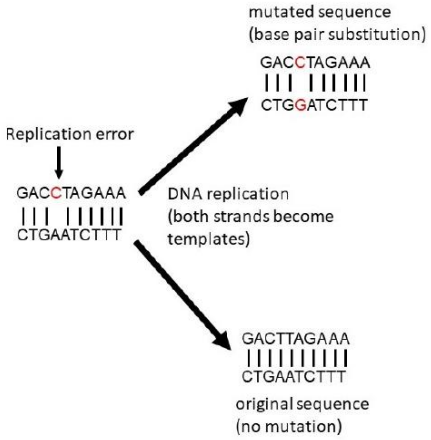


Figure **3.5.1**: Substitution Mutations in DNA. Errors in DNA replication result in substitution mutations in the DNA sequence and a change in base pair. The sequence to the left has a replication error in which a T has become an C. Following DNA denaturation, both strands become templates for DNA replication. The top strand with the replication error (red "C") will be used as a template for replication and produce the incorrect base pair of C-G (shown in red). The bottom strand has the correct sequence and the correct base pair of T-A will result following replication. Of the two replicated DNA helices, one will have a change in base pair while the other will be correct. (Substitution Mutations in DNA by Patricia Zuk, CC BY 4.0)

- **Insertion mutation:** an insertion mutation adds one or more nucleotides into a DNA sequence (Figure **3.5.2**). They are usually caused by transposable elements, or errors during DNA replication. Insertions in the coding region of a gene may alter splicing of the mRNA (**splice site mutation**), or cause a shift in the reading frame (**frameshift mutation**).
- **Deletion mutation:** a deletion mutation removes one or more nucleotides from the DNA and can occur during DNA replication (Figure **3.5.2**). Like insertions, these mutations can alter a splice site or the reading frame of the gene.

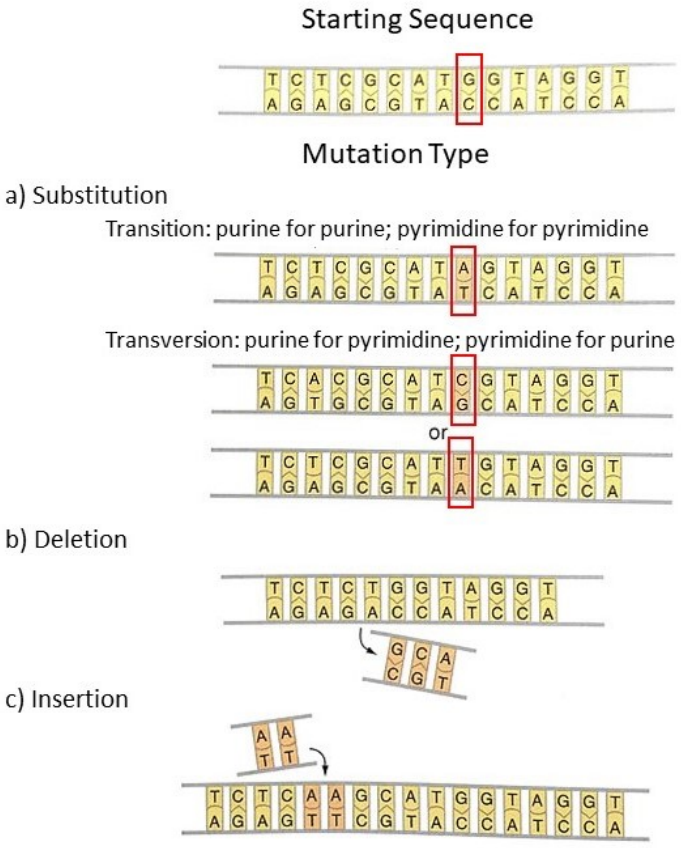


Figure **3.5.2**: Point Mutations. Small-scale mutations include substitution, deletion, and insertion mutations. Substitution mutations are classified as transition and transversion mutations. The original sequence is given at the top of the figure. For all sequences the base pair affected by the specific mutation is indicated with a box. (Point Mutations by Patricia Zuk, CC BY 4.0; adapted from Point Mutations and Their Effect by Hullo97; CC BY-SA 4.0)

Point mutations that occur in the coding region of protein-coding genes are more likely to affect the amino acid sequence of the polypeptide. These mutations can be categorized further by their effect on amino acid sequence (Figure **3.5.3**) into the following types:

• **Frameshift:** a frameshift is caused by either an insertion or deletion mutation. Due to the triplet nature of the codon, a frameshift results from the insertion/deletion of a number of nucleotides that is not divisible by three. The resulting shift can disrupt the reading frame of the coding sequence.

• **Missense:** a missense mutation changes a nucleotide and results in a different amino acid in the coding sequence

• **Nonsense:** a nonsense mutation results in a premature stop codon

• **Silent:** a silent mutation is a mutation that changes the codon within a coding sequence but does not change the amino acid. This is due to the degenerate nature of the genetic code. Silent mutations can also be found in non-coding regions of a gene, such as an intron or in a UTR, but because they are in the non-coding region of the gene, they are often simply called substitution mutations.

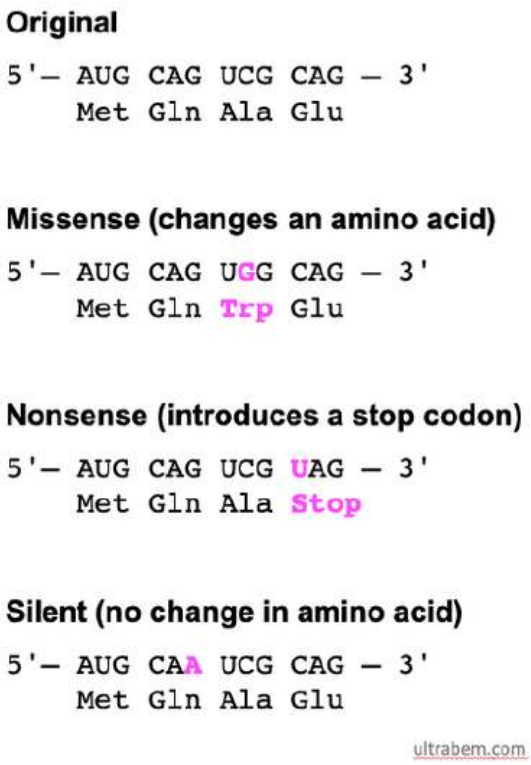


Figure **3.5.3**: DNA Point mutations. Small-scale point mutations in a coding region are classified based on their effect on the amino acid sequence. The original sequence is given at the top along with sequences produced by missense, nonsense, and silent mutations. The mutated nucleotide is indicated in pink, along with any change in the amino acid sequence. For clarity, the complementary DNA sequence is not shown. (DNA point mutations by Ultrabem; CCO)

A point mutation can be reversed by another point mutation. This type of point mutation is called a **reversion**. In a "true" reversion, the nucleotide is changed back to its original state and the correct amino restored in the polypeptide sequence. However, reversions can occur elsewhere in the coding sequence. This is known as a second-site reversion and produces a complementary mutation that restores gene function.

Large-Scale Mutations

Large scale mutations involve changes to the structure and composition of chromosomes (Figure **3.5.4**). These changes can have significant effects on the number, location, and activity of the genes found on that chromosome.

- **Chromosome Duplication:** also known as an amplification. A chromosomal duplication is a repetition of a specific chromosomal segment so that multiple copies of the genes within that segment are present.
- **Chromosome Translocation:** a mutation that exchanges chromosomal regions between non-homologous (i.e. different) chromosomes. Chromosomal translocations are usually harmless, as they do not result in the gain or loss of genetic information. However, in certain cases, the translocation might result in the fusion of two coding sequences. Such a gene fusion can increase the chances of developing cancer.
- **Chromosome Deletion:** a mutation in which a chromosomal region is deleted, leading to loss of the genes within that region. Chromosomal deletions can have a significant effect on the phenotype of an organism. In humans, several congenital diseases are associated with chromosomal deletions.
- **Chromosome Insertion:** a mutation in which a chromosomal region is inserted into another chromosome, leading to a gain of genes. This type of insertion is the result of a **translocation event**, so the insertion into one chromosome is accompanied by a chromosomal deletion in another chromosome. An alternate type of chromosomal insertion moves a chromosome region within the same chromosome. In both types, because no genetic information is lost, chromosomal insertions are usually harmless.
- **Chromosome Inversion:** a mutation that reverses the orientation of a chromosomal region. Chromosomal inversions may not have a deleterious effect in an organism as long as gene expression is not affected.

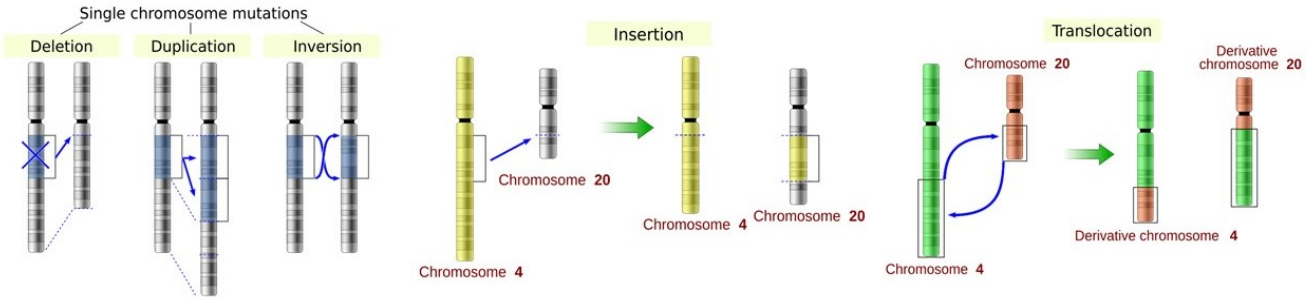


Figure 3.5.4: Large-scale mutations. Large-scale chromosomal mutations involve large chromosomal regions containing many genes. Chromosomal mutations include those involving a single chromosome (deletion, duplication, inversion) or two chromosomes (insertion, translocation). (Five Types of Chromosomal Mutations by YassineMrabet; Public Domain)

DNA Repair

DNA mutations are unavoidable. While some DNA mutations can be beneficial and underlie the evolution of a species, the majority of mutations may be harmful to an organism and must be corrected. Cells have numerous mechanisms for the correction of mutations. These mechanisms are known collectively as DNA repair.

The simplest mechanism of DNA repair is to prevent the mutation in the first place. Both prokaryotic and eukaryotic DNA polymerases have the ability to detect an incorrect nucleotide during DNA replication and replace it with the correct one. This "proofreading" capacity, however, has its limits. The correction must be done immediately. When a DNA polymerase adds the incorrect nucleotide to the 3' end of the daughter DNA strand, it can remove this nucleotide using its 3' to 5' exonuclease activity. An **exonuclease** is an enzyme that removes nucleotides *from the end of a DNA or RNA strand* (Figure 3.5.5). **3' to 5' exonucleases** remove these nucleotides at the 3' end of a nucleic acid, shortening the molecule in the 5' direction. A **5' to 3' exonuclease** would remove nucleotides from the 5' end of the nucleic acid. The DNA polymerase, when correcting a DNA replication error, will pause replication, remove the incorrect nucleotide from the 3' end and then replace it with the correct one. All prokaryotic DNA polymerases have this "proofreading" activity, along with the eukaryotic DNA polymerases delta and epsilon. This type of proofreading must be done immediately as the DNA polymerase cannot correct its mistakes once it continues to replicate the DNA.

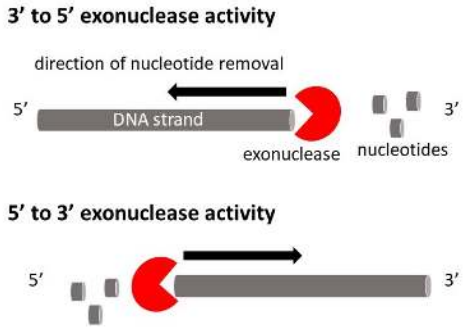


Figure 3.5.5: DNA Exonucleases. Exonucleases are enzymes that remove nucleotides from the end of DNA or RNA. A 3' to 5' exonuclease removes nucleotides at the 3' end in what is called 3' to 5' exonuclease activity. The 5' to 3' exonuclease removed nucleotides at the 5' end in what is known as 5' to 3' exonuclease activity. (DNA Exonucleases by Patricia Zuk, CC BY 4.0)

In addition to proofreading by exonucleases, prokaryotic and eukaryotic cells have additional DNA repair mechanisms. The following are summaries of common repair mechanisms found in human cells (Figure 3.5.6). These mechanisms are similar to those found in other eukaryotic cells and in prokaryotic cells. Each of these repair mechanisms involve another class of repair enzyme known as an **endonuclease**. An endonuclease is an enzyme that breaks (or "nicks") the phosphodiester bond *within the DNA or RNA strand* so that a nucleotide can be removed and replaced.

- **Base excision repair:** a repair mechanism for the correction of a G-T mismatch in DNA. A **DNA glycosylase** enzyme removes the T base, leaving the remainder of the nucleotide in the DNA strand. An endonuclease "nicks" the DNA strand by breaking the phosphodiester bond upstream of the mutation. Another endonuclease removes the sugar-phosphate remnant. A DNA polymerase replaces the removed nucleotide with the correct one. A DNA ligase replaces the phosphodiester bond.

- **Mismatch repair:** a repair mechanism for the removal of other base pair mismatches. Several protein complexes bind the parental DNA strand. One acts as an endonuclease to nick the mutated daughter DNA strand. Another acts as a helicase unwinds the DNA so that an exonuclease can remove nucleotides, creating a "gap" in the daughter strand that is filled in by a DNA polymerase. A DNA ligase replaces the missing phosphodiester bonds.
- **Nucleotide excision repair:** a repair mechanism for the removal of thymine-thymine dimers. A **thymine-thymine dimer (T-T dimer)** is a type of DNA damage produced by UV light. The UV light causes a bond to form between adjacent thymine bases (i.e. the dimer). This produces a physical distortion in the shape of the DNA helix that can prevent replication. To remove the dimer, a protein complex binds the dimer region. One of the protein complex subunits acts a helicase to unwind the DNA and give access to other components of the protein complex that will act as endonucleases to cut out the region containing the T-T dimer. This gap is filled in by a DNA polymerase and a DNA ligase replaces the missing phosphodiester bonds.

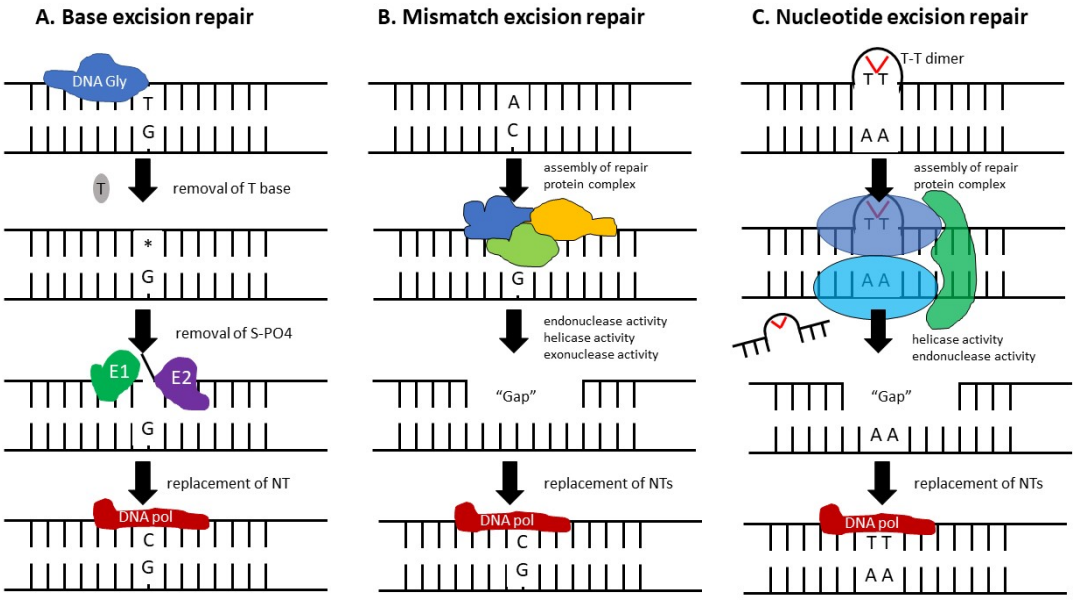


Figure **3.5.6**: DNA repair mechanisms in human cells. (A) Base excision repair is performed by the removal of the T base by a DNA glycosylase enzyme, followed by removal of the sugar-phosphate remnant by two endonuclease enzymes (E1, E2). The missing nucleotide is replaced by DNA polymerase (DNA pol) and phosphodiester bonds reformed by DNA ligase (not shown). (B) In mismatch nucleotide repair, a complex made of several proteins binds to the mismatch region. The complex acts as an endonuclease to break a phosphodiester bond in the "incorrect" DNA strand, as a helicase to unwind the DNA helix, and as an exonuclease to remove additional nucleotides to create a "gap". The gap is filled in by DNA polymerase and ligated by DNA ligase (not shown). (C) Thymine-thymine (T-T) dimers are repaired by nucleotide excision repair. A protein complex binds the T-T dimer. The complex unwinds the DNA helix (helicase activity) and removes the dimer and several surrounding nucleotides (endonuclease activity) to create a gap. As with other repair mechanisms, The gap is filled in by DNA polymerase and ligated by DNA ligase (not shown). (DNA Repair Mechanisms by Patricia Zuk, CC BY 4.0)

Key Concepts

DNA mutations can have significant effects on cellular function. Some major concepts to remember are:

- Changes in the DNA sequence, as a result of mutagens or replication errors, result in mutations
- Mutations can be classified as small-scale and large-scale
- Small-scale mutations that affect the coding region of a protein-coding gene are called point mutations
- Point mutations include missense, nonsense, and silent mutations
- Frameshift mutations are caused by insertions or deletions or nucleotides. Frameshift mutations in coding regions can alter the reading frame of translation
- Most errors in DNA replication are repaired by the proofreading capacity of the DNA polymerase
- Other mutations are repaired through DNA repair mechanisms that require multiple proteins acting in a coordinated fashion

Glossary

Deletion - a mutation that removes a nucleotide from a DNA sequence; produces a frame-shift

DNA damage - any alteration or modification to the DNA structure, which can lead to mutations. This includes single- or double-strand breaks, base modifications, and cross-linking

Endonuclease - an enzyme that hydrolyses a phosphodiester bond within a DNA or RNA sequence

Exonuclease - an enzyme that removes a nucleotide from the end of a DNA or RNA sequence; can remove nucleotides from either the 5' or 3' end

Frameshift mutation - a mutation caused by the insertion or deletion of nucleotides in a DNA codon; shifts the reading frame of the codons, potentially leading to a completely altered protein product

Insertion - a mutation that adds a nucleotide from a DNA sequence; produces a frame-shift

Inversion - a type of mutation where a section of DNA is reversed end to end; leads to changes in gene function or regulation

Mismatch repair - a process that fixes base-pairing mismatches, often caused by errors during DNA replication; involves recognition of the mismatch and excision of the incorrect base

Missense mutation - a mutation that changes the codon and the resulting amino acid

Mutagen - any physical or chemical agent that causes changes or mutations in the DNA sequence; includes UV radiation and certain chemicals

Mutagenesis - the process by which mutations are induced or occur naturally within an organism's DNA

Mutation - a change in a DNA sequence

Nonsense mutation - a mutation that changes codon to a stop codon

Point mutation - a mutation that involves a change in a single nucleotide base pair in the DNA sequence; can result in silent, missense, or nonsense mutations

Reading frame - the way of dividing a sequence of nucleotides into a set of consecutive, non-overlapping triplets called codons

Silent mutation - a mutation that does not affect the function of the protein; often produces a codon that codes for the same amino acid

Substitution mutation - a mutation where one nucleotide base is replaced by another; potentially alters the protein if the change affects the codon

Thymine dimers (T-T dimers) - a specific type of pyrimidine dimer, where two adjacent thymine bases bond together, distorting the DNA structure; typically formed by UV light exposure

Translocation - a mutation involving the rearrangement of parts between two non-homologous chromosomes; can lead to the fusion of genes or disruption of regulatory sequences

UV Radiation - a form of electromagnetic radiation that can cause DNA damage, including the formation of thymine dimers and other mutations

---

3.5: Mutations and DNA Repair is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

4: Genetic Engineering and Recombinant DNA Technology

4.1: Principles of Genetic Engineering

4.2: Creating Recombinant DNA

4.3: Applications of Recombinant DNA Technology

4: Genetic Engineering and Recombinant DNA Technology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

4.1: Principles of Genetic Engineering

Biotech Focus

In 1979, researchers at Genentech Inc. and City of Hope Hospital succeeded in cloning the human insulin gene into *E.coli* bacterial cells using the cloning plasmid pBR322. While the expression of this hormone was low, work at Genentech and at Eli Lilly eventually succeeded in increasing the yields of recombinant human insulin made by bacteria. Such work made it possible for a whole host of powerful therapeutic proteins to be made by bacteria. In 1982, recombinant human insulin was approved by the FDA for use by diabetics. In 1983, the drug **Humulin** (i.e., "human insulin") entered the market. Today, genetically modified *E.coli* and yeast strains, like *Saccharomyces cerevisiae*, are the principal sources for the therapeutic production of recombinant human insulin. However, continued genetic engineering research has created transgenic plants for the production of other recombinant proteins. Plants may represent a better option than bacteria and yeast due to their cost effectiveness, ease of production, and absence of human pathogens. For example, recombinant human insulin has been successfully expressed and produced in oilseeds of the plant *Arabidopsis thaliana*. In the future, transgenic seeds may act as "warehouses" for the stockpiling of critical therapeutic compounds like insulin.

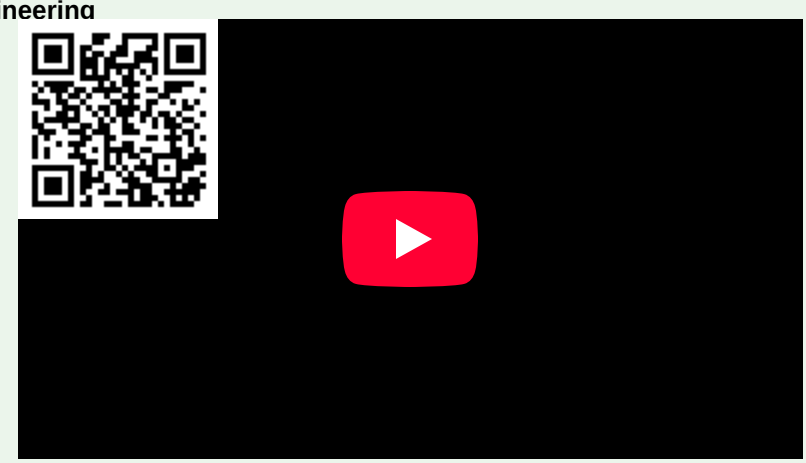
Introduction to the Principles of Genetic Engineering

Genetic engineering is the modification and manipulation of an organism's genetic information through the use of laboratory technologies. Genetic engineering alters an organism's genome through the introduction or removal of DNA, or by modifying the sequence of existing genes. Genetic changes may only be a single nucleotide in the DNA sequence, or the replacement of an entire gene. Genetic engineering is a key component of the biotechnology and is used in many settings, including research, medicine, agriculture, and industry. For more information about biotechnology, refer to Chapter 1: Introduction to Biotechnology.

Genetic engineering is often confused with breeding. In **breeding**, an organism (i.e., animal and plants) with a desired characteristic, known as the **phenotype**, is bred to another so that this characteristic is passed on to their offspring. Specific offspring, exhibiting a desired combination of parental characteristics, will be selected and bred again to either perpetuate their phenotype or improve upon the organism. In contrast, genetic engineering takes a gene directly from one organism and delivers it to another. This approach is much faster and can be used to manipulate any gene from any organism.

Concept in Action:

Video: Genetic Engineering



Learning Objectives

Genetic engineering is at the heart of biotechnology. This section will introduce you to some of the principles and tools of genetic engineering. At the end of this section, you will be able to:

- Explain the difference between genetic engineering and breeding

- List the "tools" used in genetic engineering
- Define DNA cloning and recombinant DNA
- List the major components of a plasmid
- Explain the difference between cloning and expression plasmids
- Define cosmids, and BACs
- Explain restriction enzymes and how they work

The “Tools” of Genetic Engineering

Genetic engineering utilizes several techniques in order to study and manipulate the genome of an organism. Examples of these techniques include:

- DNA cloning (i.e., recombinant DNA technology)
- Restriction digests
- Electrophoresis (DNA and protein)
- Sequencing
- PCR
- Northern, Southern, and Western blotting
- Immunofluorescence
- Transfection
- Transformation
- ELISA assays
- In vitro expression assays

The foundation of genetic engineering is the production of **recombinant DNA (rDNA)**. rDNA is created by combining DNA from different sources - often from different species - into a single piece of DNA that can be inserted into a host organism. rDNA is produced through a lab technique known as **DNA cloning, or cloning**. In a typical cloning protocol, a piece of DNA (usually a gene) is inserted into a bacterial plasmid to produce rDNA (Figure **4.1.1**). This rDNA can then be introduced into a bacterial cell so that it can be amplified by the host's replication machinery, or studied for its effect on the cell. The rDNA can also be introduced into a variety of eukaryotic cells (e.g. yeast, insect, mammalian) to study its functional effects. Because this approach is at the heart of genetic engineering, genetic engineering is often called **recombinant DNA (rDNA) technology.**

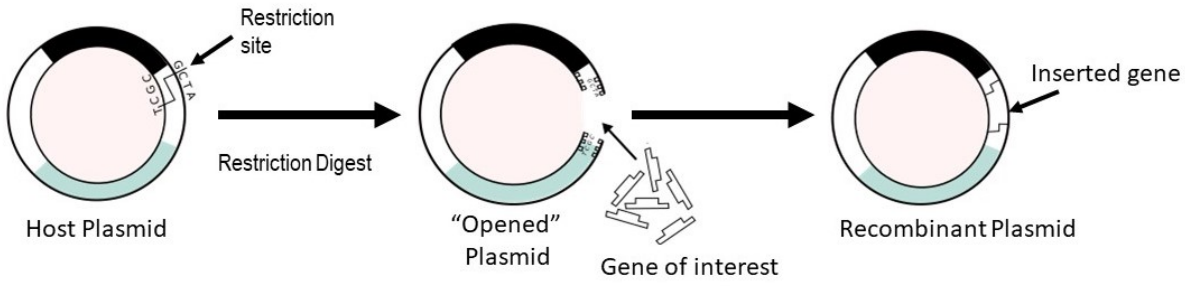


Figure **4.1.1**: Creating recombinant DNA (rDNA) through DNA cloning. Left image: A host plasmid can be cut open or "cleaved" at a specific sequence of DNA (i.e., a restriction site) using a technique called a restriction digest. Center image: The restriction digest creates an "opened plasmid" into which a gene of interest can be inserted. Right image: The plasmid containing the inserted gene is known as recombinant DNA. (Creating Recombinant DNA by Patricia Zuk, CC BY 4.0; adapted from Recombinant Formation of Plasmids by Minestrone Soup, CC BY-SA 3.0)

Plasmids, Cosmids, and BACs

DNA cloning requires the use of a DNA construct known as a **vector.** The vectors used in DNA cloning are plasmids, cosmids, artificial chromosomes, and viruses. Choosing which vector to use for DNA cloning will depend on a variety of factors, such as the size of the DNA being cloned.

The most common type of vector used in DNA cloning is the **plasmid**. As introduced in Chapter 3, a plasmid is a small, circular piece of DNA found *outside of the bacterial chromosome*. While plasmids have been identified in yeast, the term plasmid is usually

refers to bacterial plasmids. Biotechnology researchers use commercially available plasmids for many uses. These plasmids are derived from naturally occurring ones, but have been genetically engineered themselves so that they can be used in biotechnology protocols.

Plasmids can be classified into two categories:

1. **Cloning plasmids:** also known as cloning vectors; used to amplify the gene product in bacterial cells
2. **Expression plasmids:** used for the transcription and translation of the gene product in a specific cell type (e.g. eukaryotic cells)

Whether the plasmid is to be used for cloning or for expression, it typically has the following major components (Figure **4.1.2**):

- **Origin of replication (ori):** allows the vector to replicate within the host bacterial cell and controls the level of replication by the host cell's replication machinery
- **Selection marker(s):** a gene that when expressed helps select for the organism containing the plasmid; usually is a gene that confers antibiotic resistance; plasmids may have more than one selectable marker
- **Multi-cloning site (MCS):** a series of restriction sites that allows the target DNA to be inserted into the plasmid

Cloning plasmids are relatively simple in construction, containing the three components listed above. In contrast, expression plasmids are more complex because they must be constructed to allow for gene expression (i.e. transcription and translation) by the host cell. For example, the MCS in expression plasmids is flanked by a **promoter sequence** positioned upstream of the MCS and a **polyadenylation site** which is positioned downstream (Figure **4.1.2**). These sequences allow for continuous transcription of the gene that is inserted into the MCS. However, some expression plasmids contain promoter sequences that will only allow for transcription *if a specific growth condition is employed*, such as a specific temperature or the use of an inducing chemical. These promoters are called **conditional promoters**. Additional regulatory sequences are also found in expression plasmids that allow for protein translation in the host cell. Examples of such sequences would include a transcription initiation site, a ribosome binding site (RBS), and a start codon. Finally, some cloning and expression plasmids may contain "reporter genes" that facilitate the identification of host cell containing the plasmid.

The size of a plasmid can range from a few thousand kilobase pairs (kbps or kb) to 100 kb. Typical cloning plasmids are approximately 3000 to 7000 base pairs (bp) and can accommodate DNA inserts up to approximately 15,000 bp. As such, plasmids are suitable for cloning smaller DNA fragments such as genes or small gene clusters. For larger pieces of DNA, researchers can opt to use cosmids or artificial chromosomes.

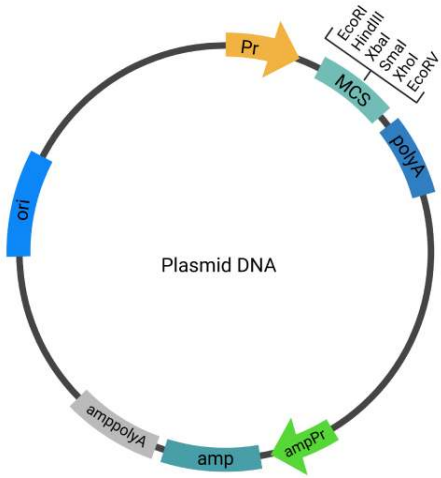


Figure **4.1.2**: The plasmid. A plasmid is a small, circular piece of DNA that can be used for the insertion of pieces of DNA. Both cloning and expression plasmids have several basic regions in common. The multi-cloning site (MCS), comprised of multiple restriction sites (e.g. EcoRI, HindIII, XbaI, SmaI, XhoI, EcoRV) is where genes of interest can be inserted. In expression plasmids, the MCS is flanked by a promoter sequences (Pr) and a poly-adenylation sequence (polyA) to help regulate the expression of the inserted gene. An origin of replication (ori) ensures replication of the plasmid by the host bacterial cell. Selection markers, like an antibiotic resistance gene, (amp for ampicillin resistance), are used to select for bacterial cells containing this plasmid. These selection markers can have their own independent promoter and termination regions for expression (ampPr, amppolyA). (Cloning Plasmid, by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Cosmids are hybrid molecules that combine the features of a plasmid and a bacteriophage. They contain plasmid sequences such as the origin of replication, the MCS, and selection markers, but also contain several regions of bacteriophage DNA. Because they contain bacteriophage DNA, they can be packaged into bacteriophage particles and replicate via the lytic cycle of the bacteriophage. Cosmids are larger than plasmids and can carry DNA inserts ranging from 30,000 to 45,000 base pairs, so they are used when larger DNA fragments need to be cloned. The presence of bacteriophage DNA helps stabilize larger pieces of DNA, making these vectors more stable than plasmids. Therefore, cosmids are particularly useful for constructing genomic libraries, mapping genes, and isolating large DNA fragments.

A **bacterial artificial chromosome (BAC)** is used to clone extremely large pieces of DNA (e.g. 100,000 to about 300,000 bp). The BACs, with their inserted DNA, can be introduced into bacterial cells. As with cosmids, BACs are used in constructing genomic libraries. Yeast and human versions of the BAC also exist (YAC and HAC, respectively).

Restriction enzymes

The insertion of DNA into a MCS requires the "opening" of the DNA with a **restriction enzyme**. A restriction enzyme (or **restriction endonuclease**) is a bacterial endonuclease that recognizes a specific sequence of DNA and "cuts" the DNA helix at specific points within that sequence. As such, think of a restriction enzyme as a "molecular scissor". The DNA sequence recognized by a restriction enzyme is a **restriction sequence** and its physical location in the DNA is called the **restriction site**. These two terms are often used interchangeably, but they are not the same thing. The restriction sequence is unique to the restriction enzyme. It range from 4 bp to 12 bp in size, and is said to be **palindromic,** meaning it reads the same in one DNA strand going left to right as it does in the other DNA strand going right to left.

All restriction enzymes make two incisions to cut open the DNA helix, one is through a phosphodiester bond on the "sense" DNA strand, and the other is through the a phosphodiester bond on the "anti-sense" strand. Restriction enzymes may cut the restriction sequence in an offset manner to create "overhangs" or "sticky ends" or they may cut the DNA helix in an aligned way to produce "blunt ends" (Figure **4.1.3**). The way a restriction enzyme cuts the DNA helix will determine how it is inserted into a vector, like a plasmid.

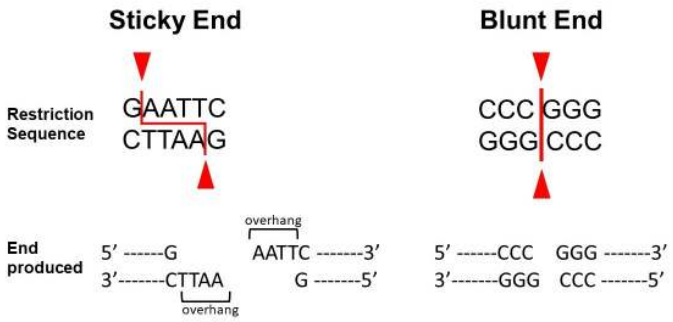


Figure **4.1.3**: Sticky vs. Blunt ends. Restriction enzymes may cut the DNA of a restriction sequence (red arrows and lines) to produce overhangs known as "sticky ends" or blunted ends known as "blunt ends". The sticky end is a single-stranded DNA overhang (e.g., TTAA and AATT) that forms because the restriction enzyme cuts two DNA strands at different locations. Sticky ends can form hydrogen bonds with complementary DNA sequences (e.g. AATT and TTAA) to reform double-stranded DNA. The blunt end has no overhangs because both DNA strands are cut at the same position (Sticky vs Blunt Ends by Patricia Zuk, CC BY 4.0)

Restriction enzymes are part of a bacterium's defense mechanism. Bacteria infected with bacteriophage DNA use their restriction enzymes to cut the "phage" DNA, rendering it harmless. As such, restriction enzymes are isolated from bacteria. The first restriction enzyme to be studied was isolated from *E.coli* bacteria and was named **EcoRI**. "Eco" indicated the bacteria the enzyme was isolated from (i.e. *E.coli*). "R" stood for the strain of *E.coli* and the Roman numeral "I" indicated it was a type I restriction enzyme. A similar nomenclature scheme is used today to identify all restriction enzymes currently available. To date, over 3,000 restriction enzymes have been isolated and studied, and more than 800 of these are available commercially. A few commonly used restriction enzymes are given in the Table **4.1.1** below.

Table **4.1.1**: **Common Restriction Enzymes and Restriction Sites**

Enzyme	Source	Restriction Site	Cut
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
BglII	*Bacillus globigii*	5'AGATCT 3'TCTAGA	5'---A GATCT---3' 3'---TCTAG A---3'
EcoRI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
EcoRV	*Escherichia coli*	5'GATATC 3'CTATAG	5'---GAT ATC---3' 3'---CTA TAG--5'
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
KpnI	*Klebsiella pneumoniae*	5'GGTACC 3'CCATGG	5'---GGTAC C---3' 3'---C CATGG---5'
NotI	*Nocardia otitidis*	5'GCGGCCGC 3'CGCCGGCG	5'---GC GGCCGC---3' 3'---CGCCGG CG---5'
PstI	*Providencia stuartii*	5'CTGCAG 3'GACGTC	5'---CTGCA G---3' 3'---G ACGTC---5'
SacI	*Streptomyces achromogenes*	5'GAGCTC 3'CTCGAG	5'---GAGCT C---3' 3'---C TCGAG---5'
SmaI	*Serratia marcescens*	5'CCCGGG 3'GGGCCC	5'---CCC GGG---3' 3'---GGG CCC---5'
StuI	*Streptomyces tubercidicus*	5'AGGCCT 3'TCCGGA	5'---AGG CCT---3' 3'---TCC GGA---5'
XbaI	*Xanthomonas badrii*	5'TCTAGA 3'AGATCT	5'---T CTAGA---3' 3'---AGATC T---5'
XhoI	*Xanthomonas holcicola*	5'CTCGAG 3'GAGCTC	5'---C TCGAG---3' 3'---GAGCT C---5'

Key Concepts

Genetic engineering manipulates and modifies the DNA of an organism using specific "tools" or techniques.

Some major concepts to remember are:

- The genome of a organism can be manipulated in the laboratory through genetic engineering
- Genetic engineering differs from breeding, which selectively crosses specific organisms to produce desired traits in their offspring
- Genetic engineering utilizes many different "tools"
- DNA cloning is at the heart of genetic engineering
- Genetic engineering is also known as recombinant DNA technology
- Plasmids are small, circular pieces of DNA used in cloning
- Cosmids and bacterial artificial chromosomes (BACs) are also used in cloning for large pieces of DNA
- Restriction enzymes are endonucleases that "cut" the DNA helix at specific sequences called restriction sites
- Each restriction enzyme has a unique restriction site
- Restriction enzymes either produce DNA overhangs called "sticky ends" or blunted ends called "blunt ends"

Glossary

Ampicillin resistance gene (ampR) - a selectable marker gene that provides resistance to the antibiotic, ampicillin; commonly found in plasmids

Bacterial Artificial Chromosome (BAC) - a genetically engineered DNA molecule used to clone large sequences of DNA in bacteria

Bacteriophage - a virus that infects bacteria; often used as a vector in recombinant DNA

Base pair (bp) - a unit of DNA length; consist of two complementary nucleotides (A-T or C-G)

Blunt end - produced when a restriction enzyme cuts double-stranded DNA in the middle of a restriction sequence; produces no overhangs

Cloning plasmid - a plasmid used to carry foreign DNA into a host cell for replication

Cosmid - a hybrid plasmid that contains sequences from a bacteriophage and is used to clone large DNA sequences

DNA cloning - the process of making multiple, identical copies of a piece of DNA by inserting the DNA sequence into a plasmid and transforming bacteria with this plasmid

Endonuclease - an enzyme that breaks (i.e. cuts) a phosphodiester bond found within a DNA sequence

Expression plasmid - a plasmid used for expression (i.e. transcription and translation) of an inserted piece of DNA in a host cell

Genetic engineering - the direct modification of an organism's DNA using biotechnology; often done to introduce new traits, to improve resistance to disease, or to produce beneficial products

Kilobase pair (kb) - a unit of DNA length that is 1,000 base pairs long

Plasmid - a small, circular piece of double-stranded DNA found outside of the bacterial chromosome; used in DNA cloning procedures; often used as a vector to introduce foreign DNA into cells

Polyadenylation sequence - a sequence of DNA that regulates the addition of the poly-A tail to mRNA; also called a poly-A sequence

Promoter sequence (promoter) - a sequence of DNA where the RNA polymerase binds; determines the site of transcription initiation

Recombinant DNA (rDNA) - DNA that is created in a laboratory by combining genetic material from different sources

Recombinant plasmid - a type of artificial DNA created by inserting a foreign DNA fragment into a plasmid

Restriction enzyme - an enzyme that cuts double-stranded DNA at a specific sequence; produces either a blunt end or a sticky end; also called a restriction endonuclease

Restriction sequence - the sequence of DNA cut by a restriction enzyme

Restriction site - the physical location of a restriction sequence in a DNA sequence

Selectable marker - a gene included in a plasmid that allows for the identification of successfully transformed cells; often an antibiotic resistance gene

Sticky end - a short, single-stranded overhang on a DNA fragment created by a restriction enzyme that cuts the DNA helix at different locations within the restriction sequence

Transformation - the process of inserting DNA into a bacterial host cell

Vector - a piece of DNA used to transport foreign genetic material into a host cell; includes plasmids, cosmids, BACs, and YACs

Yeast Artificial Chromosome (YAC) - a genetically engineered DNA molecule used to clone large sequences of DNA in yeast

4.1: Principles of Genetic Engineering is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

4.2: Creating Recombinant DNA

Biotech Focus

In 1973 researchers from Stanford and UCSF took a piece of DNA isolated from the African clawed frog, *Xenopus laevis*, containing a ribosomal gene and inserted it into a bacterial plasmid. They introduced the resulting **"recombinant DNA"** into *E. coli* cells and observed the replication of this recombinant DNA along with the bacterial host genome. This groundbreaking experiment was the first time an animal gene was **cloned**.

Listen to *PNAS Science Sessions* (opens in new window) to hear one of those researchers, Stanley Cohen, talk about their original paper on recombinant DNA.

Introduction to Creating Recombinant DNA

Genetic engineering is based upon the creation of **recombinant DNA (rDNA)**. The technology to create rDNA first emerged in the late 1960s, with the discovery of enzymes that could specifically cut and join double-stranded DNA molecules. Today, we know these enzymes as **restriction enzymes** (for cutting) and **ligases** (for joining). rDNA is created through a set of experimental methods that are known, collectively, as **DNA cloning**. DNA cloning (also known as molecular cloning) typically combines DNA sequences from two different organisms: the species that is the source of the DNA to be cloned, and the species that will serve as the living host for replication of the recombinant DNA. The use of the word cloning refers to the replication of cells, containing the rDNA, into a population of cells all with identical recombinant DNA molecules (ie., clones).

Learning Objectives

This section will outline the major steps in DNA cloning. At the end of this section, you will be able to:

- List the basic steps of molecular cloning
- Explain how plasmids and inserts are prepared for cloning
- Explain the differences between directional and blunt-end cloning
- Explain how a plasmid and insert are ligated together to form a piece of recombinant DNA called a recombinant plasmid
- Explain how transformation introduces recombinant plasmids into bacteria

Major Steps of DNA Cloning

DNA cloning is a multi-step process that takes many days in the laboratory to complete. The basic steps of DNA cloning are the same whether a plasmid, cosmid, or artificial chromosome is chosen as the vector. Because plasmids are more commonly used, this page will describe the steps of cloning into a plasmid. These steps are:

1. Isolation and preparation of the insert for cloning
2. Preparation of the plasmid for cloning
3. Combining the plasmid and insert DNA fragments suitably so they form the 'recombinant DNA' molecule (also called the **recombinant plasmid**)
4. Introducing this recombinant plasmid into the host cell (by transformation or transfection)
5. Selecting the host cells that have the correct recombinant plasmid
6. Confirmation of the recombinant plasmid inside the host

Preparation of the insert and plasmid for cloning

Both cloning and expression plasmids contain a specific region made up of multiple restriction sites. This region, the **Multi-Cloning Site, or MCS,** is the site of DNA insertion. In order to insert a piece of DNA into a plasmid (either for cloning or expression) both the DNA and plasmid must be "prepared" or digested with restriction enzymes. While not as important in a cloning plasmid, insertion of DNA into an expression plasmid must be done in a specific orientation so the DNA can be expressed from the nearby promoter. Thus, in both cloning and expression plasmids, insertion into a MCS is typically done using a "**directional cloning**" strategy where the plasmid and insert are cut with two different restriction enzymes. This type of restriction digest is called a **double digest** because it uses two different restriction enzymes. This digest will produce unique DNA single-stranded overhangs at each end of the insert. DNA

overhangs, complementary to the ends of the insert, will also be produced in the plasmid. As a result, there will be only one way that the DNA can be inserted into the plasmid. To learn about the restriction digest, go to Chapter 13.4: Restriction digestion of DNA.

Figure 4.2.2 and Figure 4.2.3 below shows such a directional cloning strategy using the restriction enzymes HindIII and PstI. In the first part of the procedure shown in Figure 4.2.2, both the DNA insert and plasmid are digested with HindIII and PstI. Digestion of the DNA insert with Hind III produces a 5' overhang with the sequence "AGCTT". Digestion of the insert with PstI will produce a 3' overhang of "GGTAC". When the plasmid is digested with the same two enzymes, a HindIII overhang of "TTCGA" and a PstI overhang of "CATGG" is produced (the tiny bit of plasmid DNA found between these two overhangs will be removed).

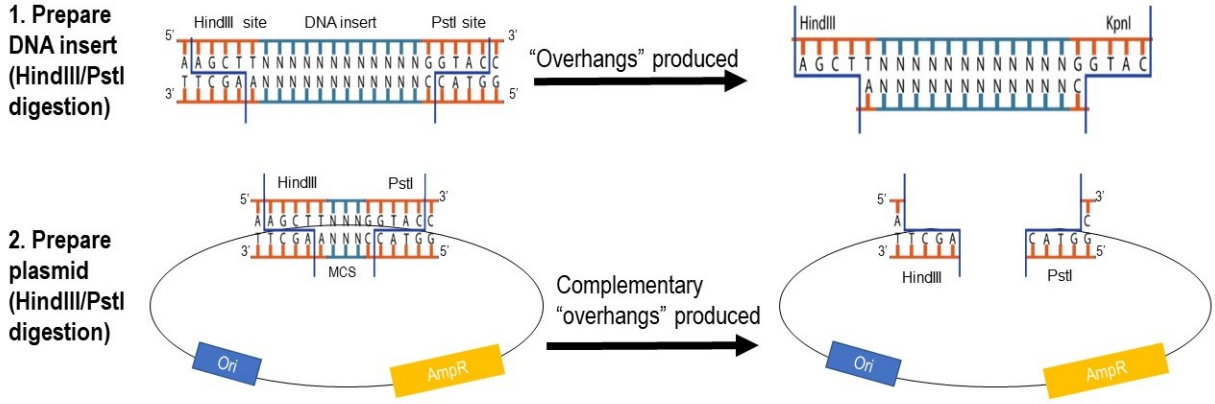


Figure 4.2.2: A directional cloning strategy: Part 1. The first steps of a directional cloning strategy using the restriction enzymes HindIII and PstI are: (1) Prepare the DNA insert (HindIII/PstI digestion) - digestion of insert DNA with HindIII and PstI produces overhangs of AGCT and GTAC, respectively; (2) Prepare the plasmid (HindIII/PstI digestion) - digestion of plasmid DNA with HindIII and PstI produces the complementary overhangs of TCGA and CATG, respectively). (Blue box = Ori; origin of replication; yellow box = AmpR; ampicillin resistance gene) (Directional Cloning Part 1 by Patricia Zuk, CC BY 4.0)

These complementary ends are critical for the correct ligation of the insert and plasmid to form a new piece of recombinant DNA.This is because the DNA insert overhangs of AGCT and GTAC are complementary to the plasmid overhangs of TCGA and CATG (Figure 4.2.3). As a result, there is only one way that the DNA insert can "fit" into the plasmid. Once the insert and plasmid are brought together, an enzyme called a **DNA ligase** will form the phosphodiester bonds required to join these pieces of DNA together. Following ligation, the HindIII and PstI restriction sites are regenerated and can be used to digest out the insert if desired.

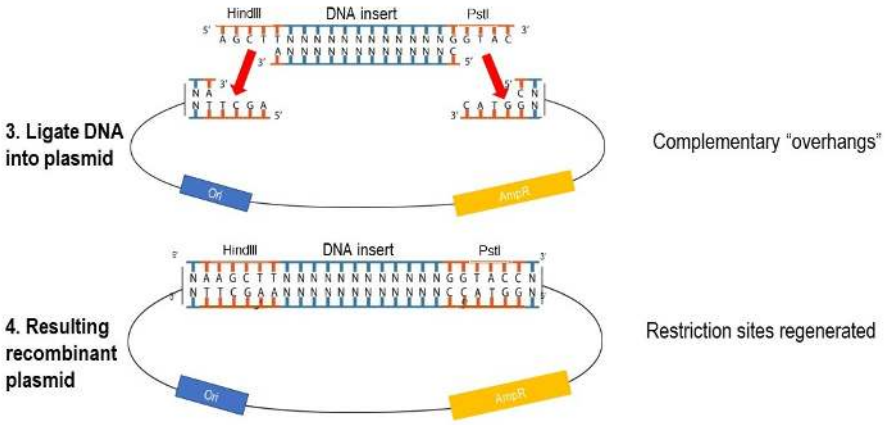


Figure 4.2.3: A directional cloning strategy: Part 2. The remaining steps of the directional cloning strategy are: (3) Ligate DNA into plasmid - ligation of DNA insert and plasmid is accomplished because the overhangs complement one another (AGCT and TCGA; GTAC and CATG). (4) The resulting recombinant plasmid contains the insert DNA and the HindIII and PstI sites are regenerated at either end. (Blue box = Ori; origin of replication; yellow box = AmpR; ampicillin resistance gene) (Directional Cloning Part 2 by Patricia Zuk, CC BY 4.0)

While directional cloning is the preferred strategy, there are times when restriction enzymes that produce a "blunt" end must be used. This technique is called **blunt end cloning**. In blunt end cloning, the restriction enzyme cuts both strands of DNA at the same position within the restriction site. As a result, no overhangs are produced and the ends are said to be "blunt" (Figure 4.2.4).

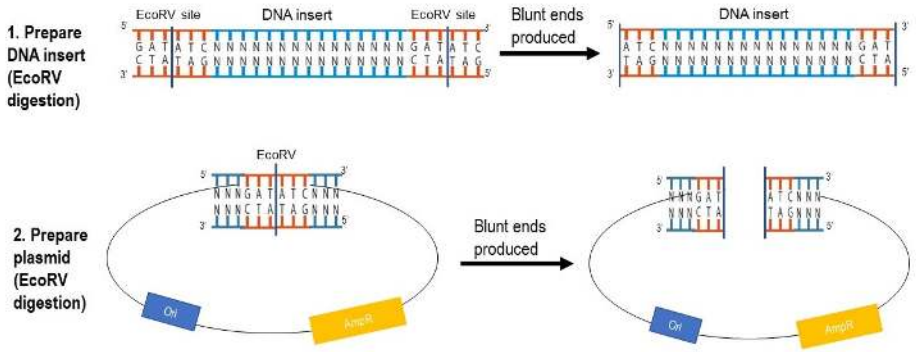


Figure 4.2.4: Blunt end cloning: Part 1. In "blunt end" cloning, both the DNA insert and plasmid are digested with a restriction enzyme that produces blunt ends. The first steps of a blunt end cloning strategy using the enzyme EcoRV are as follows: (1) Prepare the DNA insert (EcoRV digestion) - digestion of insert DNA with EcoRV produces the blunt ends of ATC and GAT at either end of the insert (sense DNA strand sequence given for clarity); (2) Prepare the plasmid (EcoRV digestion) - digestion of plasmid DNA with EcoRV produces the blunt ends of GAT and ATC (sense DNA strand given for clarity). (Blue box = Ori; origin of replication; yellow box = AmpR; ampicillin resistance gene) (Blunt End Cloning Part 1 by Patricia Zuk, CC BY 4.0)

As with directional cloning, once the DNA insert and plasmid are digested with the restriction enzyme, the two are ligated to one another by the DNA ligase. Following ligation, the two EcoRV restriction sites are regenerated and can be used to digest out the insert if desired (Figure 4.2.5).

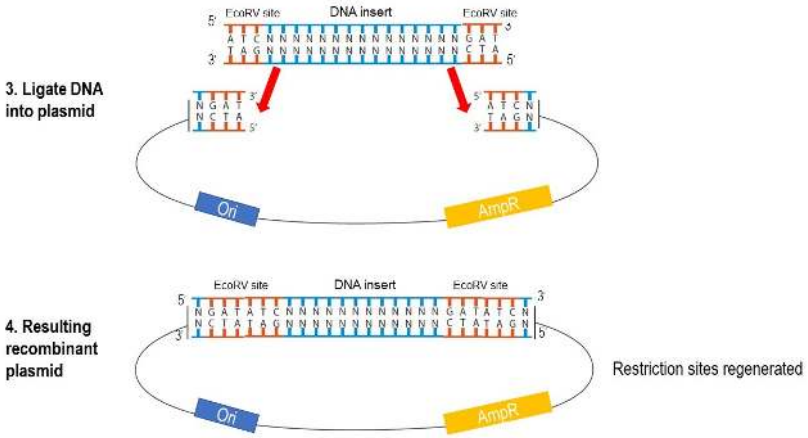


Figure 4.2.5: Blunt end cloning: Part 2. The remaining steps of a blunt end cloning strategy using the restriction enzyme EcoRV are: (3) Ligate DNA into plasmid - ligation of DNA insert and plasmid inserts the DNA into the plasmid. (4) The resulting recombinant plasmid contains the insert DNA and the EcoRV sites are regenerated at either end. (Blue box = Ori; origin of replication; yellow box = AmpR; ampicillin resistance gene) (Blunt End Cloning Part 2 by Patricia Zuk, CC BY 4.0)

Because there are no overhangs produced when using restriction enzymes that produce blunt ends (i.e., blunt end restriction enzymes), it is important that the DNA insert be ligated into the plasmid so that the restriction sites are regenerated. The regeneration of the restriction sites ensures the correct orientation of the insert so that it can be expressed, if desired. Figure 4.2.6 below shows the correct cloning orientation of a DNA insert.

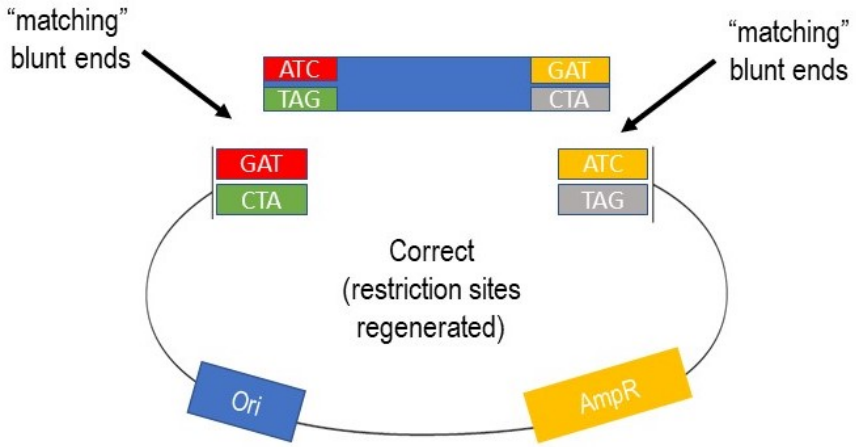


Figure **4.2.6**:The correct blunt end cloning direction. The cloning of a DNA insert with blunt ends must be performed so that the restriction sites are regenerated. The sense and anti-sense strands of the DNA insert and plasmid digested with a blunt end restriction enzyme are shown with four different colored rectangles and include their sequences. The correct orientation is shown above with the blunt ends of the DNA insert "matching" up to the blunt ends of the plasmid. This orientation regenerates the EcoRV restriction site sequence of GATATC at both ends of the cloning site. (Correct Blunt End Cloning by Patricia Zuk, CC BY 4.0)

In addition to this orientation, there are three other possibilities for cloning the DNA insert. However, none of these will regenerate the restrictions sites at either end of the DNA insert (Figure **4.2.7**). While these other orientations may permit gene expression, the protein made will be incorrect. Therefore, when using a blunt end strategy, it is vital to confirm the orientation of the insert within the plasmid. This is typically done through DNA sequencing.

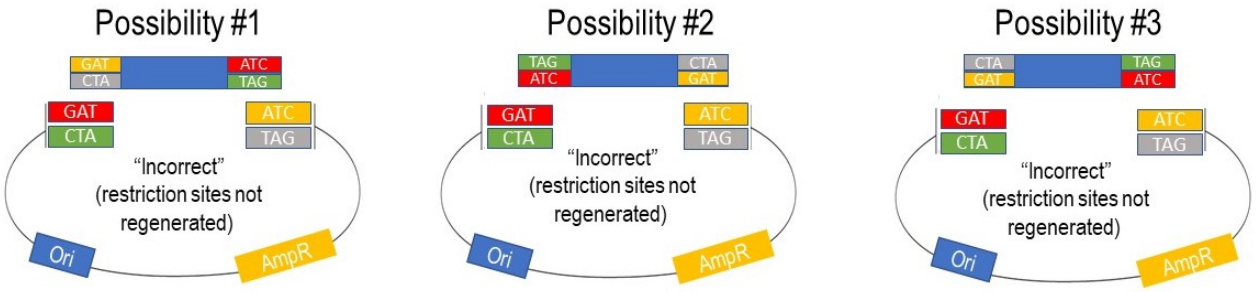


Figure **4.2.7**: Incorrect blunt end cloning possibilities. Three possible cloning orientations are shown. Each of these are incorrect because they do not regenerate the restriction site sequence of GATATC at either end of the cloning site. (Blue box = Ori; origin of replication; yellow box = AmpR; ampicillin resistance gene). (Blunt End Cloning Possibilities by Patricia Zuk, CC BY 4.0)

Concept in Action

Animation: Restriction Enzymes

Ligation of the insert and plasmid to produce the recombinant plasmid

Insertion of the DNA insert into the plasmid requires the formation of new phosphodiester bonds between the two pieces of DNA. This is done by a type of enzyme called a **DNA ligase** (or ligase). Ligation of the insert and plasmid will join these two fragments into one piece, restoring the circular nature of the plasmid and forming a piece of rDNA known as a **recombinant plasmid** (Figure 4.2.8). As described above, ligation will also regenerate the restriction sites used in cloning so that the DNA insert can be cut out again, if desired. It is important to know that DNA ligation is not 100% effective. Some plasmids will resume their circular configuration without incorporating a DNA insert. These "false positives" will need to be identified. To learn about DNA ligation, go to Chapter 13.5: Ligation of insert DNA into a plasmid.

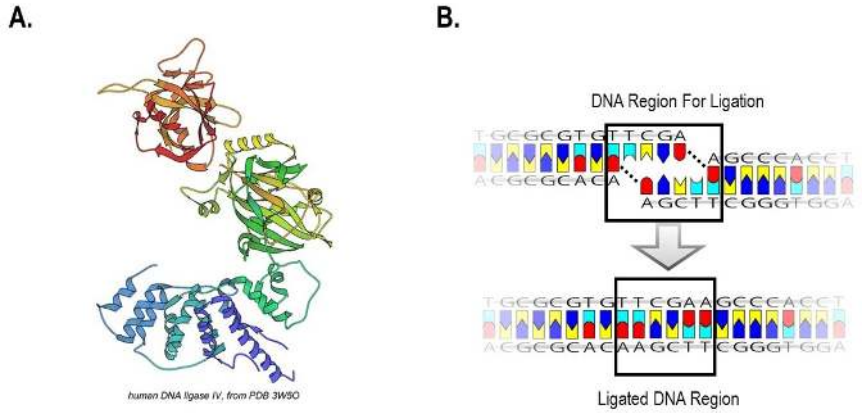


Figure 4.2.8: DNA ligation. (A) The ribbon structure of a human DNA ligase enzyme. (DNA ligase by Dcrjsr, CC BY-SA 4.0)
(B) The ligation of DNA by DNA ligase requires the formation of phosphodiester bonds between adjacent nucleotides. Once ligated, the DNA helix is one continuous piece. In the top figure, the region for ligation is shown in the black box with the forming phosphodiester bonds shown as dotted lines. In the bottom figure, the ligated region is shown in the black box. (Ligation by Madprime, CCO 1.0)

Transformation of host cells with the recombinant plasmid

Once the DNA insert has been ligated into the plasmid, the recombinant plasmid is then introduced into bacterial cells so that the bacterial host cell can replicate the recombinant plasmid along with its own DNA. This introduction is done through a technique called **bacterial transformation, or transformation** (Figure 4.2.9). Transformation relies upon the finding that bacteria will take up "naked" DNA from its surroundings if exposed to specific environmental conditions. One common method of transformation is to weaken bacterial membranes through their exposure to ice-cold salt solutions. These weakened bacteria are then mixed with recombinant

plasmids and then "heat shocked" at 42°C for a short period of time. In a mechanism not understood, this heat shock allows for the entry of the recombinant plasmids into the bacteria. To learn about bacterial transformation, go to Chapter 13.6: Bacterial transformation.

Transformation is very inefficient. The majority of bacteria will not incorporate recombinant plasmids. As such, a selection process for identifying those bacterial cells containing recombinant plasmids must be used to eliminate those bacterial cells that do not have any recombinant plasmids. This process relies upon the presence of selection markers in the recombinant plasmid. The most common type of selection marker is an **antibiotic resistance gene** - a gene that will confer resistance to specific antibiotics that bacteria are not normally resistant to. Common antibiotic resistance genes will provide resistance to ampicillin, tetracycline, or kanamycin. Bacteria that are transformed with recombinant plasmids are typically grown overnight on agar plates containing a specific antibiotic. Only those bacteria with recombinant plasmids should survive exposure to this antibiotic. These bacteria will propagate on the agar over night and form a **bacterial colony**.

As with many other molecular techniques, antibiotic selection is not foolproof. Some transformed bacteria will contain plasmids that do not contain an insert. Other bacteria may survive exposure to the antibiotic and will have no plasmid at all. As such, confirmation of the recombinant plasmid within the bacteria must be obtained. This confirmation will necessitate the isolation of the recombinant plasmid DNA from bacterial cells (Chapter 13.7: Isolation of DNA from bacteria (i.e., the “mini-prep”), followed by additional tests to confirm the presence of the insert, such as polymerase chain reaction/PCR (Chapter 13.2: Polymerase Chain Reaction (PCR) and agarose gel electrophoresis (Chapter 13.3: Agarose gel electrophoresis).

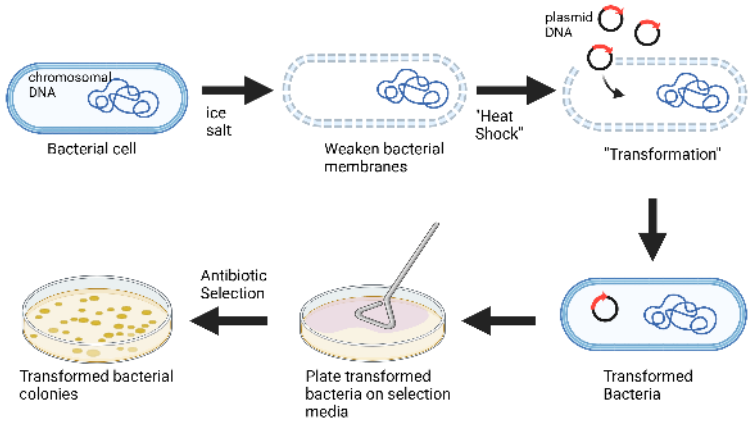


Figure **4.2.9**: Transformation of bacterial cells. Bacteria are weakened using ice-cold salt solutions prior to incubation with recombinant plasmids. "Heat Shocking" the weakened bacteria allows for the entry of recombinant plasmids. Plating of transformed bacteria onto agar plates containing an antibiotic should select for those bacteria containing recombinant plasmids. Growth of these bacteria results in the formation of colonies. (Bacteria Transformation by Patricia Zuk, CC BY 4.0; created in BioRender. Zuk, P. (2025))

Recombinant plasmids can also be introduced into mammalian cells through a process known as transfection (Chapter 13.10: Liposomal transfection into mammalian cells). Transfection typically occurs after the recombinant plasmid has been introduced into bacteria, amplified, and the presence of the DNA insert inside the recombinant plasmid has been verified.

Lab Protocols

Creating Recombinant DNA

- Lab Technique: Amplification of DNA by PCR
- Lab Technique: Polymerase Chain Reaction (PCR)
- Lab Technique: Restriction digestion of DNA
- Lab Technique: Ligation of insert DNA into a plasmid

Transformation of Bacteria

- Lab Technique: Bacterial transformation
- Lab Technique: Isolation of DNA from bacteria (i.e., the “mini-prep”)
- Lab Technique: Agarose gel electrophoresis

Transfection of Eukaryotic Cells

- Lab Technique: Liposomal transfection into mammalian cells

Key Concepts

The creation of recombinant DNA is a multi-step process that is known as DNA cloning. Some major concepts to remember are:

- recombinant DNA is created through by inserting DNA into a plasmid
- plasmid DNA is cut open by enzymes called restriction enzymes
- restriction enzymes cut the DNA helix at specific sequences called restriction sites
- many plasmids have a multi-cloning site made up multiple restriction sites
- in directional cloning, the DNA can be inserted into the cloning site of the plasmid in only one direction
- in blunt-end cloning, the DNA can be inserted into the cloning site of the plasmid in multiple directions
- insertion of DNA into a plasmid requires an enzyme called a ligase

Glossary

Antibiotic resistance gene - a gene found within a plasmid that makes the bacteria resistant to a specific antibiotic

Blunt end - produced when a restriction enzyme cuts double stranded DNA in the middle of a restriction site; produces no overhangs

Blunt end cloning - a cloning strategy that uses restriction enzymes that produce blunt ends

Directional cloning - a cloning strategy that uses restriction enzymes that produce sticky ends

DNA cloning - the process of making multiple, identical copies of a piece of DNA by inserting the DNA sequence into a plasmid and transforming bacteria with this plasmid

Ligase - an enzyme that forms a phosphodiester bond between the 3' end of one DNA fragment and the 5' end of another DNA fragment; also called a DNA ligase

Multi-cloning site (MCS) - several restriction enzyme sites clustered together in a plasmid; the site of insertion of a foreign piece of DNA

Plasmid - a small, circular piece of DNA found outside of the bacterial chromosome; used in DNA cloning procedures

Polylinker - another name for a multi-cloning site (MCS)

Recombinant DNA (rDNA) - DNA that is created in a laboratory by combining genetic material from different sources

Recombinant plasmid - a type of artificial DNA created by inserting a foreign DNA fragment into a plasmid

Restriction enzyme - a protein that cuts double-stranded DNA at specific sequences; produces either a blunt end or a sticky end

Restriction site - the sequence of DNA cut by a restriction enzyme

Sticky end - a short, single-stranded overhang on a DNA fragment created by a restriction enzyme that cuts the DNA at a staggered position

Transformation - the process of inserting DNA into a bacterial host cell

4.2: Creating Recombinant DNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

4.3: Applications of Recombinant DNA Technology

Biotech Focus

In 2023, the U.S. Department of Agriculture (USDA) reported the slaughter of approximately 32 million cattle for the commercial meat market, making the U.S. is one of the largest beef producers in the world. More and more, people are beginning to become concerned about the use of animals for meat.

Enter **recombinant DNA technology**....

Today, recombinant DNA techniques are being used to engineer growth factors and other critical substance that help animal cells grow and multiply under lab conditions. These recombinant compounds mimic the natural environment found in the animal and can support the *in vitro* development of muscle and fat cells into edible tissue - i.e., **lab-grown meat**. New companies have since been created to take advantage of this emerging technology to produce commercial-grade cultured meat with the look, texture, and taste of farm-raised beef and chicken. While it may sound "gross", lab-grown meat has the potential to reduce the environmental impact of meat production and eliminate animal cruelty. Moreover, this meat can be tailored for better nutrition (e.g., less saturated fat, more omega-3s), minimizing the health impacts of meat consumption on humans.

Lab-grown meat? "Impossible" no longer.

Introduction

Recombinant DNA (rDNA) technology (i.e., DNA cloning) is a powerful biotechnology tool that allows scientists to manipulate DNA in order to create genetic combinations that do not occur naturally. Today, rDNA is a part of everyday life, without people being aware of it. In medicine, rDNA technology has been used to genetically modify bacteria and yeast, turning them into living "factories" of therapeutic proteins, like hormones and growth factors. In agriculture, rDNA has revolutionized farming by engineering crops to be more resistant to pests and drought, in addition to enhancing their nutritional content. In the food industry, rDNA is used to produce enzymes in food processing, providing more consistent, ethical, and cost-effective alternatives to animal-derived ingredients. Genetically-engineered microbes are used to produce biofuels by converting biomass into ethanol or other renewable energy sources, supporting cleaner energy solutions. In manufacturing, recombinant enzymes like proteases and amylases are incorporated into laundry detergents to improve stain removal and reduce the need for high-temperature washing. Plants engineered through recombinant DNA are used in environmental biotechnology to clean up pollutants from soil or water. With each passing year, how rDNA technology is being applied is becoming more and more varied.

Learning Objectives

Recombinant DNA (rDNA) technology has applications in a wide variety of fields, including that of medicine. At the end of this section, you will be able to:

- Explain some common applications for rDNA technology

Applications for Recombinant DNA Technology

rDNA and Medicine

In medicine, rDNA technology drives the development of numerous treatments that can be used in healthcare and medicine. Through its combination of DNA from different sources, rDNA technology is used in the production of **therapeutic proteins**, like hormones and growth factors, the development of **vaccines** and **monoclonal antibodies**. It is also at the heart of gene therapy research, where it holds promise for treating genetic disorders by correcting defective genes. To learn more about the use of rDNA in medical biotechnology, go to Chapter 10: Medical Biotechnology.

rDNA and Genetically Modified (GM) Crops

rDNA technology is used in the creation of **genetically modified (GM) crops** in order to insert desired genes into the genome of plants. The ultimate goal of this technology is to give crops new or enhanced traits that are not naturally present. Examples of genes used in the modification of crops are those that will confer insect resistance, drought tolerance, or improve nutrition. One of the

most successful application of rDNA in the production of a GM crop is the production of "Bt crops" like Bt corn or Bt cotton. In these crops, a gene from the soil bacterium *Bacillus thuringiensis* is inserted into the plant. This gene enables the plant to produce a protein that is toxic to certain insect pests but safe for humans and animals, reducing the need for chemical pesticides. Similarly, herbicide-tolerant crops, such as glyphosate-resistant soybeans, have been engineered with rDNA to survive the application of weed-killing herbicides, making weed control more efficient. By using rDNA technology, these genetic modifications improve crop yields, reduce agricultural input costs, and contribute to more sustainable farming practices. To learn more about how rDNA technology is used in the genetic modification of plants go to Chapter 9: Agricultural and Environmental Biotechnology.

rDNA and Food Processing

rDNA technology is a key part of the food industry and its efficient, safe and ethical production of enzymes and additives. Instead of extracting these substances from animal or plant sources, scientists can insert the genes responsible for producing them into microbes like bacteria or yeast. These microbes can be used to easily and quickly mass-produce the desired compounds through **fermentation**. A well-known example is recombinant **chymosin**, an enzyme used in cheese-making. Traditionally obtained from calf stomachs, chymosin is now produced using genetically-modified *E. coli* or yeast that express the bovine chymosin gene. As a result, rDNA technology has made commercial cheese production more consistent, cost-effective, and suitable for vegetarians. In addition, rDNA is used to produce other enzymes like amylases, lipases, and proteases, which are used in commercial baking, brewing, and juice clarification. The end result of rDNA technology in food processing is improvements in food quality, shelf life, and processing efficiency while reducing reliance on animal sources and environmental impact.

rDNA and Biofuel Production

Today, rDNA technology is being used extensively to genetically-modify bacteria, yeast, or algae, to efficiently convert plant **biomass** into **biofuels** like bioethanol or biodiesel. One common approach is the introduction of the gene for **cellulase** into industrial strains of *Saccharomyces cerevisiae* (i.e., brewer's yeast). The end result is a microbe capable of digesting **cellulose**—a component of plant cell walls that yeast can't normally can't break down—into simple sugars that are then fermented into bioethanol. Similarly, algae can be engineered with rDNA to enhance their production of lipids, which can then be processed into biodiesel. These approaches allow for the enzymatic breakdown and fermentation of biomass like agricultural waste (e.g., cornstalks, rice husks), forestry scraps (e.g., wood chips, sawdust), and municipal wastes (e.g., sewage) into biofuels, making biofuel production more sustainable, cost-effective, and offering cleaner alternatives to fossil fuels. The use of genetically-modified organisms in biofuel production is also decreasing the impact of biomass on the environment. For more information about microbes and biofuel production, go to Chapter 7: Microbial Biotechnology.

rDNA and Industrial Enzymes

rDNA technology is used in the production of **industrial detergents** by enabling the large-scale, cost-effective production of critical enzymes that improve cleaning performance. As an alternative natural sources for these enzymes, rDNA enables the genetic modification of microbes like *E. coli*, *Bacillus subtilis*, or yeast. These engineered microbes then produce specific enzymes through fermentation, which are harvested and added to detergents. Common recombinant enzymes used in industry include **proteases**, for the break down of protein-based stains (e.g., blood, egg), **amylases** to break down starches, **lipases,** for the break down fats and oils found in grease stains, and **cellulases** that remove "microfuzz" and brighten fabrics. Because these recombinant enzymes work more effectively at lower temperatures than their "natural" counterparts, they make detergents more energy-efficient and environmentally friendly. rDNA also allows manufacturers to design enzymes that are more stable in harsh washing conditions like high pH or the presence of bleach. To read more about industrial biotechnology, go to Chapter 11: Industrial Biotechnology.

rDNA and the Environment

rDNA has become an important part in **bioremediation** and its cleaning up of the environment. Genetically-engineered microbes and plants have been found to be very efficient at breaking down, absorbing, or neutralizing environmental pollutants such as heavy metals, oil spills, pesticides, plastics and industrial waste. These microbes can be combined with unmodified or "natural" organisms to enhance certain cleanup processes. For example, bacteria like *Pseudomonas putida* has been genetically-modified using rDNA to degrade common industrial pollutants, such as toluene and xylene. rDNA has been used to engineer microbes with enzymes that break down plastic, offering a potential solution to plastic pollution. Finally, genes that help bacteria tolerate or accumulate heavy metals like mercury, arsenic, or lead, can also be inserted, allowing these bacteria to clean-up contaminated

water or soil. By tailoring the engineering of microbes to clean up specific contaminants, rDNA technology makes bioremediation faster, more targeted, and cost-effective. To learn more about how rDNA technology is used in bioremediation go to Chapter 9: Agricultural and Environmental Biotechnology.

Key Concepts

Recombinant DNA technology can be applied to a vast number of scientific fields, including agriculture, medicine, and industry. Some major concepts to remember are:

- common genetically-modified microbes include bacteria, yeast, and algae
- genetically-modified microbes are used to create therapeutic proteins, produce enzymes, and generate biofuels
- the use of genetically-modified microbes in the production of biofuels is helping to decrease the disposal of large amount of biomass
- genetically-modified crops are revolutionizing the agriculture field by making crops more tolerant to pests, pesticides, and harsh environmental conditions
- genetically-modified organisms are a key part of bioremediation, making this area more cost-effective and efficient

Glossary

Amylase - an enzyme that breaks down starches into simple sugars like glucose

Biofuel - a fuel made from biological materials (i.e., biomass); includes bioethanol and biodiesel

Biomass - organic material from plants or animals that is used as a renewable energy source

Bioremediation - the use of living organisms, like bacteria or plants, to clean up environmental pollutants

Cellulase - an enzyme that breaks down cellulose into glucose

Cellulose - a complex polysaccharide made up of repeating glucose molecules; found in the cell wall of plants and algae

Chymosin - an enzyme used in cheese-making to curdle milk

Genetically-modified (GM) crop - a crop whose DNA has been altered using genetic engineering techniques to introduce traits like pest resistance, herbicide tolerance, or improved nutritional content

Fermentation - a biological process where microbes (like bacteria and yeast) convert sugars into other products like alcohol, gases, or acids

Lipase - an enzyme that breaks down fats (i.e., lipids) into glycerol and fatty acids

Monoclonal antibodies - antibodies produced by clones of a single immune cell known as a B cell

Proteases - enzymes that break down proteins into amino acids

Recombinant DNA (rDNA) - DNA formed by combining genetic material from different sources

Therapeutic protein - a protein used as a treatment for disease, often produced using recombinant DNA

Vaccine - a biological preparation that stimulates the immune system to protect against a specific disease

4.3: Applications of Recombinant DNA Technology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

5: Bioinformatics- Genomics and Proteomics

5.1: Introduction to Bioinformatics
5.2: Genomics
5.3: Genome Sequencing
5.4: Comparative Genomics
5.5: Functional Genomics
5.6: Proteomics

5: Bioinformatics- Genomics and Proteomics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.1: Introduction to Bioinformatics

Biotech Focus

Bioinformatics and "personalized medicine" has become a model of healthcare that is tailor-made to each person's unique genetic make-up. Because of its ability to analyze vast amounts of data obtained from procedures, like genome sequencing or microarray analysis, bioinformatics allows scientists to search for mutations or gene variants that could affect a patient's response to a particular drug or modify the disease prognosis. By knowing a patient's genetic profile doctors may be able to predict their patient's susceptibility to certain diseases, provide them proper medication and with the proper dose to reduce side-effects.

Introduction

Bioinformatics emerged in the late 20th century as an interdisciplinary field that combines biology, computer science, mathematics, and statistics to analyze and interpret biological data (Figure 5.1.1). With advancements in genomics, proteomics, and other data-rich technologies, managing the vast amounts of data generated by these fields has become key. In response, bioinformatics develops methods and software to understand this data. Experts working in this field write algorithms, create databases, develop statistical techniques, and utilize powerful tools for analyzing sequences, structures, and functions of DNA, RNA, and proteins. Simply put, researchers working with massive datasets that require computational tools to manage and interpret data turn to bioinformatics. It is through bioinformatics that we can begin to understand complex biological systems and drive discoveries in areas like genetics, molecular biology, medicine, and evolutionary biology.

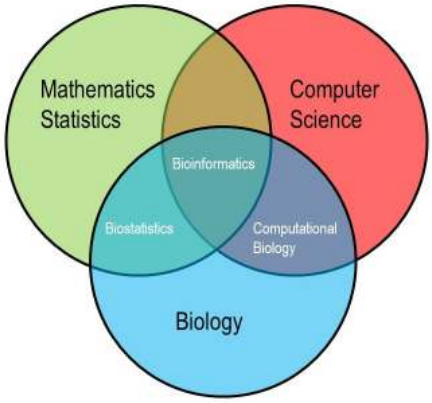


Figure 5.1.1: Bioinformatic fields. The interdisciplinary nature of bioinformatics can be shown using a Venn diagram. In this Venn diagram, bioinformatics can be found at the intersection of mathematics and statistics, computer science, and biology. (The Fields of Bioinformatics, by Patricia Zuk, CC BY 4.0)

Learning Objectives

The field of bioinformatics combines biology, mathematics, statistics, and computer science. At the end of this section you will be able to:

- Define and explain bioinformatics
- Explain some of the applications of bioinformatics
- List some tools used in bioinformatics

Applications of Bioinformatics

The primary goal of bioinformatics is to increase our understanding of biological processes through the analysis of raw biological data. While this is not unique, what sets bioinformatics apart from other similar fields is its development and use of computationally-based approaches to achieve this goal. Major research efforts in the field of bioinformatics include sequence

alignment, genome assembly and analysis, drug discovery and design, protein structure prediction, gene expression prediction, protein-protein interactions, systems biology, and evolution modeling.

Genomics and Personalized Medicine

Genomics is the study of an organism's entire **genome** - the complete set of DNA found within a cell. Bioinformatics plays a pivotal role in genomics, especially in the sequencing and analysis of genomes. With the advent of advanced DNA sequencing technologies, like **next-generation sequencing (NGS)**, entire genomes can be sequenced in a day. Because of this, bioinformatics tools are needed in order to assemble the resulting DNA sequences into genomes, in addition to annotating genes, and identifying genetic variations. This genomic information is crucial for understanding genetic diseases and developing personalized medicine approaches. Bioinformatic data can reveal how a patient will respond to certain drugs, such as chemotherapies, enabling clinicians to personalize a patient's treatment regime and prescribe the most effective treatment. Moreover, bioinformatics is integral to **genome-wide association studies (GWAS**), which identify genetic variants linked to complex diseases like cancer, diabetes, and heart disease. By analyzing large datasets from thousands of individuals, researchers can discover new genetic markers and understand the biological pathways involved in these diseases.

Proteomics and Drug Discovery

Proteomics is the large-scale study of the **proteome,** the entire set of proteins expressed by a genome (Figure 5.1.2). Proteomics allows researchers to study the structure and function of proteins, along with their interactions that are central to nearly all biological processes. Unlike the static nature of the genome, the proteome is dynamic and changes in response to different conditions. Bioinformatics is a key tool used in analyzing the proteome. Techniques like **mass spectrometry** generate vast amounts of protein data, which bioinformatics interprets. Through its study of protein interactions, pathways, and functions, bioinformatics allows us to better study disease mechanisms and identify potential targets for new or existing drugs. Through its analysis of 3D structure, bioinformatics, combined with computational docking simulations, allows for the design of small molecules that can better interact with specific protein targets, leading to the development of more efficacious drugs. Bioinformatics also aids in virtual screening, where large chemical libraries are computationally tested to identify compounds that are likely to bind to a target protein.

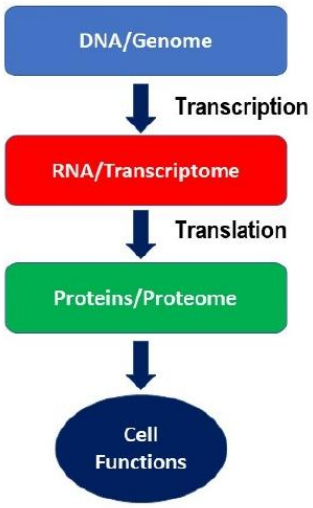


Figure 5.1.2: The relationship between the genome and the proteome. The complete set of DNA in an organism is known as the genome. Transcription of the genome into RNA creates the transcriptome. Translation of RNA into proteins produces the proteome. Proteins are responsible for cell functions. (Genome vs. Proteome by Patricia Zuk, CC BY 4.0)

Evolutionary Biology and Comparative Genomics

Bioinformatics has revolutionized evolutionary biology by allowing researchers to compare the genomes of different species. Through tools like multiple sequence alignments and phylogenetic tree-building software, scientists can trace the evolutionary history of genes and species, identifying conserved elements and evolutionary innovations. **Comparative genomics**, which

compares genomes across species, helps scientists discover insights into evolutionary relationships, identify conserved genes that have remained unchanged across species, and explore genetic adaptations that have enabled organisms to survive in changing environments. This comparative approach can also provide insights into human evolution and health through its study of genetic similarities between humans and closely related species, such as chimpanzees and other primates. Comparative genomics has been invaluable for evolutionary biology by offering a deeper understanding of how genetic diversity contributes to the complexity of life.

Systems Biology and Big Data

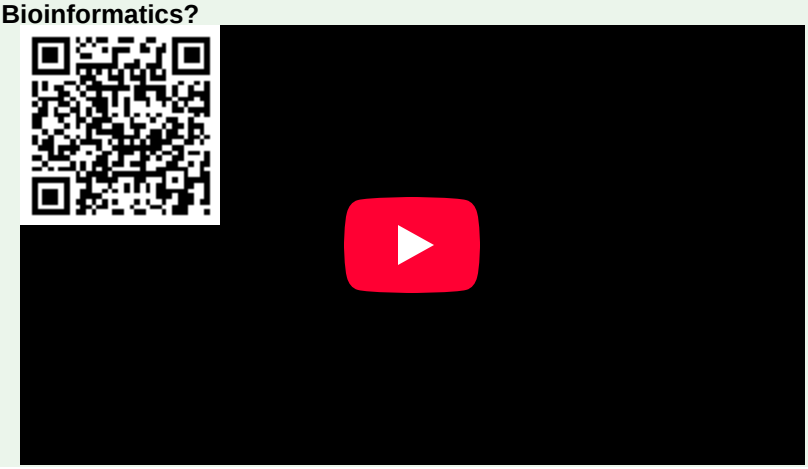
Bioinformatics is central to **systems biology**, which tries to understand biological systems as *whole networks* rather than isolated components. In systems biology, bioinformatics tools are used to integrate data from various sources, such as genomics, transcriptomics, proteomics, and metabolomics, in order to build models of cellular processes. These models are critical to helping researchers understand how the components of a biological system interact with one another and lead to insights into disease mechanisms, drug responses, and metabolic pathways. With the rise of "big data" in biology, bioinformatics has become essential for managing, processing, and analyzing the vast datasets that forms the core of systems biology. Data integration, machine learning, and artificial intelligence (AI) are becoming more and more common in systems biology in order to uncover patterns in these datasets. This makes system biology and bioinformatics powerful tools for testing theories about health and disease and may lead to novel discoveries in fields such as cancer research, neurobiology, and microbiome studies.

Tools of Bioinformatics

At its core, bioinformatics relies on biological databases and computational tools to analyze and interpret data. Common activities in bioinformatics include mapping and analyzing DNA and protein sequences, aligning DNA and protein sequences to compare them, and creating and viewing 3-D models of protein structures. **Sequence alignment algorithms** are used to compare genetic sequences to identify homologous regions. **Phylogenetic tree-building algorithms** are used to infer evolutionary relationships among species. **Machine learning techniques**, such as neural networks and support vector machines, are increasingly being applied to predict gene functions, classify proteins, and identify disease-associated genetic variants. **Databases** like GenBank, EMBL, and UniProt store genetic, protein, and other biological information, allowing researchers to retrieve, compare, and analyze sequences from various organisms. Tools like BLAST (Basic Local Alignment Search Tool) are used for sequence alignment, identifying similarities between genetic or protein sequences, and inferring evolutionary relationships. Finally, bioinformatics tools like PyMOL and Rosetta are used to also model and predict the 3D structures of proteins and nucleic acids.

Concept in action:

Animation: What is Bioinformatics?



Key Concepts

Bioinformatics is the scientific field that uses powerful computer programs to interpret large amounts of complex biological data. Through bioinformatics, both scientists and clinicians can now utilize massive and complex databases in the hopes of making advances in health and human welfare.

Some important concepts to remember:

- bioinformatics involves the collection, storage, and analysis of biological data generated from genomic and proteomic analysis
- bioinformatics develops computer programs to manage and use this biological data
- bioinformatics creates algorithms and statistical approaches to find patterns and relationships within large databases, thus allowing us to understand complex biological systems as whole

Glossary

Algorithm - a step-by-step computational procedure used to analyze data

Bioinformatics - an interdisciplinary field that combines biology, computer science, mathematics, and statistics to analyze and interpret biological data

Comparative genomics - a field in genomics that compares genomes across species

Database - a structured collection of data

Genome - the totality of genetic information found within the cell

Genome-Wide Association Studies (GWAS) - research methods used to identify genetic variants across the entire genome that are associated with specific traits or diseases

Genomics - the study of the genome

Machine learning techniques - algorithms and methods that allow computers to learn patterns from data and make predictions or decisions without being explicitly programmed for a specific task

Mass spectrometry - an analytical technique used to identify, characterize, and quantify molecules by measuring their mass-to-charge ratio (m/z)

Next-generation sequencing (NGS) - a sequencing technology that enables the sequencing of an entire genome in several hours

Phylogenetic tree building - the process of creating a phylogenetic tree

Phylogenetic tree - a diagram that shows the evolutionary relationships among a group of organisms, genes, or proteins based on their genetic or structural similarities and differences

Proteome - the entire set of proteins expressed by a genome

Proteomics - the study of the proteome

Sequence alignment - the process of arranging two or more DNA, RNA, or protein sequences to identify regions of similarity or difference

Systems biology - a field of biology that seeks understand biological systems as a whole network rather than isolated components

5.1: Introduction to Bioinformatics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.2: Genomics

Biotech Focus

With the publishing in 1953 of James Watson's and Francis Crick's seminal paper on the structure of DNA, the field of genomics was born. Having described the fundamental structure of DNA, researchers began to study how genetic information could be stored, transmitted, and expressed. However, it wasn't until the **Human Genome Project (HGP)**, initiated in October 1990, that genomics became a distinct scientific discipline. The HGP aimed to sequence the entire human genome and identify all of its genes. It was a highly collaborative effort that brought together computer scientists, mathematicians, and biologists from 20 laboratories located all around the world. While the project was met with some skepticism at first, it's success is now well known. When it was completed in 2003, the HGP sequenced more than 3 billion base pairs or more than 90% of the human genome. A DNA sample from a single individual with blended ancestry was used to elucidate approximately 70% of the genome, with another 19 individuals being used for the remaining regions. Without a doubt, the HGP represents one of the most remarkable, collaborative efforts to have been undertaken by the global scientific community. Even today, the HGP continues to be at the center of remarkable genomic discoveries and it's impact will be measured in future treatments and therapies.

- Read this letter opposing the HGP
- Read the HGP fact sheet to learn more about how the project was conducted

Introduction

Genomics is the study of an organism's entire genetic material (i.e., the genome). First coined in 1920 by the German botanist Hans Winkler as a combination of the works *gene* and chromos*ome*, genomics involves mapping, sequencing, and analyzing the structure and function of genes, but can also include studying their evolution. The term **genome** refers to the total genetic information of an organism, including all of its coding and non-coding regions. Unlike genetics, which typically focuses on individual genes, genomics is concerned with *all of an organism's genes* and their interrelationships that influence the growth, development, and functioning of organisms.

The human genome contains over three billion base pairs and approximately 25,000 genes that encode the proteins needed to carry out the cellular processes of the human organism. In addition to these protein-coding genes, the human genome also contains genes whose RNA transcripts are not translated but carry out their functions as RNA molecules. Over 99% of the human genome is composed of "spaces", regions of DNA found within and between genes whose functions are regulatory in nature or not fully understood. Almost half of these regions are known as "jumping genes" or their remnants. These jumping genes, also called **transposons**, move about the human genome in order to introduce genetic variation to a region.

With advancements in sequencing technologies and bioinformatics, scientists can now analyze vast amounts of genetic data, making genomics an important field. Genomics insights have led to remarkable developments in fields such as medicine, agriculture, evolutionary biology, and biotechnology. This page provides an overview of key concepts in genomics, tracing its history, principles, and applications.

Learning Objectives

By using genomics, researchers can begin to understand how an organism's genes determines its function. At the end of this section, you will be able to:

- Explain what a genome is
- List the types of genomics used today
- List some technique used in structural genomics
- Explain how structural genomes are created through mapping
- Define and explain recombination and recombination frequency

Types of Genomics

- **Structural genomics:** studies DNA sequences and the content of genomes
- **Functional genomics:** studies the organization and function of genes with a genome
- **Comparative genomics:** compares the content and organization of genomes across species
- **Epigenomics:** studies the epigenetic modifications to the genome
- **Metagenomics:** studies the structure and function of microbial communities by analyzing DNA extracted from environmental samples
- **Pharmacogenomics:** studies how genes affect the response to a medication

Structural Genomics, Genetic Maps, and Physical Maps

Structural Genomics is the branch of genomics that focuses on the physical structure of genomes. It includes determining the sequence of a gene, mapping this gene to its location on a chromosome and studying the three-dimensional structure of the protein encoded by that gene. For more information about chromosome structure, go to Chapter 3.1 DNA Structure. By creating a detailed blueprint of a genome's architecture, researchers can begin to understand the relationship between genes and proteins and how this relationship contributes to an organism's structure and function.

Structural genomics integrates several techniques, such as:

- **DNA sequencing**
- **Gene mapping**
- **X-ray crystallography**
- **Nuclear magnetic resonance (NMR) spectroscopy**

One of the key applications of structural genomics is in predicting the structure and function of a protein based on its DNA sequence. By determining the structure of proteins encoded by a genome, scientists can better understand their biological functions, especially for proteins with no known role. Structural genomics will often rely on comparative genomics, because the structure and function of a protein from one organism can often provide insights into a poorly understood protein from another. In addition to its application in basic biological research, structural genomics can also play a critical role in drug discovery and design. Because protein structure is closely linked with function, structural genomics has the potential to identify novel protein regions that can be potential targets for drug discovery. Through their understanding of the structure of proteins that play critical roles in disease pathways, researchers can design therapeutic molecules, such as drugs, that specifically target these proteins, leading to the development of new disease therapies.

Genetic Mapping

One of the first steps in structural genomics is to prepare a map of the genome. **Genetic maps** are a rough approximation of the location of genes or other molecular markers on a chromosome. Genetic maps are based on **genetic recombination,** a phenomenon that "swaps" the gene alleles in one segment of a chromosome (e.g. alleles A, B, and C) with the gene alleles found the same segment of the homologous chromosome (e.g. alleles a, b, and c). This recombination is the result of **"crossing-over"** that occurs during Meiosis I (Figure **5.2.1**). For more information on meiosis, check out this OER chapter on Meiosis. As a result of recombination, two of the four gametes will contain a "parental" chromosome containing the original gene alleles and the remaining two gametes will contain a "recombinant" chromosome where the alleles have been combined into new combinations. Genetic recombination is responsible for genetic diversity.

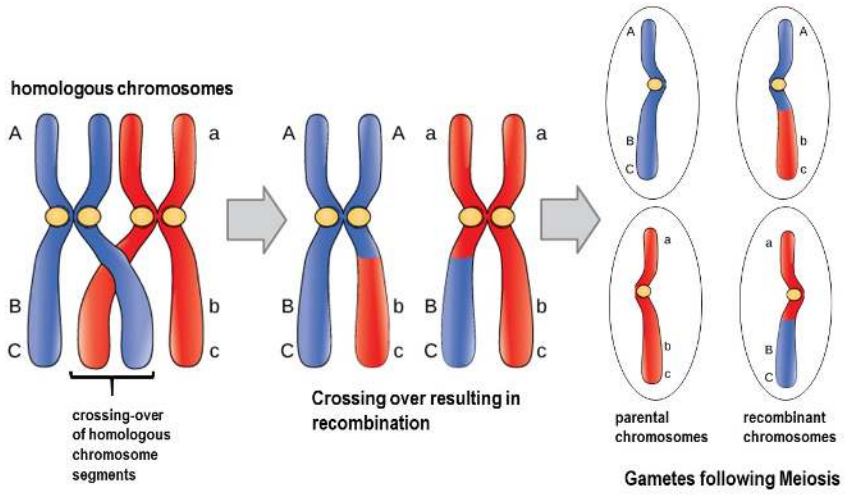


Figure **5.2.1**: Crossing-over. Left image: Crossing over can occur between homologous chromosomes. A chromosome with the alleles A, B, and C (blue chromosome) undergoes crossing over with its homologous chromosome with the alleles a, b, and c (red chromosome). The regions crossing over are called homologous chromosome segments because they have the same genes. Center image: Crossing over results in recombination. The innermost chromatid from the chromosome on the left has the alleles "Abc". The innermost chromatid from the chromosome on the right has the alleles "aBC". Right image: Of the four gametes produced at the end of meiosis, 50% of them will have a "parental" chromosome whose alleles are the same prior to meiosis (solid red chromosome with "abc" alleles and solid blue chromosome with "ABC" alleles). The other 50% of them will have a "recombinant" chromosome" with new combinations of alleles (i.e., red and blue chromosomes with alleles "Abc" and "'aBC"). (Crossing over by Patricia Zuk, CC BY 4.0; adapted from Figure 17 02 01 by CNX OpenStax, CC BY 4.0)

To make a genetic map using recombination, specific genetic crosses are performed and the number of offspring that show the parental phenotypes are recorded along with the number of offspring that show new phenotypic combinations of their parents. These unique offspring are called **recombinants**. From this data, **recombination frequency** can be calculated.

Recombination Frequency (RF) = (# of recombinant progeny/total # of progeny) x 100%

Concept in action

Video: Recombination

A recombination frequency of 1% would mean that out of 100 progeny, 1 of them would be a new combination of the parental phenotypes. The remaining 99 of them would have the phenotype of either one of their parents. If the recombination frequency between two genes is 50% or more, the two genes are located on two different chromosomes or are located *far apart on the same chromosome*. If the recombination frequency is less than 50%, the two genes are located on the same chromosome and are said to be **"linked"**. For linked genes, the *recombination frequency is a representation of the physical distance of the two genes on the chromosome*. This is why genetics maps are often called **linkage maps**. Distances on genetic maps are measured in **centimorgans**

(cM) or map units where *1% of RF is equal to 1 cM or 1 map unit*. The lower the recombination frequency, the closer the two genes will be on the chromosome with regard to one other and the less likely these two genes will be separated to different homologous chromosomes by crossing-over. With enough genetic crosses and calculations of RF, scientists can determine the order of genes on a chromosome and their relative positions with respect to one another. Figure **5.2.2** is an example of such a map, with colored bands showing the locations of multiple genes on chromosome 11 with respect to one another. The further two bands (i.e. genes) are away from one another, the greater the RF will be.

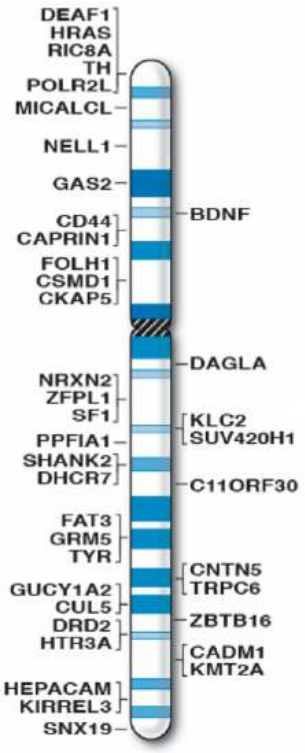


Figure **5.2.2**: A map of chromosome 11. The centromere is shown as a black band with the p arm above the centromere and the q band below the centromere. The blue bands represent the locations of several genes relative to one another. (Human Chromosome 11, by Butler et al., CC BY-SA 4.0)

Physical Maps

A significant problem with genetic maps is that they do not always accurately correspond to the actual physical distance between genes (i.e, base pairs). This is because genetic maps are based on rates of recombination, which can vary from one region of the chromosome to another. A **physical map** is based on direct analysis of DNA and gives the number of base pairs found between genes or molecular markers. A number of techniques exist for creating physical maps, such as restriction mapping, which determines the position of restriction sites within the DNA; sequence-tagged site (STS) mapping which locates positions of short unique sequences of DNA on a chromosome; fluorescent *in situ* hybridization (FISH) which physically locates markers on DNA using fluorescent probes; and DNA sequencing. The most detailed physical maps are produced through **DNA sequencing**. For more about DNA sequencing, go to Chapter 5.3 Genome Sequencing.

Key Concepts

Genomics is an interdisciplinary field of molecular biology that focuses on the structure, function, mapping, and evolution of genomes. Some important concepts to remember are:

- an organism's genome is it's complete set of DNA and includes all of its genes
- genomics characterizes and quantifies all of an organism's genes and studies the relationships between these genes
- there are numerous types of genomics, including structural, functional, and comparative
- structural genomics produces chromosomal maps giving gene location
- functional genomics studies the relationship between gene structure and its function

- comparative genomics compares the genomes of organisms in the hopes of elucidating gene function
- the study of genomes involves several molecular techniques including high-throughput DNA sequencing and bio-informatics so that the genome can be assembled and its functions analyzed
- genetic maps, or linkage maps, use recombination frequency to determine the order and relative position of genes on a chromosome
- recombination frequency calculates the fraction of total progeny that show new combinations of parental phenotypes
- physical maps use techniques like DNA sequencing to determine the physical distance (in base pairs) between genes on a chromosome
- genomic analysis has triggered revolutions in research and has led to advancements in several fields, including pharmaceuticals.

Glossary

Allele - a form of a gene found at a specific location on a chromosome

Centimorgans (cM) - the distance between two genes on a chromosome; also known as a map unit; once cM unit is equivalent to a 1% recombination frequency

Chromatid - one of two identical "arms" of a replicated chromosome

Chromosome - the most condensed and organized form of DNA found in a cell; composed of DNA and proteins; carries genetic information in the form of genes

Comparative genomics - compares the content and organization of genomes across species

Crossing-over - a cellular process that occurs during meiosis when homologous chromosomes exchange genetic material; also called recombination

Gene - a specific sequence of DNA that codes for a polypeptide or an RNA molecule

Genetic maps - a rough approximation of the location of genes or other molecular markers on a chromosome; calculated using recombination frequency

Genetic recombination - a process by which DNA sequences are rearranged, resulting in new combinations of traits in offspring; also called recombination

Genome - the total genetic information of an organism

Genomics - the study of an organism’s entire genetic material

Homologous chromosome - pairs of chromosomes that contain the same genes but may have different alleles

Linkage - the tendency of genes located close to one another on a chromosome to be inherited together

Linkage map - a map showing the order and relative distances of genes on a chromosome

Map unit - the distance between two genes on a chromosome; also known as a centimorgan; once map unit is equivalent to a 1% recombination frequency

Physical map - the location of genes on a chromosome with the actual distance between genes in base pairs

Recombination - a cellular process that occurs during meiosis when homologous chromosomes exchange genetic material; also known as crossing-over

Recombination frequency - a measure of how often a crossover occurs between two genes during meiosis; used to create linkage maps

Recombinants - the offspring produced through recombination

Structural genomics - a branch of genomics that focuses on the physical structure of genomes

5.2: Genomics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.3: Genome Sequencing

iotech Focus

Twenty years ago, the idea of sequencing an entire genome in a day seemed impossible. However in 2005, 454 Life Sciences introduced the **GS20 sequencer** capable of sequencing hundreds of thousands of DNA fragments in a matter of hours. This sequencer was the first of its kind and introduced to the world the concept of "massive parallel sequencing", now known today as **Next Generation Sequencing (NGS)**. In 2007, the GS20 was used to sequence the genome of James Watson and was hailed as a revolution in "personalized medicine". Thanks to NGS, consumers can now submit their DNA and have a copy of their genome, along with an analysis of specific genes, sent to them in weeks. In the research lab, NGS continues to advance fields such as molecular biology, genetics, genomics, evolutionary biology, and medicine.

Check out this podcast on personalized medicine and cancer from the Mayo clinic.

Introduction

Numerous scientific disciplines, including genomics, rely heavily on the precise determination of DNA sequences. The first method for sequencing DNA was developed in 1975 by Frederick Sanger and Alan Coulson, who used DNA polymerase I from *E. coli* to copy short stretches of a DNA template using radiolabeled nucleotides. These pieces were then fractionated by electrophoresis and the radiograph read to determine the DNA sequence. While successful, the process was very labor intensive as the polymerase could only produce DNA copies of only 80 nucleotides long. Nevertheless, Sanger and Coulson managed to sequence the genome of the bacteriophage phiX174 using this method.

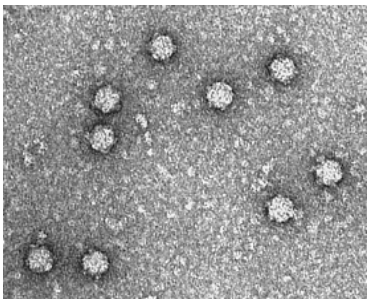


Figure **5.3.1**: An electron micrograph of the bacteriophage phiX174. (Bacteriophage Phi X174 by ShiftFn, CC BY-SA 4.0)

In 1977, Sanger and colleagues introduced what is now known as the **dideoxy chain termination method or the "Sanger sequencing method".** Modifications to their original 1975 method now allowed scientists to accurately sequence long stretches of DNA. Using the Sanger dideoxy chain termination method, Sanger was able to sequence human mitochondrial DNA (16,569 base pairs) and the lambda bacteriophage (48,502 base pairs). For his efforts, Sanger was awarded the Nobel Prize in Chemistry in 1980, along with Walter Gilbert and Paul Berg.

Learning Objectives

DNA sequences gives scientists the exact order of nucleotides in a stretch of DNA. At the end of this section, you will be able to:

- Explain the structure of a dideoxynucleotide (ddNTP)
- Explain the fundamentals of chain termination sequencing using ddNTPs
- Distinguish how shotgun and pair-wise sequencing approaches are used to sequence an entire genome
- Describe how next generation sequencing can sequence an entire genome in a day

Sanger DNA Sequencing

DNA sequencing techniques are based on a cell's DNA replication mechanism. During replication, a primer binds to the parental DNA strand. The DNA polymerase binds to the 3' end of this primer and extends from it, reading the parental DNA template and linking nucleotides to create the daughter DNA strand. The nucleotides are linked together by the DNA polymerase through the creation of phosphodiester bonds. In sequencing, a DNA polymerase binds to a **sequencing primer** that has bound to one of the two DNA strands (e.g. the anti-sense DNA strand). The polymerase extends from this primer and synthesizes a complementary daughter DNA strand. The resulting strand, comprised of the sequencing primer and the daughter DNA strand, is sent to be sequenced.(Figure **5.3.2**, left image).

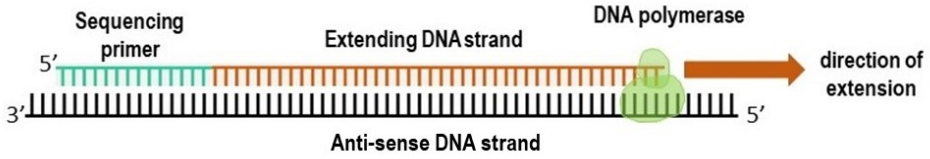


Figure **5.3.2**: In DNA sequencing, a DNA polymerase (green circles) extends from a sequencing primer (shown in green), reads a DNA template, such as an anti-sense DNA strand (shown in black), and creates a complementary daughter DNA strand (i.e. the extending DNA strand, shown in orange) that is then sequenced. The direction of polymerase extension is shown with an arrow. (DNA Extension for Sequencing by Patricia Zuk, CC BY 4.0).

As the DNA polymerase creates the daughter strand it incorporates nucleotides (i.e., dNTPs). The Sanger method uses modified nucleotides, called **dideoxynucleotides (ddNTPs),** that lack a hydroxyl (-OH) group at the 3rd carbon in the sugar (Figure **5.3.3**).

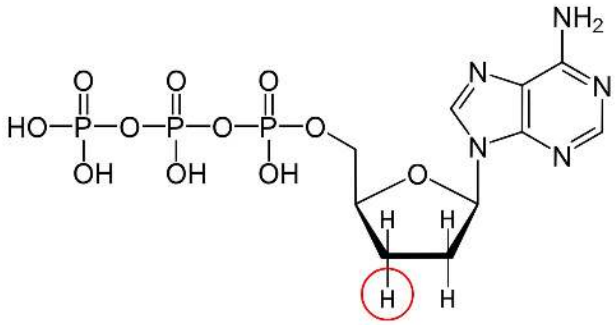


Figure **5.3.3**: The dideoxynucleotide (ddNTP). The molecular structure of a ddNTP used in Sanger sequencing shows the absence of a hydroxyl group at the 3' carbon (circled). (The ddNTP by Patricia Zuk, CC BY 4.0; adapted from Molecular Structure of ddATP by Yikrazuul, public domain)

This lack of a 3' OH group prevents formation of a phosphodiester bond with the incoming nucleotide (Figure **5.3.4**). As a result, extension of the growing daughter strand is halted when these nucleotides are incorporated (i.e., chain termination).

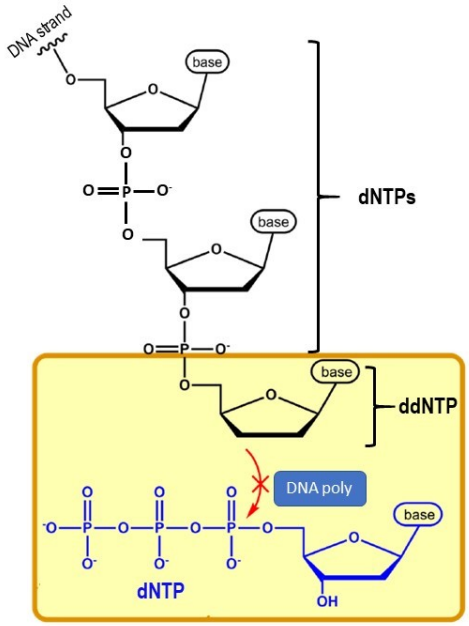


Figure **5.3.4**: In Sanger sequencing, a DNA polymerase cannot add an incoming nucleotide (shown at the bottom in blue) because the last nucleotide in the chain (shown at the top in black) is a ddNTP. This ddNTP lacks a hydroxyl group at the 3rd carbon of the sugar and cannot form a phosphodiester bond with the incoming nucleotide. The extension of the DNA chain stops at the ddNTP (i.e., chain termination). (Chain Termination by Patricia Zuk, CC BY 4.0; adapted from Dideoxy Termination of DNA Elongation by Michal Sobkowski, CC BY-SA 3.0)

The steps of Sanger sequencing are relatively straightforward and are shown in Figure **5.3.5**. For more information, go to Chapter 13.9: DNA Sequencing.

1. **DNA sequencing reaction set-up**: The DNA sequencing reaction is set up to contain a DNA polymerase, a primer, the DNA template, dNTPs (i.e. dATP, dCTP, dGTP, and dTTP), and fluorescently-labelled ddNTPs. The original labels for these ddNTPs were radioactive, but they are now coupled to fluorescent probes of different colors.
2. **Elongation from primer & chain termination by ddNTPs:** The DNA polymerase extends from the primer and copies the template strand by incorporating nucleotides (dNTPs or ddNTPs). When a ddNTP is added to the daughter DNA strand, extension is halted at that site (i.e. chain termination). The completed DNA sequencing reaction is a mixture of DNA fragments of increasing sizes, each with a fluorescently-labeled ddNTP located at the end of the fragment.
3. **Capillary gel electrophoresis separation of labeled DNA fragments:** The sample is then analyzed by a **DNA sequencer.** The sequencing reaction is loaded into a capillary gel tube that separates the fluorescently-labeled fragments according to their size and electrical charge. The smaller sequence fragments will migrate through the tube faster in comparison to those sequences that are bigger (i.e., longer). As the fragments migrate down the gel, a laser activates the fluorescent label which is picked up by a detector.
4. **Laser detection of fluorochromes and sequence compilation:** A detector picks up the fluorescent signals and converts them into a chromatograph from which the sequence can be read.

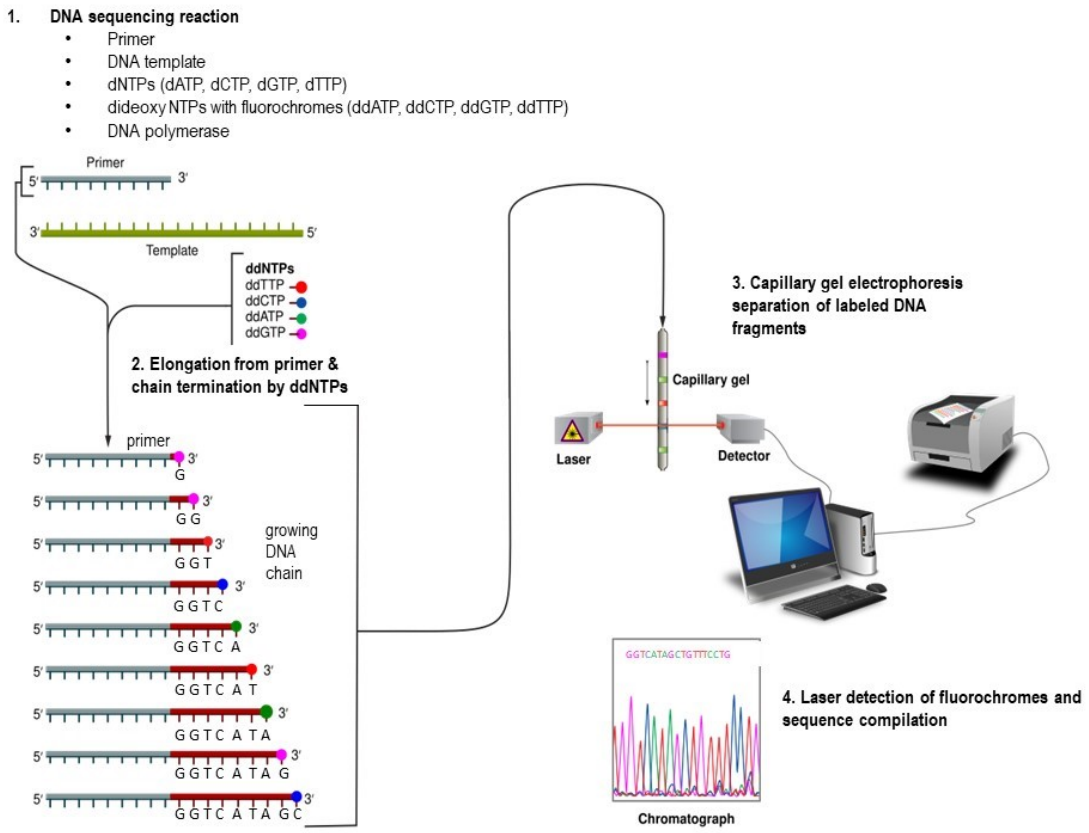


Figure 5.3.5: Sanger sequencing of DNA. The sequencing of DNA is made of the following steps that are described in the text above:
(1) DNA sequencing reaction, (2) Elongation from primer & chain termination by ddNTPs, (3) Capillary gel electrophoresis separation of labeled DNA fragments, and (4) Laser detection of fluorochromes and sequence compilation. (Sanger DNA sequencing by Patricia Zuk, CC BY 4.0; adapted from The Sanger Method for DNA Sequencing by Estevezj, CC BY-SA 3.0)

Concept in action

Animation: Sanger Sequencing

Whole Genome Sequencing

Whole-genome sequencing (WGS) is a process that determines an entire genome's DNA sequence, including nuclear and mitochondrial DNA, in addition to chloroplast DNA (if present). Genomes are cut into smaller fragments and each fragment is sequenced. Computer programs then search for regions of overlap between fragments so that they can be reassembled to create the sequence of the complete genome (Figure 5.3.6).

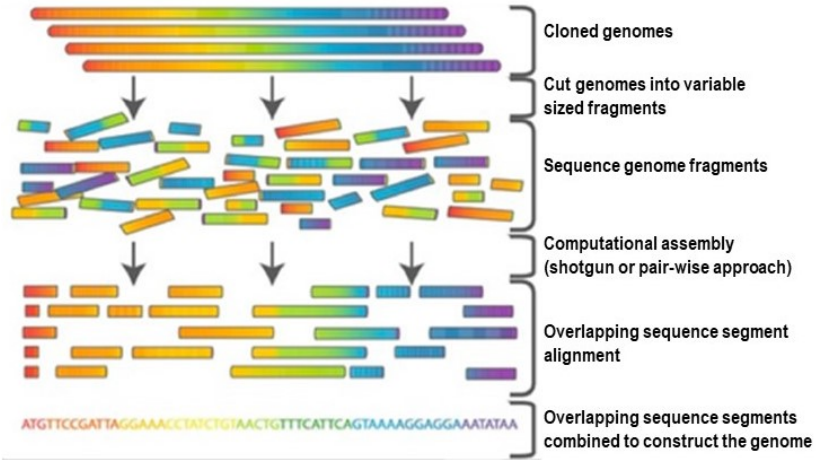


Figure 5.3.6: Whole Genome Sequencing (WGS) approach. WGS is accomplished by cutting the genome into smaller fragments, sequencing the fragments, and then aligning them based on regions of overlap between fragments. "Shotgun" or "pair-wise" approaches are used to align these regions of overlap. With enough fragments and enough overlap, the entire genome sequence can be produced. (Copyright; author via source)

WGS uses either the "shotgun" or "paired end" sequencing methods described below to generate the sequence of entire genome. Because of this, WGS was originally expensive and time consuming. As an alternative to WGS, **exome sequencing** emerged as a lower-cost option. In exome sequencing, only the exons of the DNA's coding region are sequenced. This approach is suitable for diseases in which a protein coding gene is thought to be the underlying cause. However, for determining the sequences of non-protein coding regions, WGS may be the only option. Fortunately, technology has improved the time and cost of WGS. Today, commercial companies offer WGS results in about two days for about $1,000.

Shotgun and Paired End Sequencing

In the **shotgun sequencing** method, DNA fragments are sheared or cut into many smaller pieces. Because this approach is somewhat like when several small pellets are fired from a shotgun, it was named shotgun sequencing. All of the segments are sequenced using the dideoxy chain-sequencing method and powerful computer programs align the sequences by finding regions of overlap amongst the sequences (i.e., computational assembly). With enough sequences the computer can reform the entire DNA sequence. The larger sequence that is assembled from overlapping shorter sequences is called a **contig**.

Table 5.3.1: **Shotgun Sequencing Alignment**

Strand	Sequence
Original Sequence	AGCATGCTGCAGTCATGCTTAGGCTACGCGC
First Shotgun Sequence	AGCATGCTGCAGTCATG**CT**------- ----------**CT**TAGGCTACGCGC
Second Shotgun Sequence	AGCA**TG**---------- -------**TG**CTGCAGTCATGCTTAGGCTACGCGC
Reassembled Sequence	AGCATGCTGCAGTCATGCTTAGGCTACGCGC

Shotgun sequencing assembles DNA fragments by aligning the end of one fragment with the start of another (Table 5.3.1). Originally, this was sufficient for sequencing small genomes. However, with larger genomes, such as that of a human, modifications to this method was required. In **paired end sequencing,** scientists sequence both ends of a DNA fragment and then analyze both ends for overlap with other fragments (Table 5.3.2). Paired end sequencing is more cumbersome than shotgun sequencing, and requires more sophisticated computer programs, but it produces a more accurate genome sequence because there is more available information.

Table 5.3.2: Paired-end Sequencing Alignment

Strand	Sequence
Original Sequence	------- AGCATGCTGCAGTCATGCTTAGGCTACGCGCAGCATGCTG ----------
Aligned Sequences	------**AG**CATGC**TG**---------- ----------**TG**CAGTC**AT** ----------**AT**GCTTAGGC**TA** ----------**TA**CGCGCAGCATGC**TG**---------- ----------
Reassembled Sequence	AGCATGCTGCAGTCATGCTTAGGCTACGCGCAGCATGCTG ----------

Next Generation Sequencing

While modern day Sanger sequencing instruments are still used by the majority of research facilities to quickly sequence a small DNA construct, they are limited by the relatively small number of sequencing reactions that can be analyzed at one time (e.g. up to 96 samples). **Next-generation sequencing (NGS)** is a powerful platform that enables the sequencing of *thousands to millions of DNA molecules simultaneously*, thus allowing for the sequencing of an entire genome in one day. In NGS, DNA is extracted and broken into small fragments. Short pieces of DNA, called **adaptors**, are attached to each fragment. The adaptors allow for binding of the DNA fragment to the solid support of the sequencer, called a **flow cell**. Each bound DNA fragment is amplified into multiple copies on the flow cell. The amplified fragments are sequenced base by base using different chemical or optical methods, depending on the platform (e.g., Illumina, Ion Torrent, PacBio). The sequences are assembled or aligned to a reference genome to complete the sequence. Bioinformatics tools are used to interpret the results.

NGS permits high throughput and is faster and cheaper per base than Sanger sequencing. It is capable of detecting mutations, insertions, deletions, and can quantitate gene expression levels. NGS is used in whole genome sequencing, exome sequencing, RNA sequencing, and targeted sequencing. NGS has become an integral part of personalized medicine, cancer genomics, infectious disease detection, and other areas of medical biotechnology. However, it requires powerful bioinformatics tools and expertise to interpret the massive amounts of data that the sequencer can generate. In addition, interpretation of the data can be complex. To read more about NGS, go to Chapter 10.2 Diagnostic Biotechnology.

Lab Protocols

Lab Technique: DNA sequencing

Key Concepts

Genomic sequencing can determine the complete DNA sequence of an organism's genome, including all of its genes and non-coding regions. Some important concepts to remember are:

- Sanger sequencing is also known as dideoxy chain termination method
- a dideoxynucleotide (ddNTP) lacks a hydroxyl (OH) group at the 3' carbon in the pentose sugar. This prevents the formation of a phosphodiester bond with an incoming nucleotide
- addition of a ddNTP into a growing DNA strand halts its extension at the site of its incorporation
- DNA sequencing techniques use Sanger sequencing in combination with fluorescently-labeled ddNTPs
- whole genome sequencing techniques sequence smaller fragments of the genome and then assemble the fragments into the complete genome
- Next Generation Sequencing (NGS) can sequence millions of small DNA fragments simultaneously, allowing for the sequencing of a genome in a few hours

Glossary

Adaptors - short, synthetic DNA sequences attached to a DNA fragment; used in Next Generation Sequencing

Alignment - the process of arranging DNA sequences using regions of overlap

Capillary gel - a thin, glass tube filled with a polymeric gel; used to quickly separate molecules, like DNA fragments, according to their size and electrical charge

Chain termination sequencing - a DNA sequencing method that uses dideoxynucleotides (ddNTPs) to terminate the synthesis of the complementary DNA chain; also known as Sanger sequencing

Chromatograph - a graphical representation of the DNA sequence, showing peaks for each nucleotide in the sequence

Computational assembly - the process of aligning DNA fragments into a continuous sequence (i.e., a contig) using computer alignment programs

Contig - a set of DNA sequences that overlap to provide a continuous sequence

Dideoxynucleotide (ddNTP) - a DNA nucleotide used in Sanger sequencing that lacks the 3'-OH (hydroxyl) group necessary for continued 5'-to-3' DNA synthesis; used to terminate the elongation of the complementary DNA strand

DNA polymerase - the enzyme that synthesizes new daughter DNA strands during DNA replication; used in DNA sequencing to create the DNA strand that will be sequenced

Exome sequencing - a sequencing method that sequences all of the protein-coding genes in a genome

Fluorescently-labeled - a compound that has a fluorescent dye attach to it

Genomic sequencing - a sequencing method that sequences the entire genome of an organism

Next Generation Sequencing (NGS) - a high throughput sequencing method that can sequence several genomes in a day; also known as massive parallel sequencing

Paired-end sequencing - a genomic sequencing technique that sequences random DNA fragments followed by alignment of these fragments using the start and end of each fragment

Sanger sequencing - a DNA sequencing method that uses dideoxynucleotides (ddNTPs) to terminate the synthesis of the complementary DNA chain; also known as chain termination sequencing

Shotgun sequencing - a genomic sequencing technique that sequences random DNA fragments followed by alignment of these fragments using the start of each DNA fragment

Whole Genome Sequencing (WGS) - the process of determining the DNA sequence of an organism's entire (or nearly entire) genome at a single time

---

5.3: Genome Sequencing is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.4: Comparative Genomics

Biotech Focus

The field of **comparative genomics** was born in 1986 when researchers at the University of Glasgow sequenced a protein found in a **herpes simplex virus (HSV)** and used it to identify similar genes in two other herpesviruses - the varicella-zoster virus and the Epstein-Barr virus. Their results, published in the journal Nucleic Acids Research was the first attempt to align the amino acid sequences of multiple viruses in order to identify regions of similarity. Since then, thousands of comparative genomics papers have since been published comparing a wide variety of genomes in the hopes of identifying the underlying mechanisms of biological pathways and disease.

Check out this quick video that introduces comparative genomics.

Introduction

Comparative genomics is the study of the similarities and differences in the genomes of different species. By comparing genomes, scientists can draw conclusions about the genetic basis of phenotypic traits, evolutionary relationships, and biological functions. Comparative genomics aims to identify **conserved sequences**, which are DNA or amino acids sequences that have remained relatively unchanged across multiple species (Figure 5.4.1). Identifying DNA or protein sequences that have been conserved is an important step towards understanding not only the evolutionary development of an organism but the function of the organism itself. This is because conserved sequences often play critical regulatory roles in basic biological processes, such as gene regulation or protein synthesis.

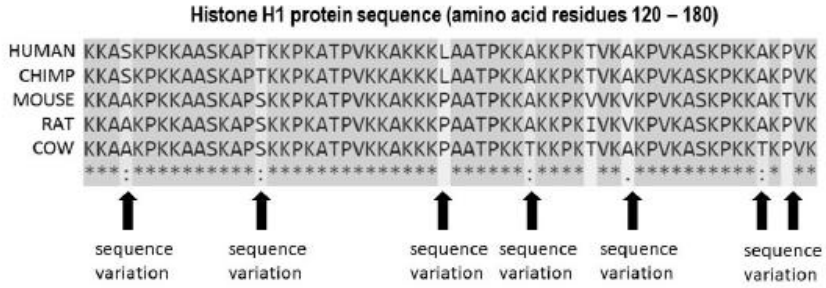


Figure 5.4.1: Amino acid sequence alignment of the histone H1 protein. The amino acid sequence from residues 120-180 of the H1 protein are compared between human, chimp, mouse, rat and cow. Amino acids that are conserved across all sequences are highlighted in grey. Variations in amino acid sequences are identified with a black arrow. (Histone Sequence Alignment by Thomas Shafee, CC BY-SA 4.0)

The analysis of conserved sequences is also key to the identification of homologous and orthologous genes (Figure 5.4.2). **Homologous genes** are genes found in different species that have evolved from a common ancestral gene and retain similar functions. **Orthologous genes** are a type of homologous gene found in different species as a result of evolution (i.e., speciation). By identifying and studying homologs and orthologs through comparative genomics, scientists can infer the functions of genes

found in newly sequenced genomes, particularly in organisms that are less studied or difficult to experiment on. Comparative genomics also helps in identifying **lineage-specific genes** that are unique to certain species and can reveal genetic innovations and adaptations as a result of evolutionary pressures.

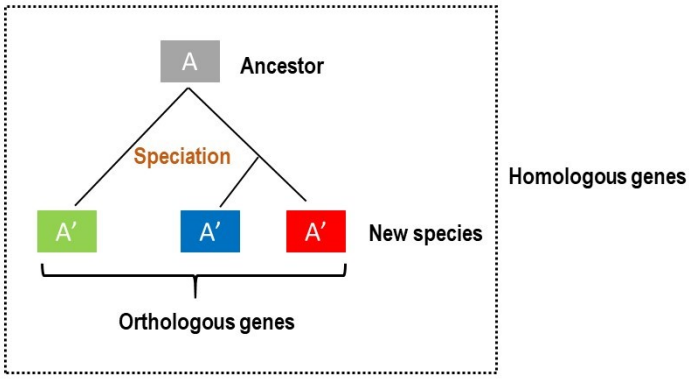


Figure 5.4.2: Homologous and orthologous genes. Homologous genes include the ancestral gene (gray box, labeled as A) and similar genes found in all descendants (all colored boxes labeled A'). Orthologous genes are homologous genes found only in the descendants (green, blue, and red boxes labeled A'). (Homologous and Orthologous genes by Patricia Zuk, CC BY 4.0)

Learning Objectives

By comparing genomes, researcher can acquire a wealth of information, including how specific genes function and how species may be related. At the end of this section, you will be able to:

- Explain conserved sequences
- Explain sequence alignment (local and global; pairwise and multiple)
- Explain BLAST alignment
- Explain whole genome alignment (WGA)
- Explain synteny

Tools and Techniques of Comparative Genomics

Comparative genomic analysis starts with a simple comparison of the general features of the genome, including its size, the number of genes, and the number of chromosomes. Table 5.4.1 presents data on several fully sequenced species used as model organisms in genetics and genomics. While the fruit fly and the flower plant known as "thale cress" have similarly sized genomes (165 vs. 157 million, respectively), the plant has nearly twice the number of genes. Moreover, while humans have nearly 2,000 times the number of base pairs versus a fruit fly (3.1 billion vs. 1.6 million), they have only twice the number of genes. It is through this simple comparative genomic analysis that we learn some powerful lessons - that genome size does not correlate with evolutionary status and the number of genes is not proportionate to the size of the genome.

Table 5.4.1: **Comparative Genomic Analysis of Several Model Organisms**

Organism	Estimated Genome Size (base pairs)	Number of Chromosomes	Estimated Number of Genes
Homo sapiens (Humans)	3.1 billion	46	25,000
Mus musculus (Mouse)	2.9 billion	40	25,000
Bos taurus (Bovine)	2.86 billion	60	22,000
Drosophila melanogaster (Fruit fly)	165 million	8	13,000
Arabidopsis thaliana (Thale cress plant)	157 million	10	25,000
Caenorhabditis elegans (Roundworm)	97 million	12	19,000
Saccharomyces cerevisiae (Yeast)	12 million	32	6,000
Escherichia coli (Bacteria)	4.6 million	1	3,200

From there, additional analysis can be performed, including:

1. **Sequence alignment**
2. **Whole Genome Alignment (WGA) and synteny**
3. **Phylogenetic reconstruction**

Sequence Alignment

At the heart of comparative genomics is **sequence alignment**. In sequence alignment, DNA, RNA, or amino acid sequences are examined for regions of similarity (i.e. conservation) (see Figure \(\PageIndex{1}\ above). Alignments are used to capture information about similar sequences such as ancestry, common evolutionary descent, or common structure and function. They can be done through **pairwise alignment** (both local and global) or **multiple sequence alignment**. In pairwise alignment, bioinformatic tools are used to identify similar regions shared between *two sequences*. **Local pairwise alignment** focuses on finding short stretches of similarity within the compared genomes. Local alignment is useful for identifying conserved sequences that may be motifs or functional domains. Algorithms like the Smith-Waterman algorithm are commonly employed for local alignments. This algorithm finds those segments in two sequences that have similarity, allowing researchers to assess evolutionary relationships or functional roles. In contrast to local pairwise alignment, **global pairwise alignment** *aligns two sequences over their entire sequence* (Figure **5.4.3**). This approach is geared towards finding overall similarity across the entire length of the genomic sequences being compared. Global pairwise alignment is particularly useful when comparing sequences that are similar in length and composition, such as closely related genes or proteins. Common algorithms for global alignment include the Needleman-Wunsch algorithm, which uses dynamic programming to find the similar segments across the entirety of the two compared sequences.

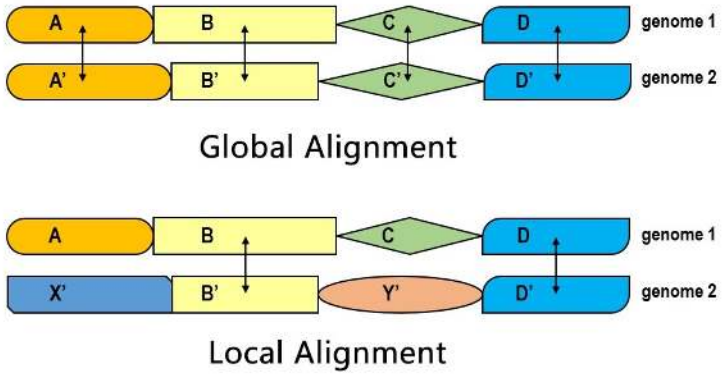


Figure 5.4.3: Local vs. Global Pairwise Alignment. The sequence of two genomes are compared using global alignment (top) and local alignment (bottom). Through global alignment, two genomes are compared in their entirety in order to find similar regions. In this figure, regions A, B, C, and D in genome 1 are compared to regions A', B', C' and D' in genome 2 in order to find sequence similarities. Using local alignment, smaller regions are compared for similarities. In the figure, only regions B and D in genome 1 are compared to regions B' and D' in genome 2. The remaining regions in these two genomes, A and C in genome 1 and X' and Y' in genome 2 are not compared and aligned. (Local vs. Global Pairwise Alignment by Patricia Zuk, CC BY 4.0; adapted from Global and Local Sequence Alignment by Yz cs5160, CC BY-SA 4.0)

In **multiple sequence alignment**, sequence from multiple species (from 4 to 70) can be compared and aligned. This tool is frequently used for identifying ancestral sequences within the genomes and is important for identifying evolutionarily conserved sequences.

BLAST Alignment

Additional algorithms have recently become more popular in comparative genomics as previous algorithms were found to be too slow. Among these are the **FASTA** and **BLAST** algorithms. The most popular algorithm is the **B**asic **L**ocal **A**lignment **S**earch **T**ool **(BLAST)** algorithm. BLAST finds regions of local similarity between sequences. The program can compare new nucleotide or protein sequences obtained after sequencing to existing sequences found in databases through their "blastn" and "blastp" algorithms, respectively. Users can upload a DNA, RNA, or protein sequence file, directly paste in a sequence or input the sequence using an identifier, such as an accession number. If desired, the user can narrow the comparison parameters by selecting a specific database for comparison, and optimizing the comparison for "highly similar sequences", "somewhat similar sequences", or "more dissimilar sequences". BLAST will then list all existing sequences that align with the inputted sequence, will indicate the degree of alignment, and will provide a visual alignment of the two sequences. Today, the BLAST algorithm has been expanded into additional applications, such as "blastx" which translates a new nucleotide sequence and then compares it to existing protein sequences. While BLAST may be intimidating at first, "how-to guides" can make learning this tool easier to manage.

Whole Genome Alignment and Synteny

As its name suggests, **whole-genome alignment (WGA)** uses sophisticated algorithms to compare multiple genomes (i.e., 4 to 70 species) and to identify similar regions of sequence within these genomes. This large-scale, holistic approach can provide a highly detailed view of how organisms are related to one another at the gene level and can provide insights into a species' evolution, gene function, and genetic diseases. Through WGA, we now know that many organisms related through evolution show similar groups of genes in the same relative positions in the genome. This situation is called **synteny**, which roughly translates from the Greek as "on the same ribbon". By combining WGA and genetic mapping, researchers can visualize synteny as a way of seeing the order of genes on chromosomes and how this order was preserved or rearranged over the course of evolution. Today, WGA and synteny is being used not only in evolutionary biology, but is being used in the study of genetic diseases.

Many of the genes of humans are syntenic with those of other animals. Perhaps the best cited example is the syntenic family of mammalian **Hox genes** that direct the development of the animal body plan. As shown in Figure 5.4.4, the order of several of these genes found in the genome of *Drosophila melanogaster* is similar to their homologs found in the mouse, *Mus musculus*, and humans, *Homo sapiens*. The similar order of genes found amongst many organisms may reflect the degree to which these genes interact with one another during development or they may give clues as how these organisms evolved.

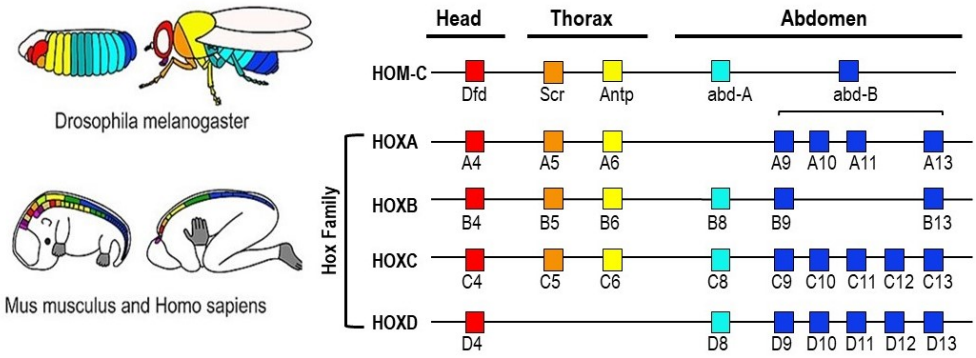


Figure 5.4.4: Hox genes and synteny in Drosophila and mammals. The locations of the homeotic genes (HOM-C) Dfd, Scr, Antp, abd-A and abd-B in the fruit fly, *Drosophila melanogaster*, are compared to several Hox family genes A through D in mouse (*Mus musculus*) and humans (*Homo sapiens*). Each gene is labeled and given a unique color that corresponds to the specific body region they control. The location of the specific body region controlled by these gene is indicated at the top of the figure as head, thorax, or abdomen. This figure indicates how the order of these genes is similar across genomes from several species. (Hox Genes by Patricia Zuk, CC BY 4.0; adapted from Synteny of Hox gene clusters by Heuber et al., CC BY 4.0)

Phylogenetic reconstruction

Another tool in comparative genomics is **phylogenetic reconstruction**. Phylogenetic reconstruction describes the evolutionary relationship between organisms in terms of common ancestors. These relationships are diagrammed through a tree known as a **phylogenetic tree**. Through their position on the phylogenetic tree, genes can easily be identified as homologous and orthologous. Today, phylogenetic reconstruction is being used more and more to help guide researchers in their selection of organisms for comparative genomic research, thus enhancing the impact of such research. As an example using the figure below (Figure 5.4.5), a genomics researcher wishing to study a biochemical pathway might choose to compare the genomes of species 'A' and 'B' since they are more closely related than species 'A' and 'C'. However, a comparison between 'A' and 'C' might be more valuable in understanding how this pathway evolved over time.

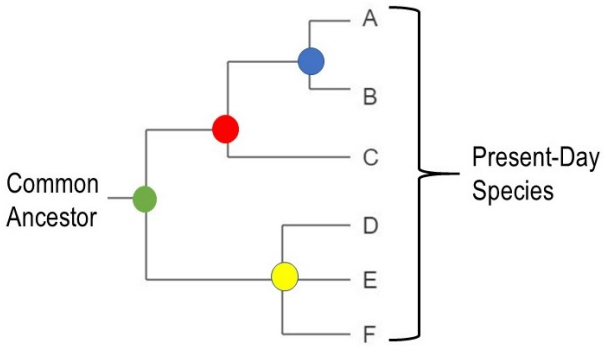


Figure 5.4.5: The phylogenetic tree. The phylogenetic tree in this figure shows the evolution of present-day species A through F from a common ancestor located at the "root" of the tree (green dot). More recent ancestors are located at each branch point (red, blue, and yellow dots). The tree shows that the most recent ancestor of species A and B (blue dot at top of tree) is different from that of species D, E, and F (yellow dot at bottom of tree). The ancestor shown with the red dot shows that species C is more related to species A and B than species D, E, and F. (Phylogenetic Tree by Patricia Zuk, CC BY 4.0)

What Have We Learned?

Investigators are increasingly using comparative genomics to explore areas ranging from human development and behavior to metabolism and susceptibility to disease. For example, by comparing the human genome with those of model organisms like mice, zebrafish, or fruit flies, scientists can study the genetic basis of diseases without the need to experiment on humans. Additionally, comparing the genomes of healthy individuals with those of individuals suffering from genetic diseases helps identify mutations or structural variations that may underlie these conditions. This knowledge is paving the way for the development of new diagnostic tools, treatments, and personalized medicine approaches based on an individual's genetic makeup.

Today, comparative genomics continues to uncover new behavioral, neurological and developmental pathways and genes that are shared or related among species. Some examples of what we have learned include the following:

- About 60 percent of genes are conserved between fruit flies and humans, meaning that the two organisms appear to share a core set of genes. Two-thirds of human genes known to be involved in cancer have counterparts in the fruit fly.
- Researchers studying milk production have mapped genes that increase the yield of high-fat milk in cows, resulting in higher production levels and potentially a significant economic impact.
- Comparisons of genomes from nearly 50 bird species has revealed a gene network that underlies singing in birds and that may have an important role in human speech and language.
- Comparisons of the genomics of various cancer types in dogs, has led to new insights into the human form of the condition. In some cases, they have mapped genes contributing to these disorders to specific chromosomes.

Key Concepts

Comparative genomics is a biological research field that compares the genome sequences of different species, from bacteria to humans. Comparative genomics identifies genes that are unique to a species or conserved across multiple species so that we begin to understand how organisms function, how they are related, and how they evolved. Some important concepts to remember are:

- comparative genomics is the study of the similarities and differences in the genomes of different species
- comparative genomics looks for conserved sequences
- conserved sequences are DNA, RNA, or amino acids sequences that have remained relatively unchanged across multiple species
- sequence alignment compares two or more sequences for the purposes of finding conserved sequences
- algorithms like FASTA or BLAST are used in sequence alignment
- pairwise alignment compares two sequences
- local pairwise alignment compares short segments of sequence
- global pairwise alignment compares significantly longer sequences
- multiple sequence alignment compares multiple sequences
- whole genome alignment compares entire genomes and can identify synteny
- synteny identifies genes that have the same chromosomal order across several species
- phylogenetic trees can identify common ancestors and patterns of evolution

Glossary

Alignment – the process of arranging DNA (or amino acid) sequences using regions of sequence overlap

Basic Local Alignment Search Tool (BLAST) - a sequence alignment algorithm used to compare nucleotide or amino acid sequences to databases

Comparative genomics - the study of the similarities and differences in the genomes of different species

Conserved sequence - a sequence of nucleotides in DNA or RNA or of amino acids in a polypeptide that is similar across multiple species

Global alignment - the alignment of long sequences of DNA, including entire genomes

Homology - any characteristic of biological organisms that is derived from a common ancestor

Homologous genes - two genes that are similar and sequence and derived from a common ancestor; also called homologs

Local alignment - the alignment of short sequences of DNA

Multiple sequence alignment - a process that compares and aligns multiple sequences

Orthologous genes - homologous genes that originated from a common ancestor and were separated into different species due to speciation; also called orthologs

Pairwise alignment - an alignment process that compares and aligns two sequences

Phylogenetic tree - a graphical representation of the evolutionary relationships between sequences or species

Phylogenetic reconstruction - describes the evolutionary relationship between organisms in terms of common ancestors

Sequence alignment - a way of arranging and comparing two of more sequences of DNA, RNA, or protein to one another in order to identify regions of similarity

Syntenic - the similarity in the gene order on chromosomes found across different species

Synteny (Chromosomal synteny) - the conserved order of genes on chromosomes between different species; may indicate their descent from a common ancestor

Whole genome alignment - the alignment of entire genomes with one another

---

5.4: Comparative Genomics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.5: Functional Genomics

Biotech Focus

In the 1980s, researchers began to observe higher-than-average rates of breast and ovarian cancers in certain families and suspected that an inherited genetic mutation might be responsible. In 1990, Dr. Mary-Claire King and her team at the University of California, Berkeley, identified the location of the **BRCA1 gene** on chromosome 17. Four years later, a team led by Dr. Mark Skolnick at Myriad Genetics successfully isolated and cloned the BRCA1 gene, leading to its classification as a tumor suppressor gene involved in DNA repair and cell cycle regulation. Mutations in BRCA1 were found to impair DNA repair processes and lead to increased cancer risk. Soon after the discovery of BRCA1, researchers, in 1995, identified the **BRCA2 gene** on chromosome 13. Like BRCA1, BRCA2 is also a tumor suppressor gene involved in DNA repair. Mutations in BRCA2 were also associated with a higher risk of breast, ovarian, and other cancers. The discovery of BRCA1 and BRCA2 was crucial in understanding the mechanisms underlying hereditary breast cancers. Since their discovery, genetic testing for these mutations has became available, offering individuals with a family history of cancer the opportunity to assess their cancer risk.

Introduction

The goal of **functional genomics** is to understand the functions of genes and proteins. Through its use of vast amounts of data generated by genome and RNA sequencing projects, functional genomics focuses on the *dynamic aspects* of the genome, such as gene expression and its regulation, as opposed to the static aspects of the genome, such as DNA sequences. Functional genomics may also include studies of the natural genetic variation that occurs during an organism's development, as well as functional disruptions such as mutations. The promise of functional genomics is its ability to provide a more complete picture of how the genome of an organism specifies its function rather than trying to extrapolate this using single genes. As a result, functional genomics data is often a part of a systems biology approach.

Learning Objectives

Functional genomics links the structure of genes to the function of proteins. At the end of this section, you will be able to:

- Explain the process of RNA sequencing
- Explain how gene expression is silenced through siRNA and miRNA
- Explain the transcriptome
- Explain mutational analysis & SNPs

Techniques of Functional Genomics

The term functional genomics is often used to refer to the *technical approaches* that are used when studying an organism's genes and proteins. These approaches include gene expression analysis, the identification of mutations and polymorphisms (e.g. SNPs), as well as the measurement of molecular activities of the transcriptome, proteome, and metabolic pathways. (e.g. transcriptomics, proteomics, and metabolomics).

Key approaches in functional genomics include:

1. **Gene expression analysis**
2. **Mutational analysis**
3. **Protein-Protein interactions**
4. **Epigenomics**
5. **Measurement of molecular activities**

Gene expression analysis: RNA Sequencing

Techniques such as **RNA sequencing (RNA-seq)** can measure gene expression levels across the genome, helping scientists understand when, where, and under what conditions genes are active. Figure **5.5.1** below summarizes the major steps of RNA-seq.

In contrast to genome sequencing, in RNA-seq, it isn't the genome that is sequenced, but the **transcriptome** - the complete range of mRNA molecules made through gene expression. Thanks to techniques like **next-generation sequencing (NGS)**, the transcriptome of a biological sample can be determined in a matter of hours. Using RNA-seq, researchers can examine alternatively spliced transcripts, post-transcriptional modifications, gene mutations and changes in gene expression over time, or measure differences in gene expression in different groups or treatments. RNA-seq can also look at different populations of RNA, including total RNA, mRNA, microRNA (miRNA), and transfer RNA (tRNA). For more information about next-generation sequencing, go to Chapter 10.2 Diagnostic Biotechnology.

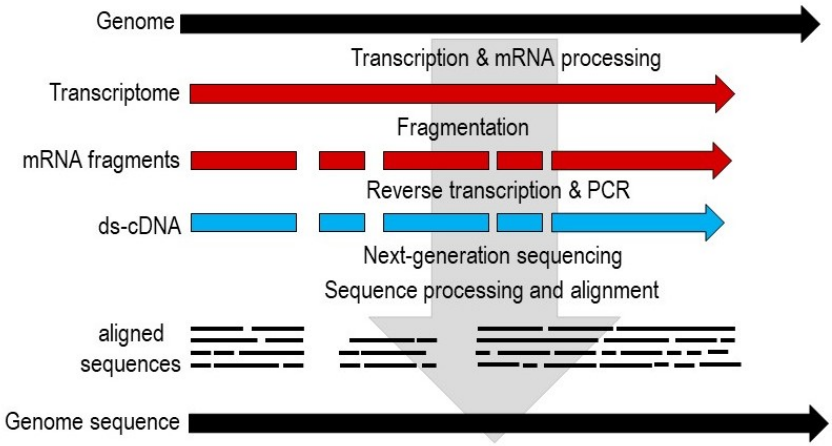


Figure 5.5.1: RNA sequencing. Transcription of the genes in the genome (black arrow at top of figure), followed by RNA processing, gives rise to the transcriptome (solid red arrow) - the complete set of mRNA found within a biological sample. In RNA-seq, the transcriptome is isolated from a sample and then fragmented into smaller mRNA fragments (dotted red arrow). The mRNA fragments are converted into complementary DNA (cDNA) through reverse transcription and then amplified through PCR to make double-stranded cDNA (ds-cDNA) fragments (dotted blue arrow). Next generation sequencing determines the sequence of each ds-cDNA fragment produced from reverse transcribing the transcriptome. Sequence processing and alignment aligns these ds-DNA sequences (dotted black lines) into the sequence of the genome (black arrow at bottom of figure). (RNA Sequencing by Patricia Zuk, CC BY 4.0; adapted from Summary of RNA seq by Thomas Shafee, CC BY 4.0)

Gene expression analysis: Gene Silencing and RNA Interference

By inhibiting or "silencing" the expression of specific genes in organisms or cells, researchers can observe the resulting changes, providing insights into a gene's role in various biological processes and diseases. There are several mechanisms that can be used in the laboratory to inhibit the expression of a specific gene. One mechanism is based on a cell's natural defense mechanism and is known as **RNA interference (RNAi)**. RNAi mechanisms within a cell target specific mRNA molecules for degradation or inhibit their expression into proteins. As such, RNAi may be a powerful tool in the discovery of drugs and their treatment of many diseases, such as cancer.

Two types of RNAi mechanisms are used in laboratories:

1. **small interfering RNA (siRNA)**
2. **microRNA (miRNA)**

siRNA and miRNA molecules are known as **non-coding RNA molecules.** Non-coding RNA is not translated into polypeptides. An siRNA molecule is a short, double-stranded RNA (dsRNA) molecule of 20 to 24 nucleotides long. miRNA is also single stranded RNA that is 21 nucleotides in length. The mechanism that siRNA and miRNA use to silence gene expression is very similar (Figure 5.5.2). siRNA genes are transcribed in the nucleus as either a long piece of double-stranded RNA (dsRNA) or as a piece of "hairpin" RNA - a section of dsDNA (i.e., the "stem") with a "loop" at the end. An miRNA gene is also transcribed in the nucleus as a long piece of dsRNA with several hairpin sections. This long piece is cut into smaller pieces known as **pre-miRNA**. Both the siRNA and pre-miRNA are exported into the cytoplasm for processing by an enzyme **Dicer**. Dicer is an RNA-specific enzyme that removes the loops from siRNA and miRNA molecules, leaving the double-stranded section. The double-stranded siRNA or miRNA molecules are then denatured into single strands through a mechanism not fully understood, but involves a protein called **Ago2**. The single stranded piece of siRNA or miRNA associates with several proteins to form an **RNA-Induced Silencing Complex (RISC).** The

bound siRNA or miRNA in the RISC complex then binds to its complementary mRNA sequence which leads to eventual silencing of the gene by the RISC.

How the siRNA and miRNA molecules result in gene silencing differs. The binding of siRNA to its complementary sequence is "perfect", with no mismatches. This leads to degradation of the bound mRNA by the RISC and silencing of the corresponding gene. However, the match between the miRNA and its complementary sequence does not have to be 100%. In fact, in animal cells, the base pairing between miRNA and mRNA is not a perfect match. This mismatch leads to an inhibition of translation because the RISC prevents the binding of the small ribosomal subunit to the mRNA. The gene is silenced as a result. Eventually, the poly-A tail is degraded which leads to the eventual degradation of the entire mRNA molecule. In contrast to animal cells, the binding of the miRNA-RISC complex is perfect in plant cells, with no mismatches. Like the siRNA mechanism, this perfect match between the miRNA sequence and its mRNA leads to direct degradation of the bound mRNA.

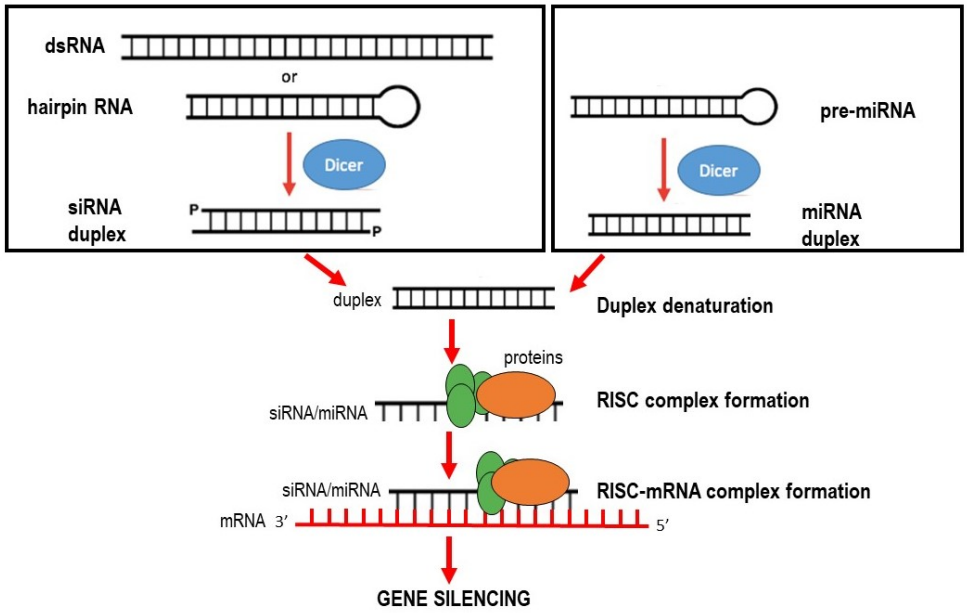


Figure **5.5.2**: Gene silencing using siRNA and miRNA. Long RNA molecules, such as double-stranded RNA (dsRNA) or hairpin RNA, are processed in the cytoplasm by the enzyme Dicer (blue circle) into smaller dsRNA molecules known as siRNA duplexes (left box at top of figure). Dicer also processes short pieces of double-stranded RNA known as pre-mRNA into miRNA duplexes (right box at top of figure). The siRNA or miRNA duplex is denatured into single stranded pieces which associate with proteins (green and orange circles) to form a RISC complex. The RISC complex containing either siRNA or miRNA then binds to a complementary sequence on the mRNA (shown in red), leading to gene silencing. (Gene Silencing by siRNA or miRNA by Patricia Zuk, CC BY 4.0)

Gene silencing in the lab using siRNA or miRNA is based on their naturally-occurring mechanisms. For siRNA silencing, an siRNA sequence is designed against the target gene and then cloned into an expression vector that can be introduced into mammalian cells either through transfection or by using an expression system that incorporates the siRNA sequence into a viral vector. The effect of silencing that gene can then be determined. As with siRNA, miRNA sequences can be cloned and introduced into mammalian cells to learn how the silenced gene functions. Anti-mRNA oligonucleotides can be designed to bind and inhibit miRNA function without the need for cloning. Finally, other compounds that inhibit miRNA function can also be considered.

Confirmation of gene silencing involves downstream protein analysis techniques, such as **SDS-PAGE electrophoresis** and **Western Blotting**. Through these techniques, researchers can detect expression of a specific protein and quantify its expression level. To learn more about SDS-PAGE, go to Chapter 13.11: SDS-PAGE. To learn about Western Blotting, go to Chapter 13.12: Western Blotting.

Concept in Action:

Animation: Gene silencing by microRNA

Mutational Analysis

Mutational analysis is used in laboratories to identify mutations in the DNA sequence of a gene so that researchers can understand how genetic alterations contribute to diseases, disorders, and even certain infections. It is a critical part of the process known as **genetic testing**. In mutational analysis, DNA is extracted from tissues such as blood, tissue, saliva, or even tumor biopsies. The gene of interest is amplified using Polymerase Chain Reaction (PCR) and the mutation confirmed using DNA sequencing methods, like Sanger sequencing and next generation sequencing (NGS). The use of NGS has become popular owing to its ability to detect a range of mutations, including single nucleotide polymorphisms (SNPs), insertions, deletions, and copy number variations. However, additional sequencing methods like Sanger sequencing are often used to confirm the NGS results. Bioinformatics tools align the sequenced DNA to the reference genome to confirm and identify the mutation(s). Functional studies can then be performed in order to assess how the mutation affects protein function, cellular pathways, or affects the phenotype of the organism. Well known genes identified using mutational analysis include the BRCA1 and BRCA2 genes involved in breast and ovarian cancer. To learn more about PCR, go to Chapter 13.2: Polymerase Chain Reaction (PCR). To learn about DNA sequencing, go to Chapter 13.9: DNA sequencing.

One of the most common mutations studied through mutational analysis is the **single nucleotide polymorphism (SNP).** A SNP is a substitution of a single nucleotide at a specific position in the genome. SNPs can occur in both coding and non-coding regions of a gene, in addition to regions of the genome outside of a protein-coding gene (Figure **5.5.3**). SNPs within a coding region of a gene can be classified as **synonymous** (not affecting the protein sequence - i.e., a "silent" mutation) and non-synonymous (affecting the protein sequence). While the synonymous mutation does not affect the sequence of the protein, it may still affect the function of the protein. The **non-synonymous** SNPs are known as **missense** and **nonsense** mutations. These mutations can have significant effects on protein function depending on what domain of the protein the mutation can be found. SNPs that are not in protein-coding regions may still affect gene expression through their effects on splicing, transcription factor binding, or mRNA stability.

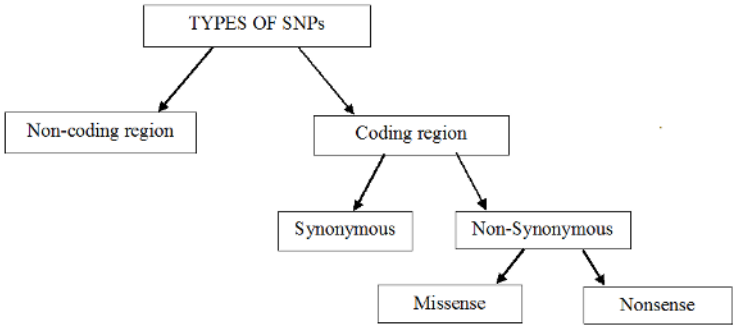


Figure **5.5.3**: Types of single-nucleotide polymorphisms (SNPs). SNPs can be classified as those found in non-coding regions and coding regions. The coding region SNPs are classified as either synonymous or non-synonymous. Non-synonymous SNPs are known as either missense or nonsense SNPs (Types of single-nucleotide polymorphisms (SNPs) by MansiG123 CC BY-SA 4.0)

Over 600 million SNPs have been identified in the human genome across the world's population. Within a single human genome, close to 4 million SNPs can be found. Many of these SNPs are found associated within protein-coding sequences. However, most SNPs are located outside protein-coding regions. These SNPs may be unique to the individual or may be found within individuals who share a similar racial profile or geographic origin. This finding is the basis for many commercial ancestry companies.

Measurement of Molecular Activities: The "Omics" Approach

In genomics, the measurement of molecular activities involves the quantitation of various molecules within a cell, like RNA transcripts or proteins. Through this work different "omics" have emerged, including including **transcriptomics** (for RNA expression), **proteomics** (for protein expression), and **metabolomics** (for studying metabolite levels). Each "omic" is related to one another and is a part of a critical flow of information in the cell (Figure **5.5.4**).

Transcriptomics studies and quantifies the **transcriptome**, the complete set of RNA transcripts, produced by the genome in a specific cell type, under particular conditions, or at specific points in time. The transcriptome includes all RNA, such as mRNA and the other non-coding RNAs like rRNA, tRNA, miRNA and snRNA. Because the transcriptome is dynamic, constantly changing based on the condition of the cell, studying it through transcriptomics can provide a detailed view of gene expression patterns. These patterns may help researchers understand how genes are regulated and function within complex biological processes, such as diseases like cancer. Transcriptome analysis can be done through microarray analysis. However, most transcriptomics is now done through RNA sequencing (RNA-seq).

Proteomics studies and quantifies the **proteome**, the complete set of proteins expressed in a cell, tissue, or organism. Proteomics uses techniques such as mass spectroscopy, two-dimensional gel electrophoresis and protein-protein interaction studies to understand how proteins function within complex biological pathways, in addition to identifying biomarkers associated with diseases, and those drugs which may be used for treatments. Finally, **metabolomics** studies and measures the **metabolome**, the complete set of **metabolites** in a biological system. Metabolites are small molecules produced or modified by metabolic processes. They include carbohydrates, amino acids, fatty acids, nucleotides, and signaling molecules. Metabolomics provides insights into the biochemical state of a cell or organism and is often used in combination with genomics, transcriptomics, and proteomics to gain a more holistic understanding of biological processes and disease mechanisms. To read more about proteomics, go to Chapter 5.6 Proteomics.

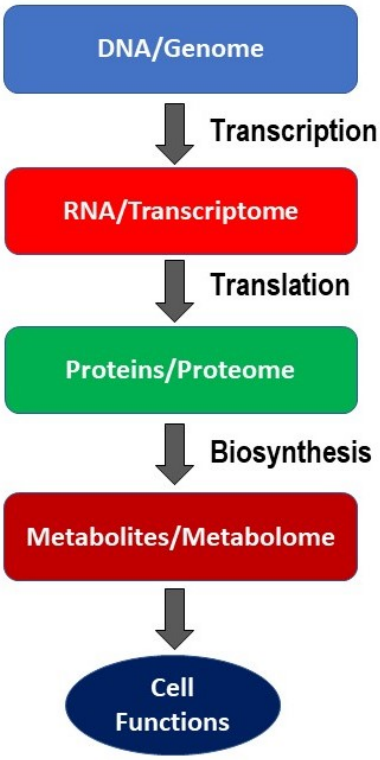


Figure **5.5.4**: The relationship between the genome, the proteome, and the metabolome. The complete set of DNA in an organism is known as the genome. Transcription of the genome into RNA creates the transcriptome. Translation of RNA into proteins produces the proteome. Biosynthetic pathways using enzymes in the proteome create the metabolites of the cell, also known as the metabolome. Cell functions are determined as a result of this relationship. (Genome vs. Proteome vs. Metabolome by Patricia Zuk, CC BY 4.0)

Other Techniques

Scientists studying functional genomics have additional techniques available to them. Studying protein-protein interactions other using methods like yeast two-hybrid screening or co-immunoprecipitation, may help identify metabolic pathways that are critical for cellular function. **Epigenomics**, the study of chemical modifications on DNA and histones (e.g. methylation) provides insight into gene regulation mechanisms beyond that of DNA sequences.

Lab Protocols

- Lab Technique: PCR
- Lab Technique: DNA Sequencing
- Lab Technique: SDS-PAGE
- Lab Technique: Western Blotting

Key Concepts

Functional genomics examines the relationship between the structure of a gene and its expression of a protein and how these genes and proteins function within an organism.

Some important concepts to remember are:

- functional genomics tries to understand the roles genes play in complex cellular processes
- functional genomics uses advanced techniques like gene silencing, mutational analysis, and transcriptomics and proteomics to examine gene expression, gene regulation,and protein interactions on a genome-wide scale

- functional genomics reveals how genes contribute to health, disease, and responses to environmental changes by analyzing how genes are turned on or off in response to various conditions
- key approaches to functional genomics include gene silencing through siRNA or miRNA, RNA sequencing, DNA microarrays, and mutational analysis

Glossary

DNA microarray - a genomic technique that can analyze the expression of multiple genes at once or detect variations in DNA sequences

Epigenomics - an area of biology that studies chemical modifications on DNA and histones

Functional genomics - the study of how the structure of the genome relates to the function of genes and their proteins

Gene silencing - the inhibition of a gene's expression through natural or artificial means; can be achieved in the laboratory using siRNA or miRNA techniques

Genetic testing - a type of medical test that examines a DNA sequence in order to identify mutations

Metabolome - the complete set of metabolites made by a cell; includes carbohydrates, amino acids, fatty acids, nucleotides, and signaling molecules

Metabolomics - the study of the metabolome

microRNA (miRNA) - a short piece of non-coding RNA that binds to a complementary sequence in the mRNA and leads to its degradation; found naturally in cells as part of its regulation of gene expression; can be produced artificially in the laboratory to silence gene expression

Mutational analysis - a technique used identify mutations in the DNA sequence of a gene

Next-generation sequencing (NGS) - an advanced sequencing technology that allows for rapid and large-scale determination of DNA sequences like those making up a genome

Proteome - the complete set of proteins expressed by a cell

Proteomics - the study of the proteome

RNA Induced Silencing Complex (RISC) - an aggregation of proteins that helps guide siRNA or miRNA molecules to their complementary mRNA sequence

RNA interference (RNAi) - a mechanism used by cells to target specific mRNA molecules for degradation; uses either siRNA or miRNA molecules to affect mRNA stability and gene expression

RNA sequencing - a genomic technique that measures the quantity and presence of RNA molecules in a biological sample

Single Nucleotide Polymorphism (SNP) - a mutation of a single nucleotide at a specific position in the genome; can be found in protein coding and non-protein coding regions of the genome

small interfering RNA (siRNA) - a short piece of non-coding RNA that binds to a complementary sequence in the mRNA and leads to its degradation; found naturally in cells as part of its regulation of gene expression; can be produced artificially in the laboratory to silence gene expression

Systems biology - a field of biology that seeks to understand biological systems as a whole network rather than isolated components

Transcriptome - the complete set of RNA transcribed by a cell; includes mRNA, tRNA, rRNA, and miRNA

5.5: Functional Genomics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

5.6: Proteomics

Biotech Focus

In 1986, Alex Ullrich, a scientist working for the biomedical company Genentech, identified the protein known as the **Human Epidermal Growth Factor Receptor 2 (HER2)** protein. Using mass spectrometry and protein profiling, researchers measured the overexpression of HER2 in certain breast cancer patients. In the mid-1990s, Dennis Slamon and his colleagues at UCLA, using proteomic techniques, developed **trastuzumab** (Herceptin), a monoclonal antibody that specifically inhibits HER2 activity. The clinical introduction of this drug has drastically improved survival rates for HER2-positive breast cancer patients. Proteomics-based assays are now used in clinical diagnostics to determine HER2 expression levels in breast cancer tissues, helping guide personalized treatment strategies.

Introduction

Proteomics is the large-scale study of proteins. Proteins are produced based on genetic information encoded in genes, but unlike genes, protein expression, structure, and function can vary greatly under different conditions. The goal of proteomics is to understand the **proteome**, the full set of proteins expressed in a biological sample, such as a cell or tissue. Unlike the genome, which is relatively stable, the proteome is highly dynamic, with protein expression levels, modifications, and interactions constantly changing. Therefore the proteome is the full set of proteins expressed *at a specific time*. Proteomics complements genomics by showing how genetic information is translated into functional proteins and how these proteins interact with one another to form complex networks within a cell. By studying these networks, researchers can identify protein complexes and elucidate their roles in signaling pathways and cellular functions. Through proteomic research, scientists can identify protein biomarkers and pathways that drive disease progression, identify potential drug targets, and optimize treatments and reduce side effects. Outside of medicine, proteomics can determine how organisms respond to environmental changes or stressors, by studying changes in protein expression and modification.

Concept in Action:

Video: Proteomics

Learning Objectives

Proteomics studies the structure and function of proteins over a large-scale. At the end of this section, you will be able to:

- Explain mass spectroscopy
- Explain 2D gel electrophoresis
- Explain liquid chromatography
- Explain protein microarrays

The Techniques of Proteomics

The techniques of proteomics can identify the proteins present in a sample, compare protein profiles between different samples, and study protein-protein interactions. Key approaches in proteomics include:

1. **Mass Spectrometry (MS)**
2. **2D Gel Electrophoresis**
3. **Liquid chromatography**
4. **Protein Microarrays**

Mass Spectroscopy

Mass spectroscopy (MS or "mass spec") is a cornerstone method for identifying and quantifying proteins by analyzing their mass and structure. Using mass spec, researchers can also learn about post-translational modifications, such as phosphorylation, methylation, or glycosylation, that occur after proteins are synthesized. These modifications are key to protein function and regulation, and proteomics allows researchers to study these modifications and understand their impact on cellular processes.

In mass spec, proteins are digested into peptides that are separated by chromatography and then ionized to convert them into charged particles (Figure **5.6.1**). These ionized peptides are separated based on their **mass-to-charge ratio (m/z ratio)** using an instrument called a mass analyzer. A detector within the mass analyzer measures the m/z ratio and abundance of each ion, creating a **mass spectrum**. The resulting spectrum for a peptide can then be analyzed to determine it's identity, which in turn identifies the protein from which it originated.

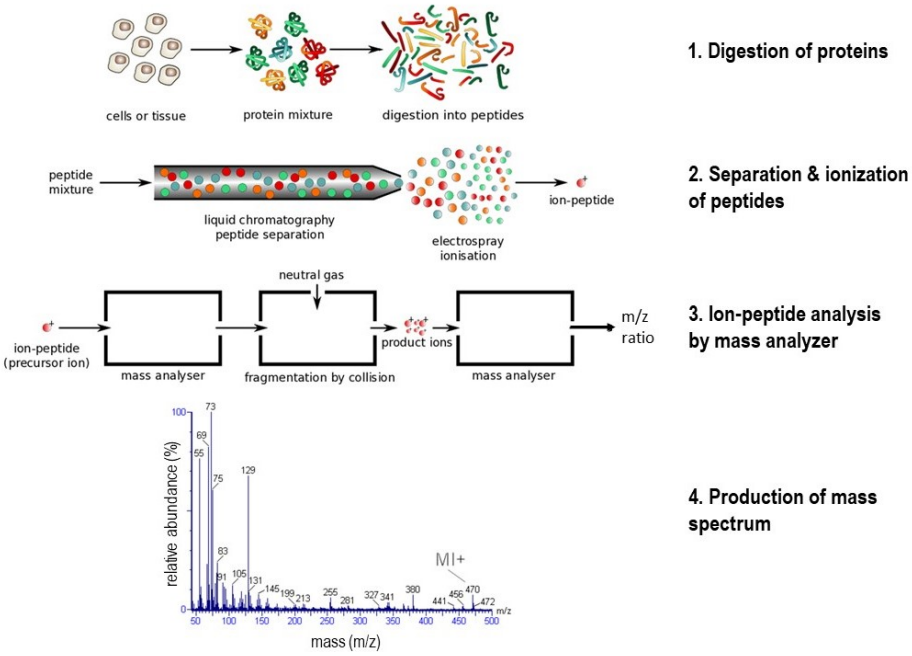


Figure **5.6.1**: The steps of mass spec analysis of the proteome. (1) Digestion of proteins: Proteins are isolated from a biological sample (cells or tissues). The protein mixture is digested into peptides. (2) Separation and ionization of peptides: The peptide mixture is subject to liquid chromatography to separate the peptides which are then ionized by methods such as electrospray ionization to form charged ion-peptides. (3) Ion-peptide analysis by mass analyzer: The ion-peptides are analyzed in several steps by a mass analyzer that determines the mass-to-charge (m/z) ratio and abundance for each peptide. (4) Production of the mass spectrum: The data is compiled into a mass spectrum graph where the identity of each peptide can be determined. (The Steps of Mass Spec by Patricia Zuk, CC BY 4.0; adapted from Mass spectrometry protocol by Phillip Hupe CC BY-SA 3.0 and Mass spec brassicasterol by Smmudge, CC BY-SA 3.0)

Two-Dimensional Gel Electrophoresis

Like mass spec, **two-dimensional gel electrophoresis (2D-GE)** separates proteins based on their size and charge. 2D-GE is often used in the study of post-translational modifications of proteins owing to its high level of resolution. In the first step, proteins undergo **isoelectric focusing**, which is separation based on their on their **isoelectric point (pI),** the pH at which the proteins net

charge is 0. Following this, the proteins are then separated by molecular weight. The separated proteins are visualized using conventional stains like Coomassie Blue or and can be extracted from the gel for further analysis by mass spec.

In 2D-GE, proteins are denatured and then applied to a gel strip containing a pH gradient. An electric field is applied, and proteins migrate to their isoelectric point where they "focus" into tight bands. The gel strips can be loaded onto polyacrylamide gels for conventional SDS-PAGE analysis (Figure **5.6.2**). While 2D-GE can separate thousands of proteins based on their pI, the process is laborious and time consuming, making mass spec a more desirable option for labs that can afford the equipment.

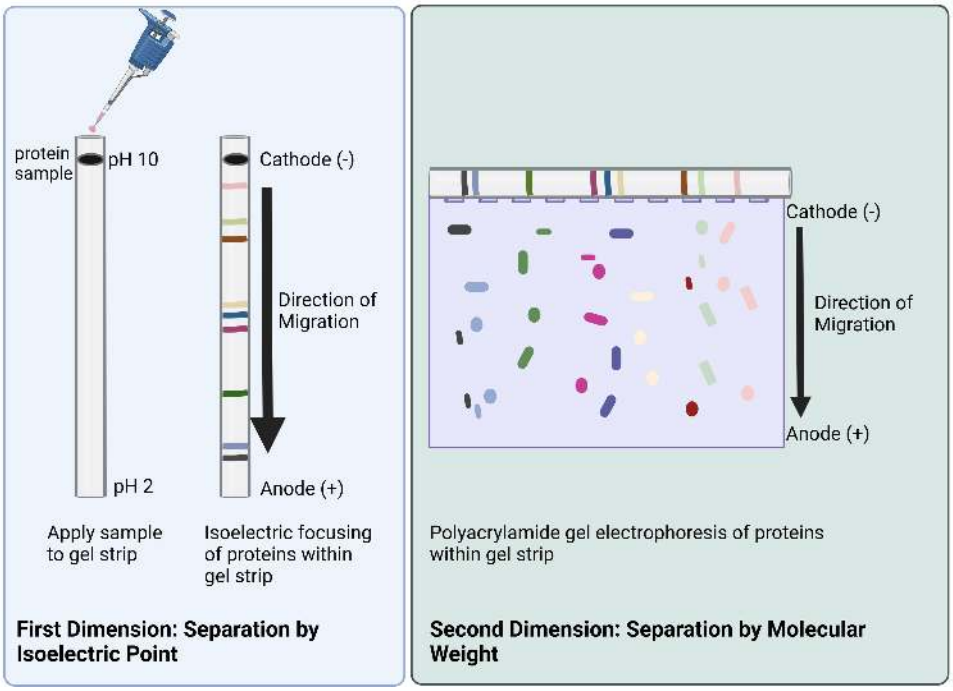


Figure **5.6.2**: 2D Electrophoresis. 2D gel electrophoresis separates proteins by size and charge using two "dimensions". First Dimension - Separation by Isoelectric point (blue box at left in figure): A protein sample (black oval) is applied to a gel strip that has a pH gradient (pH 10 at top of strip, pH of 2 at bottom of strip). An electric field results in isoelectric focusing of proteins within the gel strip. During focusing, each protein in the sample will migrate from the cathode at the top of the strip towards the anode at the bottom of the strip and stop at their isoelectric point (IP), forming a tight band. At the end of isoelectric focusing, the gel strip will contain multiple proteins, each at their individual IP (colored bands). Second Dimension - Separation by Molecular Weight (green box at right in figure): The gel strip from the first dimension is laid on top of an polyacrylamide gel and an electric field applied. The proteins will exit the gel strip and enter into the polyacrylamide gel. Once in the gel, the proteins will migrate based from the cathode at the top of the gel towards the anode at the bottom of the gel and stop at a position in the gel based on their molecular weight (colored dots). (2D Electrophoresis by Patricia Zuk, CC BY 4.0; Created in BioRender. Zuk, P. (2025))

Chromatography is a widely used technique for separating, identifying, and purifying the components of a mixture, such as proteins (Figure **5.6.3**). There are numerous types of chromatography, including liquid, gas, and thin-layer chromatography. Proteins are often separated using liquid chromatography. To separate proteins using liquid chromatography, the sample is dissolved in a liquid phase called the **mobile phase.** The mobile phase is then applied to a **stationary phase** - a solid (or liquid-coated) material that has been fixed into a column - and then allowed to move through the stationary phase. Because of their unique physical and chemical properties, each protein in the sample will interact differently with the stationary and mobile phases. This will result in different rates of movement for each protein through the stationary phase, separating the sample into individual proteins. Depending on the type of stationary phase used, proteins can be separated based on their charge, size, hydrophobicity, isoelectric point, or through their interaction with a ligand attached to the stationary phase.

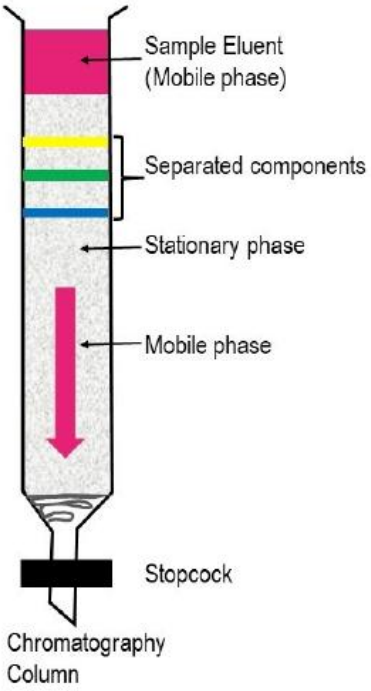


Figure **5.6.3**: Liquid chromatography can separate proteins. Separation of a protein mixture by column chromatography is based on the movement of a liquid, mobile phase through a stationary, solid phase that is formed into a column. The protein mixture is dissolved in the mobile phase (also called the sample eluent) and applied to the column. A stopcock at the bottom of the column is opened to allow for mobile phase migration down the column. As the mobile phase moves through the stationary phase (shown with an arrow), it carries the protein sample with it, resulting in the separation of individual proteins within the column (separated components). (Protein chromatography by Patricia Zuk, CC BY 4.0)

Protein Microarrays

The **protein microarray** is used for high-throughput screening of proteomes. Like DNA microarrays, the protein microarray is made of a solid support, usually a glass slide, known as a **chip**. An array of "capture proteins" (e.g. antibodies, antigens, peptides, full-length proteins) are bound to the chip. Sample proteins are labeled with fluorescent probes and then applied to the chip. The capture proteins "capture" the labeled sample proteins by binding them. Those proteins bound to the chip can then be detected and quantified using their fluorescent signal. Using the appropriate protein microarray, the proteins found within a proteome can be tested for their function, expression level, or presence/absence of modifications through their interactions with the proteins on the microarray. Protein microarrays have been key in the study of antibody-antigen interactions, inflammatory mediator production, detection of infectious diseases, and identification of disease biomarkers.

Key Concepts

Proteomics provides a deeper understanding of cellular function, bridging the gap between genetic information and biological processes, making it essential for fields like molecular biology, medicine, and biotechnology.

Some important concepts to remember are:

- proteomics studies the structure, function, and modifications of proteins using techniques such as:
  - mass spectrometry
  - 2D gel electrophoresis
  - chromatography
- the study of the proteome is critical in the construction of biochemical pathways that underlie normal cellular processes and disease like cancer, cardiovascular disease, or neurodegenerative conditions
- proteomics has led to the identification of numerous disease biomarkers and the development of therapeutic drugs

- proteomics can measure drug effects on the proteome to optimize treatments and reduce side effects
- proteomics can reveal how organisms respond to environmental changes, such as temperature shifts, pollutants, or pathogens, by studying changes in protein expression and modification

Glossary

Biomarker – a protein or molecular signature that indicates a biological state, such as disease or infection

Chromatography - a lab technique for purifying compounds in a mixture based on differences in their physical and chemical properties; main types include liquid, gas, thin-layer, and paper chromatography

Isoelectric focusing - the separation of proteins using their isoelectric point

Isoelectric point (pI) - the pH at which a proteins charge is "0"

Liquid chromatography - a separation technique based on the movement of a sample within a mobile phase through a stationary phase

Mass spectroscopy (i.e. mass spec) - an analytical tool useful for measuring the mass-to-charge ratio (m/z) of one or more molecules, like a protein, present in a sample

Mass spectrum - a plot of the relative abundance of a molecule, like a protein, as a function of its m/z ratio

m/z ratio - the mass-to-charge ratio

Protein microarray - a solid surface onto which thousands of different proteins are immobilized; allow researchers to study multiple proteins simultaneously

Proteome - the complete set of proteins expressed by a cell

Proteomics - the study of the proteome

Two-dimensional (2D) gel electrophoresis - a technique that separates proteins first based on their isoelectric point, then their molecular weight

---

5.6: Proteomics is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

6: Mammalian Cell Culture Fundamentals

6.1: Principles of Mammalian Cell Culture

6.2: Principles of Aseptic Technique

6.3: Cell Culture Equipment

6: Mammalian Cell Culture Fundamentals is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

6.1: Principles of Mammalian Cell Culture

Biotech Focus

In 1951, **Henrietta Lacks (1920 - 1951)** entered Johns Hopkins hospital for the surgical removal of cervical adenocarcinoma. Cells biopsied from her tumor were given to George Otto Gey (pronounced "Guy") and Gey, together with his wife Margaret, began culturing these cells. Despite having failed to culture other types of cells before, the carcinoma cells from Mrs. Lacks survived and rapidly grew under *in vitro* conditions. They named the cell line **HeLa**, from the letters of Henrietta Lack's name, and began to distribute these cells to other labs around the country. Today, Hela cells are one of the most commonly cultured cell lines in culture facilities. They divide - quickly and indefinitely under standard culture conditions, a hallmark of an immortalized cell line. They have been credited with numerous discoveries, including the development of the polio vaccine, and have been used in drug development, the study of cellular aging, and cancer. However, Mrs. Lacks was not asked for her permission to distribute the cells to Dr. Gey. Patient consent to use their tissues for research was not a common practice back then. Nor did Mrs. Lack's or her family receive any compensation when the cells entered the US marketplace. The commercialization of HeLa cells and subsequent profits has since raised questions about ownership and informed consent of patients.

To learn more about Henrietta Lacks and George Guy, read *"The Immortal Life of Henrietta Lacks"* (2010) by Rebecca Skloot.

Introduction

Cell culture is the growth of cells separate from the donor organism using a liquid, semi-solid or solid growth medium. A more modern definition would be the growth of cells from a multi-cellular organism under controlled conditions. Cell culture gives researchers a method for studying the behavior of cells, under experimental conditions, free from the variations that might arise within a complex, multicellular organism. Such research has elucidated the mechanisms of complex processes such as normal embryologic development, disease progression and how drugs can impact the health and function of an organism. Today, cell culture is an integral part of basic scientific research, pharmaceutical development, bioengineering labs and many other kinds of labs performing research that impacts human health. It is truly one of the most important skills a biologist can have.

The term cell culture is often used interchangeably with **organ culture** and **tissue culture**, but they are not the same thing. Cell culture is the *culture of individual cells* - taken from plant or animal. Organ culture refers to the the *culturing of mammalian tissues* isolated from an organ, or the organ itself. Finally, tissue culture is frequently associated with the *culturing of plant cells or tissues* (Figure **6.1.1**).

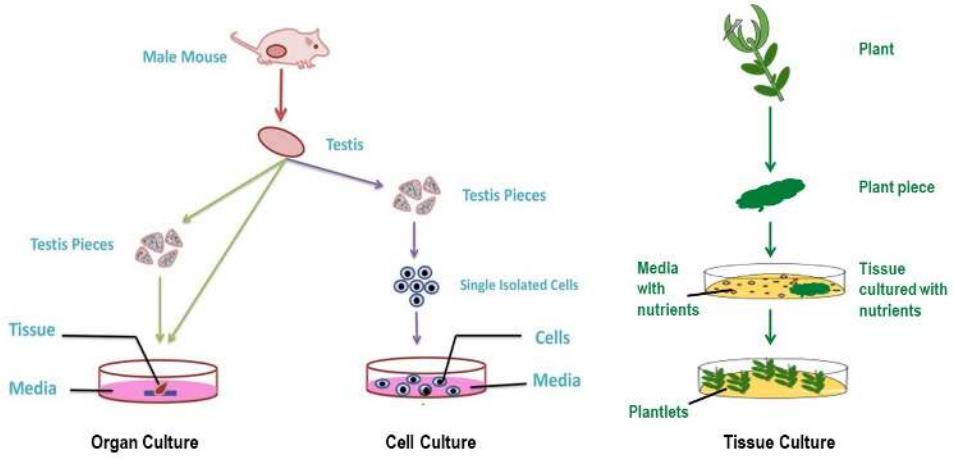


Figure **6.1.1**: Cell vs. Organ vs. Tissue Culture. In organ culture, a tissue, like the testis, is isolated from an animal (such as a male mouse) and cut into pieces (testis pieces). The resulting tissue is placed into culture dishes with media for maintenance of the tissue. In cell culture, the tissue pieces are used to isolate cells that are then placed into culture dishes with media. In tissue culture, tissue is isolated from plants (plant piece) and placed into media with nutrients. Over time, the plant cells within the tissue will differentiate and form small "plantlets". (Cell vs. Organ vs. Tissue Culture by Patricia Zuk CC BY 4.0, adapted from Testis Organ Culture by Ibtisham et al. CC BY-SA 3.0 and Simple Micropropagation Technique by MarchaelaM, CC BY-SA 4.0)

Learning Objectives

Cells and tissues can be cultured outside of the organism using *in vitro* culture techniques. At the end of this section, you will be able to:

- Define the types of cell culture: adherent, suspension, organ, and tissue
- Distinguish between primary cultures, secondary cultures, cell lines, and cell strains
- Compare a cell line with a cell strain
- Describe the two types of cell cultures
- List the conditions for cell culture
- List some cell culture reagents
- List and describe the three types of cultured cells
- List and describe the major types of culture media
- Explain biosafety considerations
- List and explain the four BSL levels

Types of Cell Culture

Adherent culture: a type of *in vitro* culture in which cells grow on a surface as a single layer of cells (Figure **6.1.2**). Most cells must grow under adherent culture conditions because they require a surface on which to adhere in order to survive and divide. The culture surface is provided in the form of specially-treated and sterilized **cell culture plastic-ware**. It provides the cell with a charged surface that is essential for its adherence. Normal cells grown under adherent conditions will stop growing once they are surrounded on all side by other cells, produced a **monolayer** of cells. This inhibition is called **contact-growth inhibition** and adherent culture can also be known as monolayer culture. Transformed cells will not demonstrate this property. Nearly all normal vertebrate cells grow under adherent conditions.

Suspension culture: a type of *in vitro* culture in which the cells grow suspended within the culture medium; no adherence is required (Figure **6.1.2**). While not as common as adherent culture, there are cell types, such as immortalized cells and certain types of immune cells, that will grow suspended in culture medium. This type of culture requires specialized equipment in order to keep

the cells suspended so that they receive adequate oxygen and nutrients and do not settle out due to gravity. Most suspension cultures will either use **roller balls** or **"spinner" flasks**. Roller balls are placed on machines that continuously turn the bottle, while magnetic stirrers within spinner flasks keep the medium and cells suspended.

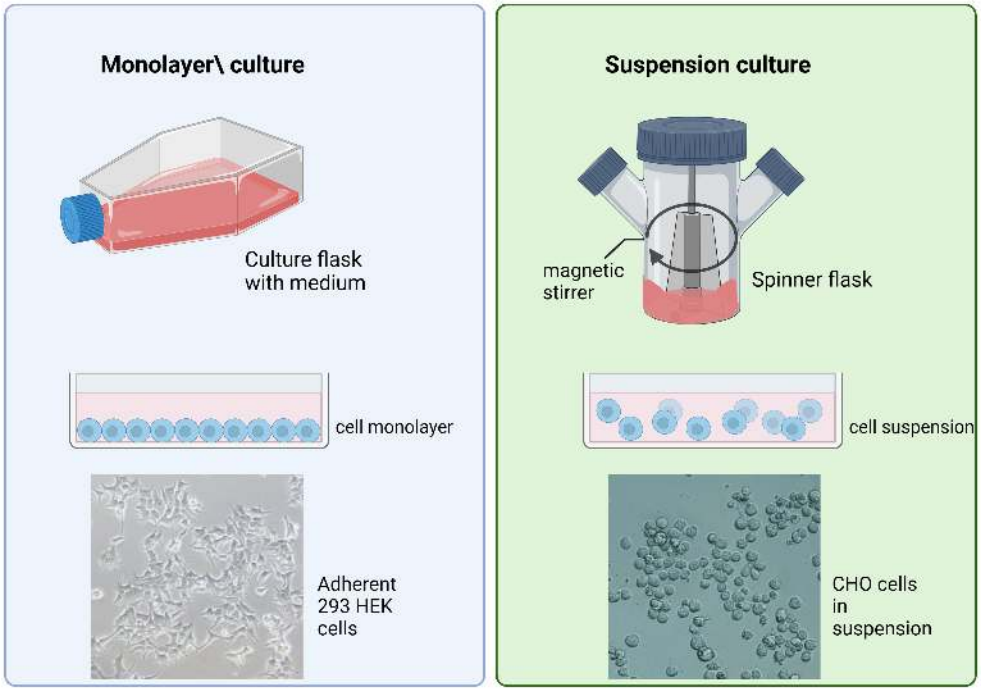


Figure **6.1.2**: Adherent vs. Suspension Culture. In adherent culture (blue box on left), cells are grown specially-treated plastic-ware, like a culture flask, whose surfaces promote cell adherence (culture flask with medium). The cells in adherent culture will grow as a cell monolayer. Adherent 293 HEK cells are shown. In suspension culture (green box on right), cells can be grown in vessels known as Spinner flasks, whose magnetic stirrer keeps the cells suspended and exposed to oxygen and nutrients. The cells in suspension culture are non-adherent and "float" within the medium (cell suspension). CHO cells in suspension are shown. (Types of Culture by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025); photo credits: HEK 293 cells by Panzer VI-II public domain; CHO cells by Jamiri, CC BY-SA 3.0)

Organ culture: a type of *in vitro* culture that grows either whole organs or parts of organs. One of the major advantages of organ culture is its maintenance of tissue architecture and function within the organ. Organ culture is complex and requires delicate handling *in vitro* as it is essential that the tissue is never disrupted or damaged. The media used for a growing organ culture are generally the same as those used for tissue culture. Organ culture technology has contributed to advances in embryology, inflammation, cancer, drug development, and stem cell research.

Tissue culture: a type of *in vitro* culture that maintains and/or grows plant cells, tissues, or organs. Tissue culture of plants is widely used to create exact clones of a plant. Plant cells, pieces of leaves, stems or roots, given the required nutrients and plant hormone, are often used to generate a new plants through tissue culture.

Cell Culture Types

Cell culture is an integral part of basic scientific research, pharmaceutical development, bioengineering labs, and many other kinds of labs performing research that impacts human health. As a result, the types of cells that are cultured are extremely varied but can be placed into one of four major categories based on their morphology (i.e., their appearance) in culture:

1. **Epithelial-like:** cells attach to a substrate and appear flattened and polygonal in shape
2. **Lymphoblast-like:** cells do not attach but remain in suspension with a spherical shape
3. **Fibroblast-like:** cells attach to an substrate appears elongated and bipolar

Table \(\PageIndex{1}\ below lists some of the more commonly cells cultured cells.

Table **6.1.1: Commonly Culture Cells**

Cell Abbreviation	Cell Name	Description
3T3	"**3**-day **T**ransfer **3**00,000 inoculum"	mouse fibroblast cell line; derived from Swiss albino mouse embryonic fibroblasts; original culture "transferred" every "3" days at a density of "300,000" cells per culture; used to derive additional 3T3 cell lines
3T3-L1	**3T3** cell, subclone **L1**	mouse fibroblasts (immortalized pre-adipocyte cell line); derived from 3T3 cells
MC-3T3	"**M**ouse **C**alvarial" from **3T3** cells	mouse fibroblasts (immortalized pre-osteoblast cell line); derived from mouse calvaria (i.e., bone)
CHO	"**C**hinese **H**amster **O**vary"	hamster epithelial cells (immortalized cell line); numerous subclones available
L6		rat myoblasts (immortalized cell line)
PC12	**P**heo**c**hromocytoma-12	rat neuroblastic cells (non-immortal cell line); derived from pheochromocytoma cells in adrenal medulla
COS	"**C**V-1 in **O**rigin, carrying the **S**V40 genetic material"	monkey kidney fibroblasts (immortalized cell line); produced by transforming CV cells with SV40 viral DNA; numerous cell lines available (e.g. COS-1, COS-7)
MDCK	**M**adin **D**arby **C**anine **K**idney	canine epithelial cells (non-immortal cell line)
Saos-2	"**Sa**rcoma **Os**teogenic"	human fibroblasts (pre-osteoblast cell line); derived from osteosarcoma patient's bone
HEK 293	"**H**uman **E**mbryonic **K**idney" experiment number **293**	human epithelial cell line (immortalized); derived from human fetal kidney tissue; produced by transfecting HEK cells with adenoviral DNA
H1, H9		human Embryonic Stem (ES) cell lines; derived from inner cell mass of human blastocysts
MCF-7	**M**ichigan **C**ancer **F**oundation-7	human breast cancer cell line (immortalized); derived from metastatic breast tumor
HeLa*	"**He**nrietta **La**cks"	human epithelial cells (immortalized cell line); derived from metastatic cervical tumor

Primary vs. Secondary Cultures vs. Cell Lines

Many cells used in *in vitro* culture are derived from either animal or plants tissues. The process of isolating these cells is well-defined and will result in a primary culture. From this culture, others can be derived, such as a cell line or cell strain.

A **primary culture** can be prepared from a tissue that is removed from an organism. The tissue can be dissected out and cut into small pieces or treated with enzymes to release cells from the tissue. The resulting cellular mixture is then placed in suitable culture environment. For an adherent primary culture, the non-adherent cells will be removed when the medium is changed and the

adherent cells can be expanded. A primary culture is a **heterogeneous** (i.e. mixed) population of many different cell types because of its origin from a multi-cellular tissue. The cells in the primary culture, known as **primary cells**, have a finite life span in culture, unless they are immortalized (see below). Sub-culturing of these primary cells can expand their numbers and lead to either a secondary culture of a cell line. Many primary cell types will not divide i*n vitro* (e.g. macrophages and neurons). As such, these cells can be maintained *in vitro* for a limited amount of time.

A **cell line** is produced by sub-culturing (or passaging) a primary cell culture (see Chapter 13.13: Cell Culture). A cell line may be referred to as a **secondary culture**. Dissociating the primary cells from the culture dish and transferring them to a new one will kill those cells that are not as "hardy" as the others, removing them from the subsequent culture. As a result, a cell line is more **homogenous** (i.e. uniform) than the primary culture. Most cell lines are produced by passaging multiple times in order to increase the removal of contaminating cell types. While the composition of a cell line is relatively homogenous, the cells of a cell line are not all identical to one another. As with primary cultures, cell lines have a finite life span in culture unless immortalized.

A **cell strain** is produced from either a primary culture or a cell line. The cells are diluted over and over again until a single cell is achieved. This single cell is expanded into a cell population known as a cell strain. In most cell strains, a single cell with a specific trait is selected and expanded. The expansion of these single cells in number is called **cloning** as every cell in a strain originates from the same "starting" cell. The cloning process can produce multiple cell strains (or **sub-clones**), with each strain having a unique set of characteristics. Because the cell strain is derived from a single cell, it is a homogenous population, with each cell in the culture being of the same type. However, the cells of a homogenous cell strain are not identical to one another as genetic changes can occur as they are maintained *in vitro*.

Cell line and cell strain are frequently used interchangeably but they not the same thing. A cell line is produced from a primary culture, while a cell strain is produced from a single cell taken from a primary culture or cell line. Cell strains have a finite life-span in culture and do not change their properties over time. Because they are often isolated from cell lines, cell strains are also more homogenous and specialized then the cell line. Table **6.1.2** below compares a cell line with a cell strain.

Table **6.1.2**: Comparison of Cell Line with Cell Strain

Feature	Cell Line	Cell Strain
Definition	A population of cells derived from a primary culture	A population of cells derived from a primary culture or cell line and have been selected for a specific trait (or traits)
Origin	Usually created by subculturing cells from a primary culture to eliminate contaminating cell types	Derived from a single cell through a process called "cloning"
Composition	Can be heterogenous	Homogenous
Lifespan	Can be finite or immortal in culture	Typically finite in culture

Immortalized Cells

Cell lines and cell strains that can only be cultured for a limited time-span before the cells die are known as **finite**. Through a process called **transformation**, the genetic make-up of a cell line or cell strain can be altered and the cells can be maintained indefinitely in culture. These cells are said to be **immortalized.** Because they can be cultured continuously, immortalized cell lines and cell strains are also called **continuous cell lines** or **continuous cell strains**. Immortalized cell lines can be produced from cancerous primary cultures, from cell lines in which genetic mutations have occurred naturally during culture, or following the direct introduction of oncogenes into the cultured cell. The genetic changes of transformation “turn off” the cellular mechanisms that control cell death, permitting continuous growth of the cells in culture. With their infinite life-span in culture, immortalized cell lines and cell strains are used extensively in biotechnology and biomedical applications. Table **6.1.3** below outlines some of the advantages and disadvantages of immortalized cells.

Table **6.1.3**: Advantages and Disadvantages of Immortalized Cells

Advantages	Disadvantages
fast growth & unlimited proliferation; decreased need to produce primary cultures	genetic changes during transformation lead to altered gene expression and cellular functions; cells cannot be used for the study of normal physiology
easy to maintain; more robust under suboptimal conditions	transformation produces cells with characteristics of cancer cells (e.g. altered chromosome number, uncontrolled proliferation); limits relevance for certain non-cancerous processes
reduced dependence of serum and substrate - grow more easily in suspension conditions (loss of anchorage dependence)	cells can accumulate additional genetic mutations over time that can compromise research results
ability to grow at higher cell density (loss of contact inhibition)	may exhibit reduced resemblance to tissue of origin, such as loss of tissue-specific gene expression; cells cannot be used for highly specialized studies
homogenous; similar cultures produced with each passage; results in consistent experimental results	cross-contamination with other cells lines common
well-studied and widely used in research; extensive data available on their growth, behavior, genetic profile, and functional characteristics	prone to contamination with mycoplasma
cost-effective; unlimited growth and ease of culture make cells more economical for large-scale experiments or industrial applications	

Cell Culture Conditions & Nutrients

The culturing of cells requires well-defined and precise external conditions that mimic those found in the cell's *in situ* environment. The exact values for the conditions listed below can vary according to the type of cell being cultured.

- Temperature
- CO2 concentration
- pH
- Humidity

In addition to these physical conditions, cells also require essential nutrients. As with the external conditions listed above, the concentrations and types of the nutrients given below will vary based on the cell being cultured.

- Amino acids
- Carbohydrates
- Vitamins
- Minerals
- Growth factors and hormones

Cells are provided these nutrients through their **culture medium**. For animal cell culture, the culture medium is used to change the physical and chemical properties of the environment surrounding the cells. Specifically, the culture medium is critical for the maintenance of pH & bicarbonate levels, salt concentration, and osmolarity.

Culture Medium

The two basic types of culture medium are natural and synthetic.

- **Natural culture medium** is made up of biological substances
  - Concentrated plasma "clots" – plasma separated from blood serum
  - Tissue extracts – extracts from chicken embryos, bone marrow, liver, and spleen
  - Biologic fluids – lymphatic fluid

- **Synthetic culture medium** is made up of a **basal medium** and **supplements**

Basal medium (Figure **6.1.3**) provides the cells with a “baseline” of amino acids, salts, glucose, vitamins, and minerals for their viability and growth. Basal medium can be as simple as a balanced salt solution or a more complex formulation, like BME, DMEM, or Ham's medium, that can be purchased from a variety of cell culture companies.

Figure **6.1.3**: Basal Medium for Cell Culture. Basal medium contains glucose, plus essential amino acids, salts, vitamins, and minerals for cell viability and growth *in vitro*. (DMEM cell culture medium by Lilly_M, CC BY-SA 3.0)

Table **6.1.4** shows the compositions of four of the more common basal media: Basal Medium Eagle (BME) or Eagle's Medium, Minimal Essential Medium (MEM), alpha-Minimal Essential Medium (a-MEM), and Dulbecco's Modified Eagle's Medium (DMEM).

Table **6.1.4**: Composition of Common Basal Media

Component	Basal Medium Eagle (BME) i.e. Eagle's Medium	Minimal Essential Medium (MEM)	alpha-Minimal Essential Medium (a-MEM)	Dulbecco's Modified Eagle's Media (DMEM)
Vitamin B12			1.36	
Thiamine (Vitamin B1)	1	1	1	4
Sodium pyruvate			110	110
Sodium phosphate (NaH_2PO_4-H_2O)	140	140	140	125
Sodium chloride (NaCl)	6800	6800	6800	6400
Sodium bicarbonate ($NaHCO_3$)	2200	2200	2200	3700
Riboflavin (Vitamin B2)	0.1	0.1	0.1	0.4
Pyridoxal hydrochloride	1	1	1	4
Potassium chloride (KCl)	400	400	400	400
Phenol red	10	10	10	15
Niacinamide (Vitamin B3)	1	1	1	4
Magnesium sulfate ($MgSO_4$)	97.67	97.67	97.67	97.67
Lipoic acid			0.2	
L-valine	23.5	46	46	94
L-tyrosine	26	52	52	104
L-tryptophan	4	10	10	16
L-threonine	24	48	48	95
L-serine			25	42
L-proline			40	
L-phenylalanine	16.5	32	32	66
L-methionine	7.5	15	15	30
L-lysine	36.47	73	73	146
L-leucine	26	52	52	105
L-isoleucine	26	52	52	105
L-histidine		42	42	
L-histidine	8		31	42

Component	Basal Medium Eagle (BME) i.e. Eagle's Medium	Minimal Essential Medium (MEM)	alpha-Minimal Essential Medium (a-MEM)	Dulbecco's Modified Eagle's Media (DMEM)
L-glutamine	292	292	292	584
L-glutamic acid			75	
L-cysteine	16	31	31	63
L-cysteine			100	
L-aspartic acid			30	
L-asparagine			50	
L-arginine	21	126	126	84
L-alanine			25	
i-Inositol	2	2	2	7.2
Glycine			50	30
Folic acid (Vitamin B9)	1	1	1	4
Ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)				0.1
D-Glucose (dextrose)	1000	1000	1000	1000
D-calcium pantothenate	1	1	1	4
Choline chloride	1	1	1	4
Calcium chloride ($CaCl_2$)	200	200	200	200
Biotin (Vitamin B7)	1		0.1	
Ascorbic acid (Vitamin C)			50	

Culture supplements complete the basal medium by providing additional nutrients, proteins, amino acids and vitamins, and also the appropriate pH. The most commonly added supplements are **serum and antibiotics.** Serum provides the cells with key proteins, minerals, lipids, hormones, growth factors, and adhesion factors. Antibiotic supplementation (i.e. penicillin and streptomycin) decrease the chances of microbial proliferation when combine with proper aseptic technique. Synthetic medium may also contain additional amino acids for growth and proliferation, like L-glutamine, cysteine and tyrosine. Fungal contamination can be decreased through the supplement amphotericin B.

Depending on the type of cells being cultured, there are several types of synthetic culture media that can be used.

- **Serum-containing synthetic medium:**
  - basal medium + serum + antibiotics (optional)
  - serum is usually obtained from animal sources (e.g. horses, fetal cow) that have been raised and slaughtered for this purpose - ethical issues
  - chances of microbial contamination can be high
  - serum properties can vary from batch to batch and company to company - results in inconsistent cultures

- human serum can be used – expensive and highly regulated due to disease transmission

- **Serum-free synthetic medium:**
  - uses **crude protein fractions** such as bovine serum albumin, or globulins to replace serum
  - provides more consistent results
  - only specific cell types can grow using this kind of medium
  - protein fractions are still obtained from animals
  - cells may require additional growth factors and hormones to optimize growth
- **Chemically-defined synthetic medium:**
  - type of serum-free medium
  - uses highly purified protein fractions - e.g. recombinant proteins
  - concentrations of these fractions are known and optimized for cell growth
- **Protein-free synthetic medium:**
  - type of serum-free medium
  - undefined components such as peptide fractions are used
- **Xeno-free synthetic medium:**
  - no animal components used
  - human sourced components are used as supplements - e.g. human serum albumin used as a source of protein
  - typically used for the maintenance and growth of lymphocytes
  - expensive and highly regulated due to disease transmission

Biosafety Considerations

Biosafety is a concept that refers to the protection of human health and the environment from the possible adverse effects of pathogenic and/or genetically modified organisms and micro-organisms that are used in research and development (R&D) and biotechnology. Biosafety is important when working with cell cultures because certain cell cultures may contain pathogens that may be harmful to humans or the environment. Therefore, it is the responsibility of all lab personnel to ensure that each lab member is operating safely and that accidents do not occur.

The typical hazard encountered in a cell culture lab is a biohazards. To mitigate exposure, culture labs rely upon two very important aspects: personal protective equipment and biological safety.

Personal Protective Equipment

Personal protective equipment (PPE) protects the user against specific hazards in the laboratory. Safety glasses, lab coats and gloves are examples of PPE that are commonly used in labs. Safety glasses are important pieces of PPE and should be selected based on the specific risks anticipated (e.g. splash risk, shortwave, ultraviolet radiation). Because cell culture is done using a biosafety cabinet, eye protection when culturing cells is not necessary. However, outside of the biosafety cabinet, eye protection may be necessary depending on the lab protocol being performed.

Besides eye protection, it is important to limit exposure of the skin to microbes and chemicals using gloves. The type of glove used is like safety glasses – it is dependent upon the hazard encountered in the culture lab. Most gloves used in culture labs are either **latex, nitrile, or vinyl**. Latex is the simplest material and the most common kind of glove. However, many people may exhibit skin sensitivity to latex. Some disposable latex gloves are also flammable. Nitrile gloves may be used as an alternative in the culture lab. Nitrile is thicker than latex, does not irritate most user's skin and provides a more substantial barrier.

Wearing a lab coat protects clothing and is often worn in a cell culture lab. Many cell culture labs have dedicated lab coats for culture work. In addition to a lab coat, the clothing worn must be appropriate for the lab. Open toed shoes are not allowed in a lab. Long pants are also required. While not necessary, long-sleeved shirts may also be worn. However, short-sleeves are fine if wearing a lab coat.

Biological Safety

The safety of personnel working in a culture lab is described by set of bio-containment precautions known as **Biosafety Levels (BSLs)**. The Centers for Disease Control (CDC) have established four BSLs, classified as *BSL-1 through BSL-4*, that describe the

risk level associated with a particular biological agent along with the corresponding microbiological practices, safety equipment, and facility safeguards lab personnel shall use with handling such an agent (Table **6.1.5**. Biological labs are classified using these BSLs.

Table **6.1.5**: Biosafety Levels

BSL Level	Description	Biologic Agents	Practices & Precautions
BSL-1	Risk level: Minimal Facility requirement: Standard laboratory Special Equipment needed: No	Non-pathogenic, well characterized microbes Agents not known to cause illness or disease is health adults e.g. E.coli	Standard microbiologic practices, PPE (gloves, lab coats)
BSL-2	Risk level: Moderate Facility requirement: Limited access lab, biohazard signs Special Equipment needed: Yes	Pathogenic microbes Agents could cause mild to serious illness e.g. Influenza, herpes, common cold	Biosafety cabinet for aerosols, enhanced PPEs, sharps precautions
BSL-3	Risk level: High Facility requirement: Controlled access lab, negative air pressure ventilation Special Equipment needed: Yes	Pathogenic microbes - indigenous or exotic Agents cause disease that could be lethal if untreated e.g. SARS-CoV1, SARS-CoV2, tuberculosis, avian influenza, plague	Biosafety cabinet for all activities, respirators, air is HEPA-filtered upon lab exit, decontamination system, personnel shower out of lab
BSL-4	Risk level: Extremely High Facility requirement: Isolated facility, full-suit positive pressure Special Equipment needed: Yes	Pathogenic microbes - dangerous and exotic Agents cause disease that have no treatment e.g. Ebola virus, Marburg virus, smallpox virus	Biosafety cabinet for all activities, full-body air-supplied suits, air is HEPA-filtered upon lab exit, extensive decontamination of lab personnel and lab materials. chemical and personnel showers out of lab

BSL-1

- the basic level of protection
- appropriate for labs working with well-characterized agents that do not cause disease in healthy humans
- no specialized equipment needed
- labs must have a door to limit access to lab
- precautions: standard microbiological practices (i.e., wash hands, area decontamination with bleach or isopropanol); personal protective equipment (PPE) recommended but not required
- type of lab found in high schools and colleges
- example of agents: E.coli, bacillus, saccharomyces

BSL-2:

- involves use of biological agents that pose a moderate risk to personnel and environment
- biological may cause human disease by ingestion or through percutaneous or mucous membrane exposure; treatment is readily available if exposed
- BSL-2 labs are also able to work with samples contaminated with fluids like blood and can also conduct experiments that involve recombinant DNA

- precautions: BSL-1 precautions plus BSL-2 specific requirements
  - use of personal protective equipment like gloves and lab coats
  - use of a biosafety cabinet for cell culture
  - use of other physical containment equipment to limit aerosol transmission or splashing of infectious materials
  - lab must have a door to limit access to lab
- personnel must undergo specific training in handling pathogenic agents
- the lab classification for most cell culture labs in biotechnology and pharmaceutical companies, national labs, research universities and hospital
- examples of agents: influenza, salmonella, staphylococcus, hepatitis

BSL-3

- involve use of pathogens with significant risk to personnel and environment
- pathogens have a known potential for aerosol transmission that may cause serious or potentially lethal infections in humans
- precautions: BSL-1 and BSL-2 precautions plus BSL-3 specific requirements
  - use of biosafety cabinet for cell culture
  - the lab entrance must be separated from other areas of the building
  - the lab must have two sets of self-closing doors and sealed windows to minimize escape of aerosols
  - the lab must have a specialized ventilation system with filtration to prevent airborne circulation of pathogens
  - lab personnel are monitored medically on a regular basis and offered relevant immunizations
  - lab personnel must wear solid-front protective clothing (e.g. gowns that tie in the back) and face shields or eye protection
- personnel undergo an enhanced level of training in handling pathogenic agents
- examples of agents: SARS-CoV, mycobacterium, West Nile virus, additional viruses

BSL-4

- involve use of pathogens with significant risk to personnel
- pathogens pose a high risk of life-threatening disease by infectious aerosols for which no treatment is available
- precautions: BSL-1 through BSL-3 precautions plus BSL-4 specific requirements
  - the lab entrance must be separated from other areas of the building
  - the lab entrance uses airlocks to prevent escape of aerosols
  - the lab must have a specialized ventilation system with filtration to prevent airborne circulation of pathogens
  - work must be done either in a Class II or Class III biosafety cabinet
  - personnel using the Class II cabinet must wear a full-body, positive-pressure suit with HEPA-filtration
  - personnel leaving the lab must pass through a chemical shower for decontamination of pressure suit, then enter a room to remove the suit, and then take a shower before donning clothes
  - all materials leaving the lab must undergo rigorous decontamination, including lab waster, air, and water
- personnel require exceptional level of training
- examples of agents: Ebola virus, smallpox virus, Marburg virus

Lab Protocols

- Chapter Lab Technique: Cell Culture

Key Concepts

In vitro cell culture is used to grow cells outside of the donor organism by using a liquid, semi-solid or solid growth medium.

Some important concepts to remember are:

- The growth of cultured cells, tissues, or organs requires precisely controlled physical conditions and chemical reagents
- The types of cells that can be cultured are wide and varied and depend on the goal(s) of the experiment
- *In vitro* culture can also be used to maintain tissues and organs outside of the organism, through the process of organ culture
- Primary cultures are established following the removal of cells from a tissue and placement into culture

- Sub-culturing of a primary culture produces a cell line with increased homogeneity when compared to the primary culture
- Isolation of single cells from a cell line, followed by *in vitro* expansion produces cells strains with specific characteristics
- While the most common cell types grown *in vitro* are non-pathogenic, biosafety considerations during culture are essential.

Glossary

Adherent cells - cells that must attach to a surface (e.g. a culture dish) to grow

Basal medium - a liquid mixture of amino acids, salts, glucose, vitamins, and minerals essential for mammalian cell viability and growth

Biosafety - the principles and protocols used to prevent the unintentional exposure of workers to biological agents or toxins; used also to reduce the risk of their accidental release into the environment

Biosafety level (BSL) - a classification system used to define the containment precautions required when working with biological agents that may pose a potential risk to human health and the environment; there are biosafety levels (BSL-1 to BSL-4), each with increasing degrees of containment, protective measures, and operational requirements

BME (Basal Medium Eagle) - a common basal medium used for cell culture; also called Eagle's medium

Cell culture - a laboratory technique used to maintain and/or grow eukaryotic cells *in vitro*

Cell line - a population of cells derived from primary cultures that can grow and multiply *in vitro*

Cell strain - a population of cells derived from a single cultured cell that can grow and multiply *in vitro*

Culture medium - a mixture of basal medium and supplements used to support the viability and growth of eukaryotic cells *in vitro*

DMEM (Dulbecco's Modification of Eagle's Medium) - a common basal medium used for cell culture; a modified version of Eagle's medium

Immortalized cells - eukaryotic cells that grow and multiply indefinitely *in vitro* due to genetic transformation

In vitro - refers to biological or chemical process that occurs outside of an organism and in a controlled laboratory environment like a test tube or culture dish; Latin for "in glass"

Organ culture - a laboratory technique used to maintain and/or grow whole organs or pieces of an organ *in vitro*

Primary culture - a population of eukaryotic cells derived from a tissue and maintained *in vitro*

Personal protective equipment (PPE) - specialized clothing or equipment designed to protect individuals from exposure to hazards that could cause injury or illness

Serum - the fluid portion of blood plasma that remains after the blood has clotted and the clotting factors have been removed; contains various proteins, hormones, electrolytes, and nutrients that can support cells *in vitro;* often added to basal medium and supplements to create culture medium

Supplements - additional factors added to basal medium to support cell growth in vitro and/or prevent contamination; often added to basal medium in combination with serum to create culture medium

Tissue culture - a laboratory technique used to maintain and/or grow tissues *in vitro*; usually refers to the culture of plant tissues *in vitro*

6.1: Principles of Mammalian Cell Culture is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

6.2: Principles of Aseptic Technique

Biotech Focus

In 1912, **Alexis Carrel** established the first cell line derived from fragments of explanted chicken embryo heart. This cell line could be sub-cultured maintained in vitro and even sub-cultured multiple times but his cultures eventually become contaminated with bacteria. In response, Carrel developed a series of procedures that minimized this contamination and, in doing so, introduced the concept of **aseptic technique** to cell culture. Through aseptic technique, cultures can now be maintained in the absence of microbe contamination.

Introduction

Successful *in vitro* cell culture depends on keeping the cultured cells free from contamination by micro-organisms, thus ensuring the safety and integrity of the process, products, the environment, and the user. Sources of contamination in the culture lab include: non-sterile supplies, media, and reagents, airborne micro-organisms, unclean incubators, and dirty work surfaces. The easiest way to eliminate a contamination is to prevent one in the first place. Prevention can be achieved through a combination of aseptic and sterile techniques. **Sterile technique** eliminates contamination through sterilization. **Aseptic technique** maintains the sterility of something that has been sterilized.

Learning Objectives

Preventing microbe contamination of cell cultures through proper aseptic technique is essential. At the end of this section, you will be able to:

- List and describe the types of microbe contamination
- Explain autoclaving and filtration
- Describe the fundamentals of aseptic technique

Types of Contamination

Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories. Cell culture contaminants can be divided into two main categories: 1) **chemical contaminants,** such as impurities in media, sera, and water, endotoxins, plasticizers, and detergents, and 2) **biological contaminants,** such as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines. Chemical contaminants can significantly alter the chemical properties of the culture and quickly kill cells. Unfortunately, chemical contaminants can be difficult to detect as they are often invisible. Biological contaminants are more common in the culture lab and are easier to detect using simple methods, such as microscopy. The biological contaminant competes with host cells for nutrients. They also secrete acidic or alkaline by-products which can inhibit growth of the culture cells or kill them. Microbes can degrade essential culture components, like arginine and purine, inhibiting synthesis of histones and nucleic acids by the cultured cell. Finally, microbes produce significant amounts of hydrogen peroxide, which is directly toxic to cells.

The major types of biological contamination are:

1. **bacterial**
2. **fungal: mold**
3. **mycoplasma**
4. **eukaryotic: yeast & cell lines**

Bacterial Contamination

Bacteria are a large and ubiquitous group of unicellular microorganisms. They are typically a few micrometers in diameters, and can have a variety of shapes, ranging from spheres to rods and spirals. Because of their ubiquity, size, and fast growth rates, bacteria are one of the most commonly encountered biological contaminants in cell culture. Fortunately, bacterial growth can be prevented with good aseptic technique and the with addition of **antibiotics**, such as penicillin and streptomycin, to the growth medium.

Bacterial contamination is easily detected by visual inspection of the culture within a few days of it becoming infected. Infected cultures usually appear cloudy (i.e., turbid), sometimes with a thin film on the surface. Sudden drops in the pH of the culture medium is also frequently encountered. Under a low-power microscope, the bacteria appear as tiny, moving granules between the cells, and observation under a high-power microscope can resolve the shapes of individual bacteria.

Mycoplasma Contamination

Mycoplasma are simple bacteria that lack a cell wall and they are considered the smallest self-replicating organism. Because of their extremely small size (typically less than 1 µm), mycoplasma are very difficult to detect until they achieve extremely high densities and cause the cell culture to deteriorate; until then, there are often no visible signs of infection. Some slow growing mycoplasma may persist in culture without causing cell death, but they can alter the behavior and metabolism of the host cells in the culture. Chronic mycoplasma infections might manifest themselves as decreased rate of cell proliferation, reduced saturation density, and agglutination in suspension cultures. The only assured way of detecting mycoplasma contamination is by the testing of cultures periodically using assays such as fluorescent staining, ELISA, PCR, or microbiological assays.

Yeast Contamination

Yeasts are unicellular eukaryotic microorganisms found in kingdom Fungi that range in size from a few micrometers (typically) up to 40 µm (rarely). Like bacterial contamination, cultures contaminated with yeast become turbid, especially if the contamination is in an advanced stage. There is very little change in the pH of the contaminated culture until the contamination becomes heavy, at which stage the pH usually increases. Under microscopy, yeast appear as individual ovoid or spherical particles. In cultures with significant numbers of yeast, the cultures can actually smell like bread.

Mold Contamination

Molds are eukaryotic microorganisms in found kingdom Fungi that grow as multicellular filaments called **hyphae**. A connected network of these multicellular filaments are referred to as a colony or a **mycelium**. Similar to yeast contamination, the culture medium becomes turbid upon advanced contamination and the pH of the culture

remains stable in the initial stages, then rapidly increases as the culture become more heavily infected. Under microscopy, the mycelia usually appear as thin, wisp-like filaments, and sometimes as denser clumps of spores. Spores of many mold species can survive extremely harsh and inhospitable environments in their dormant stage, only to become activated when they encounter suitable growth conditions such as those encountered during cell culture.

Cross-Contamination

While not as common as microbial contamination, extensive cross-contamination of cell cultures with HeLa and other fast growing cell lines is a clearly-established problem with serious consequences. Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique are practices that will help you avoid cross-contamination. The main elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

Sterilization: Autoclaving and Filtration

There are two main methods for sterilization in a culture lab: **autoclaving and filtration**. Autoclaving is used to sterilize solid materials, such as culture glassware. Both autoclaving and filtration can be used to sterilize most culture liquids. An **autoclave** (Figure **6.2.1**) uses pressurized steam to destroy microorganisms, and is the most dependable system for the sterilization of laboratory glassware, media, and reagents, in addition to the decontamination of laboratory waste.

Figure **6.2.1**: The autoclave. (Sterilisator by KOchstudiO, CC BY-SA 3.0)

Not everything used in a cell culture lab can be autoclaved. For example, culture media, serum and antibiotics cannot be autoclaved because the high heat will destroy their component parts. For these heat-sensitive solutions, sterilization is typically done through **filtration** using a **vacuum-driven filtration system** (Figure **6.2.2**). When filtering a liquid, there are two important considerations: pore size and filter composition. A filter with a pore size of 0.2 µm will effectively remove the most common microorganisms, including fungus, yeast and bacteria. Viruses can be removed using filters with smaller pore sizes (20 to 50 nm). However, the smaller the pore size, the lower the flow rate through the filter. The composition of the filter is also important because it may bind certain constituents of the liquid being filtered (e.g. proteins) or may not be very chemically resistant. Typical cell culture filters are made of polyethersulfone (PES) owing to their low protein-binding capacity.

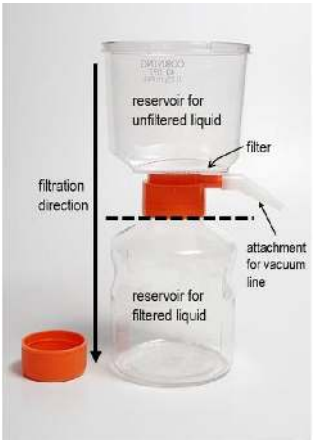


Figure **6.2.2**: A vacuum-driven filtration unit. The top of the filtration unit is a reservoir for the unfiltered liquid. A filter is found at the bottom of the reservoir. The bottom of the unit is a sterile reservoir for the filtered liquid. An attachment for a vacuum line is found attached to the upper reservoir chamber. The vacuum pulls the liquid from the upper reservoir, down through the filter, and into the bottom reservoir (filtration direction shown with an arrow). The upper reservoir and filter can be unscrewed and discarded and a sterile lid attached for storage. Filtration units of various sizes are available depending on the amount of liquid needing to be filtered. Bottle-top filters (not shown in figure), comprised of the just the upper reservoir, filter, and vacuum attachment (i.e., the area of figure above dotted line), can be used as an alternative. These units simply screw on top of sterile glass or plastic bottles. (Filtration unit by Patricia Zuk, CC BY 4.0; adapted from Bottle top disposable filter by Lilly_M, CC BY-SA 3.0)

Concept in Action

Video: Aseptic Technique

Aseptic Technique Fundamentals

Aseptic technique is a set of procedures that is designed *to create a barrier between micro-organisms in the environment and the sterile cell culture*. Good aseptic technique reduces the chance of contamination and maintains the sterility of the culture environment and reagents.

Aseptic technique requires that the user do the following:

1. Wash their hands prior to beginning culture work
2. Wear the appropriate personal protective equipment (PPE) when working in the lab - e.g. gloves, lab coat
3. Use sterile culture reagents (e.g. culture medium, saline, serum, antibiotics) or properly sterilize them prior to use
4. Decontaminate the biosafety cabinet before and after use
5. Maintain all cell culture equipment properly (e.g. incubators, biosafety cabinet) and confirm their sterility on a regular basis

Good Personal Hygiene & PPE

In cell culture, the chief source of contaminants, such as bacteria and yeast, is hands. Therefore, washing hands before cell culturing is essential. The use of PPE, such as gloves and lab coats, will also reduce the probability of contamination from shed skin, as well as dirt and dust from clothes. The use of PPE will also provide protection from potentially hazardous materials.

The Sterile Work Area

The **biosafety cabinet (BSC)** is a sterile work area for cell culture. However, it must be used properly in order to maintain this sterile environment. To learn more about the BSC, go to Chapter 6.3 Cell Culture Equipment.

The following list should be followed when using a BSC:

- The BSC should be located in an area that is exclusively for cell culture
- The BSC area should be free from drafts from doors, windows, and other equipment, and with no through traffic
- The work surface inside the BSC should be uncluttered and contain only the items required for a particular procedure; place those items needed inside the BSC prior to the procedure and remove them when complete
- The stainless steel work surfaces inside the BSC should be disinfected thoroughly or autoclaved on a regular basis
- Raise the glass sash and turn on the BSC blower at least 30 minutes before use to allow for adequate recirculation of air through the BSC
- Prior to use, wipe the BSC's work surface with 70% ethanol; continue to wipe the surface during use and especially after any spillage
- Between use, the ultraviolet light may be used to sterilize the air and exposed work surfaces in the BSC; do not use the UV light when personnel are near the BSC
- Do not use a Bunsen burner inside a BSC

Aseptic Handling Principles

- Using 70% ethanol, wipe off the outside of materials that have been removed from the water bath, refrigerator or other non-sterile areas before placing them in the BSC
- Do not spray flasks and dishes that have been removed from an incubator with 70% ethanol before transferring them to the BSC unless contamination of the outer surface is suspected
- Use only sterile glassware (i.e., autoclaved) or disposable plastic-ware in sealed packages
- Avoid pouring media and reagents directly from bottles or flasks; use sterile glass or disposable plastic serological pipettes and a pipette-aid
- To ensure sterility, open sterile culture plastic-ware packages and culture reagents in the BSC immediately before use and close immediately after
- Seal open plastic-ware packages in their plastic packaging with tape or place them in re-sealable bags to prevent contamination

- To prevent contamination, open culture dishes immediately before use and close immediately after
- Upon removing a culture cap or culture dish lid, place it on the BSC work surface, with the opening facing up
- Perform all experiments as rapidly and aseptically as possible to minimize contamination
- If the sterility of a pipette or culture dish is suspected, discard and obtain an aseptic replacement
- If the sterility of a reagent such as medium is suspected, sterilize it by filtration or autoclaving
- If the sterility of a culture is suspected, properly dispose of it

Key Concepts

Aseptic technique refers to the practices and procedures used in cell culture that are designed to maintain a sterile environment and prevent contamination of cultures by microorganisms

Some important concepts to remember are:

- cell culture contaminants can be chemical and biological (i.e. micro-organisms)
- common micro-organisms that contaminate cell cultures are bacteria, yeast, molds, and mycoplasma
- the most common source of biological contamination is the user
- biological contaminants compete with cultured cells for nutrients
- contamination can be minimized through good aseptic technique
- sterility can be achieved through autoclaving and filtration, the use of good personal hygiene and proper PPE

Glossary

Aseptic technique - a series of procedures designed to ensure the sterility of the culture environment and reagents

Autoclave - laboratory equipment that uses high pressure stem to sterilize materials

Biological Safety Cabinet (BSC) - laboratory equipment used to aseptically work with biological materials; designed to protect users from exposure to potential biohazards through its filtering and circulation of air inside of the cabinet

Contamination - the presence of unwanted microorganisms (bacteria, yeast, mold) in a cell culture or cross-contamination from other cell lines

Decontamination - the process of removing or neutralizing contaminants to make an object or surface safe for handling

Disinfection - the process of reducing or eliminating microbes on surfaces

Filter - a device or material used to separate, remove, or block unwanted particles, substances, or impurities from a fluid or other materials

Filtration - a physical separation process that separates solid matter from a fluid using a filter with a complex structure through which only the fluid can pass

Personal Protective Equiment (PPE) - protective clothing, gloves, masks, and eyewear that is used to prevent contamination and exposure to biological or chemical hazardous materials

Sterilization - a process that kills or removes all microorganisms and other biological agents from a surface, fluid, or object; common methods include autoclaving and filtration

6.2: Principles of Aseptic Technique is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

6.3: Cell Culture Equipment

Biotech Focus

In the 1800s, as the field of cell culture was being developed, researchers began searching for the ideal *in vitro* environment in which to maintain their cell culture stocks. The earliest cell **incubator** was a simple bell jar into which a lit candle was placed next to the cell culture. The jar was then placed into a heated oven. Laboratory incubators were first properly introduced during the second half of the twentieth century, when doctors realized that they could be used to identify pathogens from the bodily fluids of patients. During the late 1960s, the first dedicated, commercial CO2 incubator was developed by New Brunswick Scientific (NBS). These incubators maintained a temperature of 37ºC and CO_2 concentrations capable of supporting cell growth. The modern incubator is not that different from these first commercial attempts. However, they have had additional improvements added, including better control of CO_2, temperature, and humidity levels. Today, the iculture incubator has become an indispensable piece of equipment for the fields of cell biology, microbiology and molecular biology.

Introduction

The major requirement of any cell culture laboratory is *the need to maintain an aseptic work area*. If possible, a separate culture room is preferred. This room should be small, with no windows that can open, and ideally one door so that air drafts are minimized. The equipment used in culturing cells should be placed inside of this room and their use should be dedicated to cell culture only. Much of the equipment of cell culture is unique and is designed to provide the aseptic conditions critical to successful cell culture.

Learning Objectives

Cell culture equipment is used to ensure the integrity of the aseptic work area. At the end of this page, you will be able to:

- List the basic equipment of the cell culture lab
- Compare the three biosafety cabinet classes
- Explain how the Class II biosafety cabinet works
- Compare the four types of Class II biosafety cabinets
- Explain how the cell culture incubator works
- Explain the major types of cell counting systems
- Explain how the inverted microscope works

The Basic Equipment of the Culture Lab

The major focus of cell culture equipment is to provide a sterile environment so that contamination of cell cultures does not occur. The typical cell culture lab will contain the following equipment:

1. **Cell culture hood (i.e., the biosafety cabinet)**
2. **Incubator**
3. **Water bath**
4. **Centrifuge**
5. **Refrigerator (4°C) and freezer (–20°C)**
6. **Cell counting system**

7. **Inverted microscope**
8. **Liquid nitrogen (N2) freezer or cryopreservation container**
9. **Sterilizing equipment (i.e., autoclave)**
10. **Aspiration pump (peristaltic or vacuum)**

The Biosafety Cabinet

The cell culture facility requires a place to aseptically manipulate cells. This is achieved using a **biosafety cabinet** (BSC; i.e. a cell culture hood). The BSC is an *enclosed, ventilated workspace for safely working with biological materials that require a defined biosafety level*. There are three main types of BSCs, with each providing different levels of protection to the user, the environment, and the biological product. Table **6.3.1** compares the three types of BSCs.

Table **6.3.1**: **Biosafety Cabinet (BSC) Classes**

Class of Biosafety Cabinet	Personnel Protection	Product Protection	Environmental Protection	Use
Class I	Yes Inward air flow through sash opening	No Unfiltered room air is drawn across the work surface	Yes Exhaust air is HEPA-filtered	Not used for bioagents May be used to enclose equipment or procedures with aerosol potential
Class II (Type A1, A2, A3, A4)	Yes Inward air flow through sash opening	Yes HEPA-filtered air is drawn down onto the work surface and room air is blocked from the work surface	Yes Exhaust air is HEPA-filtered	Most common class of BSC Used to handle specimen material, biological toxins, cell and tissue culture, and biohazard materials
Class III	Yes Complete containment of interior work area	Yes HEPA-filtered air is supplied to the work surface; room air is completely blocked from the work surface	Yes Exhaust air is double HEPA-filtered	Provides the highest level of containment for handling of extremely dangerous microorganisms

It is important not to confuse a BSC with a laminar flow hood. A **laminar flow hood** draws air in from the environment, passes it through a HEPA filter and then moves the cleaned air across the work surface from back to front, protecting the materials in the hood from contamination. However, this curtain of air is blown out the hood at the worker placed in front of the hood. Therefore, the laminar flow hood provides no protection to the worker using the hood. In contrast, the air flow inside of a BSC is designed to protect the materials inside the cabinet, in addition to the worker seated in front of it.

Class II Biosafety Cabinets

The most commonly used type of BSC in the cell culture laboratory is the **Class II cabinet** (Figure **6.3.1**). The interior of this class of BSC, including the work surface, is made of stainless steel and can be disassembled for sterilization through autoclaving. The front of the work surface is a large grill that collects air from the environment. A grill located at the back of the work surface collects air from the interior of the cabinet. At the front of the class II BSC is a glass sash that can be raised and lowered. When working in the cabinet, the sash is partially raised to allow for manipulation of the materials inside the hood but limit the entrance of room air with contaminants. A HEPA filter, exhaust manifold and a blower motor are inside the top of the cabinet. Inside the cabinet are visible and ultraviolet (UV) light bulbs. The UV light can be turned on for short periods of time to help sterilize the work surface when it is not in use. A control panel at the front of the cabinet turns on the lights and the blower motor for airflow. It also provides key information about the status of the cabinet.

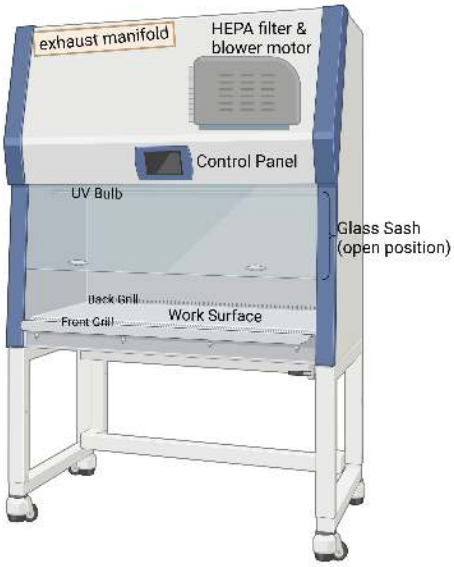


Figure **6.3.1**: The Class II Biosafety Cabinet (BSC). The Class II BSC provides the user with an aseptic work surface for the sterile manipulation of cultured cells, tissues, and organs. The front of this BSC has a glass sash which can be raised to an open position in order to access the work surface. The work surface is flanked by a front and back grill for the capture of contaminated air. The contaminated air enters the grills and is directed through a HEPA filter located at the top of the BSC. The air is filtered and then blown back into the BSC to protect both the materials on the work surface and the user. A portion of the filtered air is exhausted out of the BSC either into the room through an exhaust manifold. A UV bulb can be used to sterilize the work surface when the BSC is not in use. The control panel of the BSC provides the user with the cabinet's functions and status. (The BSC by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

The Class II BSC creates a unique pattern of airflow within the cabinet that protects both the material inside of the cabinet and the worker using it (Figure **6.3.2**). Contaminated room air is drawn in through the open sash into the front grill. The air flows beneath the work surface and up the back of the cabinet. It enters a HEPA filter at the top of the cabinet where the air is filtered. A portion of the filtered air is exhausted out to the environment and the remainder is blown down over the work surface from the front of the cabinet to the back. This air then flows through the back grill to mix with the room air flowing up to the HEPA filter. The air pattern over the work surface creates a “curtain” to protect the worker and efficiently moves potentially contaminated air out of the work area.

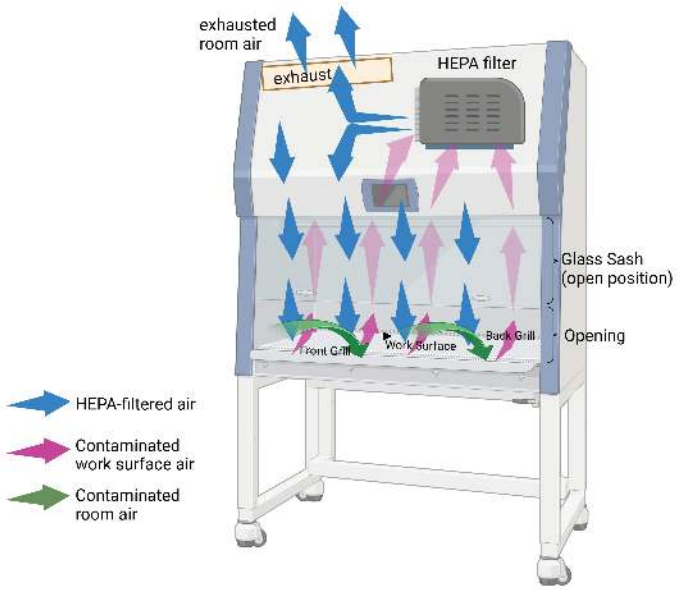


Figure **6.3.2**: Airflow in the Class II Biosafety Cabinet (BSC). "Dirty" air from the room (curved, green arrows; contaminated room air) enters the BSC through the front grill, passing under the work surface and up the back of the cabinet to a HEPA filter located at the top of the cabinet. The filtered air is blown down over the work surface (blue arrows, HEPA-filtered air)), protecting the materials in the cabinet and the user. Contaminated air from the work surface enters the back grill (purple arrows; contaminated work surface air) to mix with the room air traveling to the HEPA filter. A portion of the filtered air is returned to the environment-(exhausted room air). (Class II BSC by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Concept in Action

Animation: Airflow Through a Class II Biosafety Cabinet

There four types of Class II biosafety cabinets are classified as A1, A2, B1 and B2, with each type having unique specifications (Table **6.3.2**). Most cell culture facilities use a either a type A1 or A2 biosafety cabinet because they are cheaper to operate since they do not require a hard connection to the exhaust system of the room's building.

Table **6.3.2**: Class II Biosafety Cabinet Types

Type II Classification	HEPA-Filtered Air Recirculation	Ducting	Use
A1	Most is recirculated over the work surface (70% recirculated; 30% exhausted to room); airflow rate of 75 linear feet per minute	Cabinet is not connected to building ventilation system; filtered air exhausted to environment	Use with biological materials only; do not use with chemicals
A2	Most or all is recirculated over the work surface (70% to 100% recirculated; 0 to 30% exhausted to room); airflow rate of 100 linear feet per minute	Cabinet is not connected to building ventilation system; filtered air exhausted to environment	Use with biological materials; can be used with materials containing *minute amounts* of volatile chemicals if air is exhausted to environment
B1	Most is exhausted to environment (30% recirculated; 70% exhausted to room through a ducting system); airflow rate of 100 linear feet per minute	Cabinet is directly connected to building ventilation system	Use with biological materials and *minute amounts* of volatile or toxic chemicals
B2	All is exhausted to environment (0% recirculated; 100% exhausted to room through a ducting system); airflow rate of 100 linear feet per minute	Cabinet is directly connected to building ventilation system	Use with biological materials and *small amounts* of volatile or toxic chemicals

The Culture Incubator

Cultured cells are maintained and grown within an **incubator** that provides the cells with a sterile, humidified, and warm environment (Figure 6.3.3). The incubator is like a biosafety cabinet in that it relies upon a flow of HEPA-filtered air to prevent contamination. Air inside the incubator is filtered through a HEPA filter, warmed by the incubator and then moved through the interior of the incubator before being pulled back through the HEPA filter for filtration and recirculation. The majority of the air that moves through the incubator is taken from the environment and is a mixture of oxygen (~20%), carbon dioxide/CO2 (~0.04%), and nitrogen (~78%). The CO2 levels found in this air are inadequate for cell culture. Therefore, additional CO2 is injected into the incubator from a cylinder of compressed CO2 gas. CO2 levels for most cell cultures will range between 5% and 10%, depending on the needs of the cultured cell. Sensors inside of the incubator constantly measure the CO2 concentration and adjust the levels by injecting additional compressed CO2. Humidity levels inside of the incubator can be monitored and adjusted by the incubator. However, most incubators provide humidity to the air using a pan of sterilized water placed at the bottom of the incubator.

The air of the incubator is typically warmed to 37°C as most mammalian cells grow well at this temperature. To keep the temperature inside of an incubator stable, the incubator is “jacketed”, meaning that there is an inner chamber (where the cells are incubated) surrounded by an outer chamber (the "jacket"). The jacket prevents significant drops in temperature when the door is opened and closed. There are two kinds of jackets: water and air. The inner chamber of the air-jacketed incubator (also known as a direct heat incubator) contains air, warmed to 37°C. This type of incubator is light and cost-effective in comparison to the water-jacketed incubator. In a water-jacketed incubator, the jacket is filled with sterile water and heated to 37°C. This jacket keeps the temperature of the incubator more stable versus an air-jacketed incubator, as the water jacket will return the inner chamber's temperature to its set point much faster (i.e. within 10 minutes for water-jacketed vs. 1 hour for air-jacketed). However, the water jacket is an excellent source of contamination by micro-organisms like fungus. In addition, a water-jacketed incubator is extremely heavy and must be drained in order for the incubator to be moved.

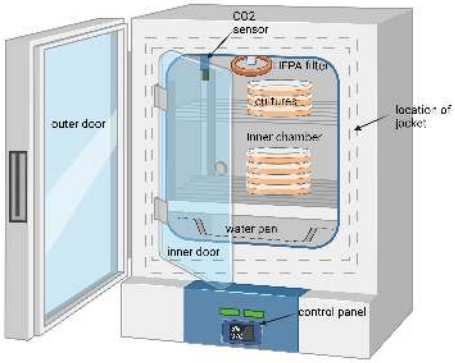


Figure **6.3.3**: The Cell Culture Incubator. Cell cultures are maintained within the inner chamber of the incubator that is made of stainless steel. A HEPA filter at the top of the inner chamber removes contaminates from the air circulating through the incubator. A CO_2 sensor located in the inner chamber monitors the CO_2 and adjusts levels through the injection of compressed CO_2 gas (not shown). Humidity is provided by a water pan placed at the bottom of the inner chamber. Temperature and CO_2 levels are set through a control panel. Temperature is kept stable either by a water or air "jacket" inside the body of the incubator (location is shown with dotted lines). An inner door made of glass allows the user to open the outer door and view the samples without exposing the inner chamber to contaminated room air. (Cell Culture Incubator by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Cell counting systems

Cell counting systems are used to determine the number of cells within a culture. The most common and inexpensive type of counting system is the **hemacytometer**. Originally designed and used for counting red blood cells, the hemocytometer is used for the manual counting of numerous types of cells. Refer to Lab Technique: Cell Culture for a protocol on the use of a hemocytometer. The hemocytometer consists of a thick glass microscope slide with a rectangular indentation that creates a counting chamber of 10^{-4} mL (Figure **6.3.4**). This chamber is engraved with a laser-etched grid of perpendicular lines. Cells are added to the counting chamber and visualized under the microscope. Those falling over certain parts of the counting grid are counted. With this cell count and the volume of the counting grid (i.e. 10^{-4} mL), the number of cells per mL can be determined. This concentration can be used to determine the total number of cells within the culture.

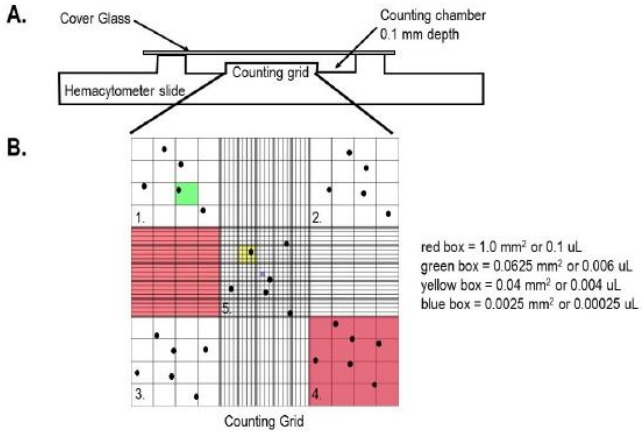


Figure **6.3.4**: The Hemacytometer. The hemacytometer consists of a heavy glass slide with a depression in the center that is covered with a glass coverslip (i.e., Cover Glass). A series of grids are etched into the center of the hemacytometer (Counting Grid). The raised edges of the hemocytometer hold the coverslip off the depression and create a counting chamber with a depth of 0.1 mm (A). Cells are added to this chamber and their numbers found over the counting grid are counted using specific areas of this grid. The Counting Grid (B) consists of nine, 1 x 1 mm squares, each with an area of 1 mm^2 and a volume of 0.1 µL or 10^{-4} mL (red boxes). Each of the outer 'corner' squares (numbers 1 through 4) are subdivided into 16 smaller squares of 0.25 x 0.25 mm, each with a total area of 0.0625 mm^2 and a volume of 0.006 µL (green box). The 5 'inner' squares of the counting grid form a "cross". The central square in the middle of this cross (number 5) is further subdivided so that each of its 0.25 x 0.25 mm squares (yellow box) is subdivided into twenty, 0.05 x 0.05 mm squares. Each of these squares (blue box) has an area of 0.0025 mm^2 and a volume of 0.00025 µL. The cells (black circles) are counted in squares 1 through 5 and the average number of cells determined. This number is multiplied by 10,000 (and any dilution factor used) to obtain the number of cells in 1 mL. (The Hemacytometer by Patricia Zuk, CC BY 4.0; adapted from Hemacytometer grid by Zephyris, CC BY-SA 3.0)

Automated cell counters use advanced technology to accurately and efficiently count and analyze cells isolated from a culture. Most counting systems are image-based, using cameras and microscopes to capture high-resolution images of the sample. Software processes the images to identify and count cells based on their size, shape, or staining. Other systems use measures changes in electrical resistance when the cells pass through a small aperture or channel cells through a micro-fluidic device where they are imaged, electrically measured, or optically analyzed. Automated counting systems eliminate the manual labor and potential for human error associated with traditional methods like hemacytometry. They are significantly more accurate and faster and easier to use. However, they are markedly more expensive that hemacytometers.

Inverted microscope

An **inverted microscope** is a type of light microscope where the optical arrangement is reversed compared to a conventional (upright) microscope. The light source, with its condenser, is located above the stage, while the objectives are positioned below the stage (Figure **6.3.5**). This design is ideal for observing living cells in their culture dishes, culture flasks, or multi-well plates.

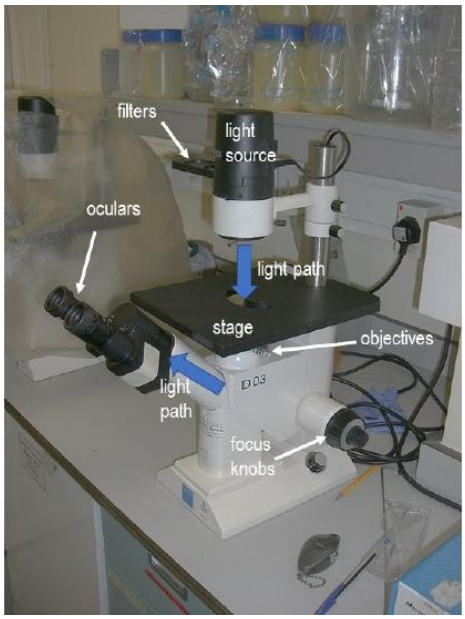


Figure **6.3.5**:The Inverted Microscope. The inverted microscope has its light source, condenser, and filters located above the stage and its objectives below the stage. This arrangement allows for the visualization of cells in a variety of culture dishes when they are placed on the stage. Light passes from the light source, through the sample, into the objectives and up into the oculars or eyepieces (shown with blue arrows). (The Inverted Microscope by Patricia Zuk, CC BY 4.0; adapted from An inverted microscope by Zephryis, CC BY-SA 3.0)

Cryopreservation Systems

Cell culture facilities will often remove cells from culture in order to store them over the long-term. Storing cultures allows the facility to remove cells from culture when they are not needed and re-introduce them when they are. This saves time, materials, and money. Long-term storage of cell cultures is done at extremely low temperatures and is referred to a **cryopreservation**. The simplest system for cryopreservation is a **liquid nitrogen tank** or **Dewar.** The liquid nitrogen inside of the tank (approximately -320°F or -196°C) provides the optimal temperature for long-term storage of cells. Modern tanks can hold hundreds of cryopreservation samples. The samples are placed into boxes and the boxes are stacked onto racks that are lowered into the tank. The bottom of the tank holds several inches of liquid nitrogen that produces a vapor layer that is cold enough to keep the samples frozen. The liquid nitrogen inside of the tank is replenished on a regular basis.

Additional Culture Equipment

1. Water bath: necessary to warm reagents used in cell culture
2. Centrifuge: for the collection of culture cells
3. Refrigerator (4°C) and freezer (–20°C): for storage of culture reagents
4. Sterilizing equipment (i.e., autoclave)
5. Aspiration pump (peristaltic or vacuum)

Key Concepts

The equipment of a cell culture facility includes specialized equipment like the biosafety cabinet and incubator, in addition to more common types of lab equipment like water baths, microscopes, and centrifuges.

Some important concepts to remember are:

- cell culture equipment is used inside of a dedicated cell culture room where the conditions help to maintain the sterile nature of the culture
- the biosafety cabinet (BSC) creates a flow of filtered air that prevents culture contamination and protects the user from exposure biohazards
- a BSC is not a laminar flow hood

- a cell culture incubator creates and maintains the optimal temperature, humidity, and carbon dioxide levels necessary for *in vitro* growth of cells
- an inverted microscope allows for the visualization of live culture cells

Glossary

Air filter - a device that removes airborne particles and contaminants from the air

Biosafety cabinet (BSC) - laboratory equipment used to aseptically work with biological materials; designed to protect users from exposure to potential biohazards through its filtering and circulation of air inside of the cabinet

Contamination - the presence of unwanted microorganisms (bacteria, yeast, mold) in a cell culture or cross-contamination from other eukaryotic cell lines

Cell counter - laboratory equipment used to determine the number of cells in a sample; can be manual (i.e. hemacytometer) or automated

Cryopreservation - a process that preserves biological samples, like cultured cells, by freezing and storing them at very low temperatures

Dewar - a specialized container used for holding a cryogen, such as liquid nitrogen; cell culture Dewars are modified to store hundreds of cryo-vials containing frozen cell samples

Hemacytometer - a counting-chamber device designed and used to visualize and count cells

HEPA (high efficiency particularte air) filter - the efficiency standard of air filters that are used to remove particulate matter from the air

Incubator - laboratory equipment used to grow and maintain microbiological cultures or cell cultures under optimal temperature, humidity, and carbon dioxide levels

6.3: Cell Culture Equipment is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

7: Microbial Biotechnology

7.1: Microbe classification

7.2: Microbial Identification

7.3: Microbial Cultivation and Manipulation

7.4: Industrial Applications of Microbial Biotechnology

7.5: Medical Applications of Microbial Biotechnology

7: Microbial Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

7.1: Microbe classification

Biotech Focus

Extremophiles are microorganisms that thrive in extreme conditions where most other life forms would perish. Traditionally, these hardy microbes are found in environments like deep-sea hydrothermal vents, Antarctic ice, or highly acidic hot springs. However, recent studies have revealed a surprising habitat for certain extremophiles: common household appliances. Household appliances such as dishwashers, washing machines, and even coffee makers create unique microenvironments that can mimic some extreme conditions. Dishwashers, for instance, generate high temperatures, rapid temperature shifts, and fluctuating pH levels from detergents—all challenging environments that some extremophiles can endure. These extremophiles, adapted to the challenging conditions of household appliances, hold significant potential for biotechnological innovation. They could be used in many domains, for example, to produce enzymes that could withstand extreme conditions, or to develop methods for sustainable waste management, demonstrating how even the most commonplace settings can contribute to groundbreaking science.

To know more about the study, read The Microbiome of Things: Appliances, Machines, and Devices Hosting Artificial Niche-Adapted Microbial Communities

Introduction

Microbes are microscopic organisms (i.e. microbes) that are too small to be seen by the naked eye. They include bacteria, archaea, fungi, but also viruses. Such a broad definition means that many different types of microbes exist, with different structures, sizes and metabolism. Despite their small size, microbes play critical roles in natural ecosystems. They are also invaluable tools in biotechnology.

Learning Objectives

Microbes are an incredibly important part of biotechnology. At the end of this section, you will be able to:

- Distinguish between prokaryotic and eukaryotic cells, including listing their common structures and how they differ
- List the major microbes used in biotechnology
- Describe the basic morphology of bacterial cells and some of their major structures, including the cell wall
- Explain the "extremophile" nature of archaea
- Describe some basic viral structures

Prokaryotes vs Eukaryotes

All living things are made of one or more cells. Two general types of cells can be found: **prokaryotic (lacking a nucleus)** and **eukaryotic (possessing a nucleus**). Prokaryotic organisms are unicellular, meaning they are made of one cell only (Figure **7.1.1**). In contrast, eukaryotic organisms can be unicellular, such as yeast, or multicellular, such as humans.

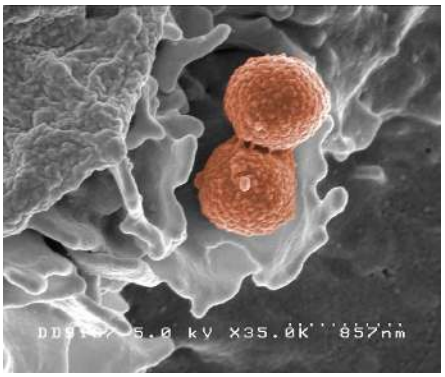


Figure **7.1.1**: Methicillin-resistant *Staphylococcus aureus*. A scanning electron micrograph shows two methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial cells (colorized orange) interacting with a human neutrophi (gray). Note the difference in size between the smaller two bacterial cells and the much larger neutrophil (eukaryotic). (Methicillin-Resistance Staphylococcus aureus, by NIAID, CC BY 2.0)

Both prokaryotes and eukaryotes share the same basic elements:

1. a **cytoplasm:** a gel-like substance mainly composed of water, proteins and salts
2. a **cell membrane:** a phospholipid bilayer that surrounds the cytoplasm
3. one or more **chromosomes:** an organized form of DNA; the number is specific to the species
4. **ribosomes:** a non-membranous organelle used for protein synthesis

However, they also differ in many ways. Table **7.1.1** below lists some of these differences.

Table **7.1.1**: Prokaryotes vs. Eukaryotes

Feature	Prokaryotes	Eukaryotes
Nucleus	No	Yes
DNA organization	DNA in the form of a circular chromosome known as a genophore	DNA in the form of circular or linear chromosomes; multiple chromosomes found within the nucleus
Membrane-bound organelles	No	Yes (mitochondria, ER, Golgi, lysosomes, peroxisomes, vacuoles, chloroplasts etc....)
Cell division mechanism(s)	Binary fission, budding (asexual reproduction)	Binary fission, budding, mitosis, and meiosis
Cell wall composition	Peptidoglycans (bacteria), pseudopeptidoglycans (archaea)	Cellulose (plant cells), chitin (fungal cells), no cell wall in animals cells
Ribosome composition	70S: 30S and 50S subunits	80S: 40S and 60S subunits
Examples	bacteria, archaea	fungi, plants, animals

Microbial Biotechnology: Cell Types

Microbial biotechnology is the use of microbes for industrial, environmental, and pharmaceutical applications. Over the years, advances in genetic engineering, molecular biology, and bioinformatics have expanded the scope of microbial biotechnology, allowing us to harness these organisms for innovations in a wide range of areas such as agriculture, medicine, bioenergy, and environmental management. What microbes are used in biotechnology is dependent upon the application. For example, bacteria are widely used in biotechnology due to their rapid growth and the ability to manipulate their genetic material easily.

The microbes used in biotechnology are:

1. **Bacteria:** single-celled prokaryotic organisms that lack a nucleus and membrane-bound organelles
2. **Yeast:** single-celled eukaryotic organisms that are classified as fungi
3. **Archaea:** single-celled prokaryotic organisms that are genetically distinct from bacteria

4. **Viruses:** small, infectious organisms that can only replicate inside of a host cell

Bacteria

Bacteria are the oldest and most diverse form of life and comprise the domain Bacteria. They can be found in many shapes and sizes depending on the bacterial species (Figure **7.1.2**).

The most common bacterial shapes are:

1. **coccus:** spherical shape (plural = cocci), e.g., *Streptococcus mutans*
2. **bacillus:** rod shape (plural = bacilli), e.g., *Esherichia coli (E.coli)*

While these two are the most common shapes seen, there are additional bacterial shapes including spiral shaped (or **spirillum**), stalked forms, filamentous shapes, and a comma-shaped cell called **vibrio**.

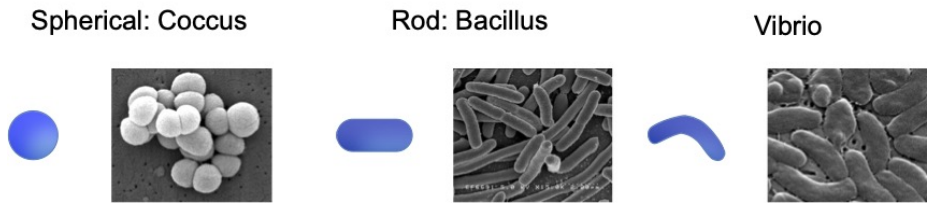


Figure **7.1.2**: Common bacterial shapes. Left: the spherical bacterial cell is called coccus (plural: cocci). Center: the rod shaped cell is called bacillus (plural: bacilli). Right: the bent rod shaped cell is called vibrio. (Electron microscopy image credits: Left: Betsy Crane; Center: NIAID; Right: Janice Haney Carr; all images are public domain)

Bacterial species can also be characterized by the arrangement of their cells (Figure **7.1.3**). For example, they can found as single cells, in pairs (diplo), in chains (strepto) or in clusters (staphylo). The names of bacteria often reflects their shapes and arrangement, such as the genus *Staphylococcus* (spherical cells arranged in clusters).

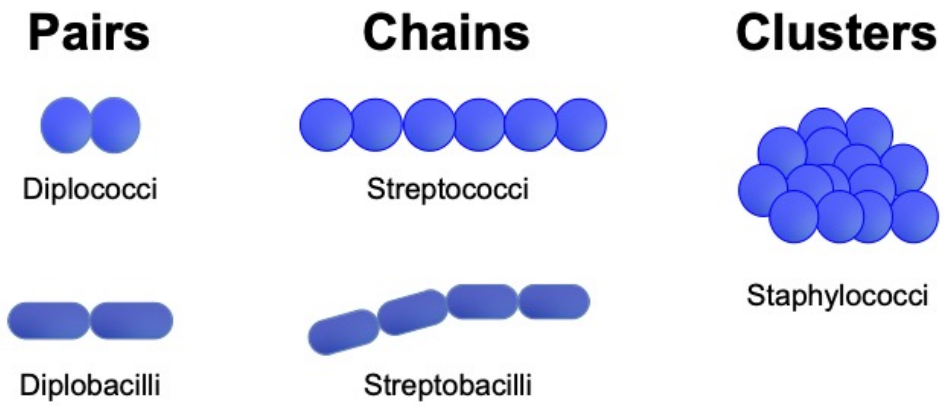


Figure **7.1.3**: Bacterial cell arrangements. Left: cells can be arranged in pairs. Cocci found in pairs are called diplococci (top), bacilli in pairs are called diplobacilli (bottom). Center: cells can be arranged in chains. Cocci grouped as a chain are called streptococci, bacilli in chains are called streptobacilli. Right: only cocci can be arranged in clusters and are called staphylococci. (Bacterial Cell Arrangements by Kareen Martin, CC BY 4.0)

The genome of most bacteria is a circular chromosome, called a **genophore**. Because bacteria lack a nucleus, the bacterial chromosome is found in a region of the cytoplasm called the **nucleoid**. In addition to the bacterial chromosome, some bacteria have pieces of extra-chromosomal DNA called plasmids. A **plasmid** is a small, circular double-stranded piece of DNA that replicates independently from the bacterial chromosome and can be passed on from one bacterium to another bacterium. While the genes found in a plasmid are not necessary for the normal metabolism of the bacteria, they confer advantageous traits, such as antibiotic resistance. Plasmids are a valuable tool in molecular biology and genetics, they can be easily manipulated and used as vector to transfer and express genes into a host organism. They can be used to study the effect of the product of a gene or to create genetically modified organism. For more about the bacterial chromosome and plasmids, go to Chapter 3.1 DNA Structure. The bacterial genome can easily be altered either by exposing them to physical or chemical mutagens. They also have the ability to acquire DNA from their environment. Because of these properties, bacteria constitute an invaluable tool in biotechnology.

Although both bacteria and eukaryotic cells possess ribosomes that are responsible for the synthesis of proteins, the bacterial ribosome is smaller and simpler than their eukaryotic counterpart. Prokaryotic ribosomes are called **70S ribosomes**, whereas eukaryotic ribosomes are called 80S ribosomes. The "S" is short for **Svedberg unit** and it is a measure of how fast a particle settles when being centrifuged. The sedimentation rate of a particle depends on a combination of characteristics, such as size, shape, and density. The 70S ribosome indicates that this particle sediments slower that the eukaryotic 80S ribosome, which is due to its smaller size. Prokaryotic ribosomes not only differ from eukaryotic ones in size, but also in their overall structure and composition. These differences make prokaryotic ribosomes sensitive to a wide range of antibiotics and allow for the treatment of bacterial infections without damaging eukaryotic cells.

Most bacteria are surrounded by a **cell wall** composed of a complex polymer called **peptidoglycan** (Figure **7.1.4**). Peptidoglycan is composed of repeating disaccharide units. The two sugars of this unit are called **N-acetyl glucosamine (NAG)** and **N-acetyl muramic acid (NAM)**. The NAM monomer within the disaccharide is modified by the addition of 5 to 6 amino acids (i.e., "peptido"). The NAG/NAM disaccharides link together to form long chains of sugars (i.e. "glycan") that crosslink to one another through the peptides. The peptidoglycan-rich cell wall forms a rigid layer that protects and maintains the shape of the bacterium. Numerous antibiotics are effective against bacteria through their ability to affect the assembly or integrity of the peptidoglycan cell wall.

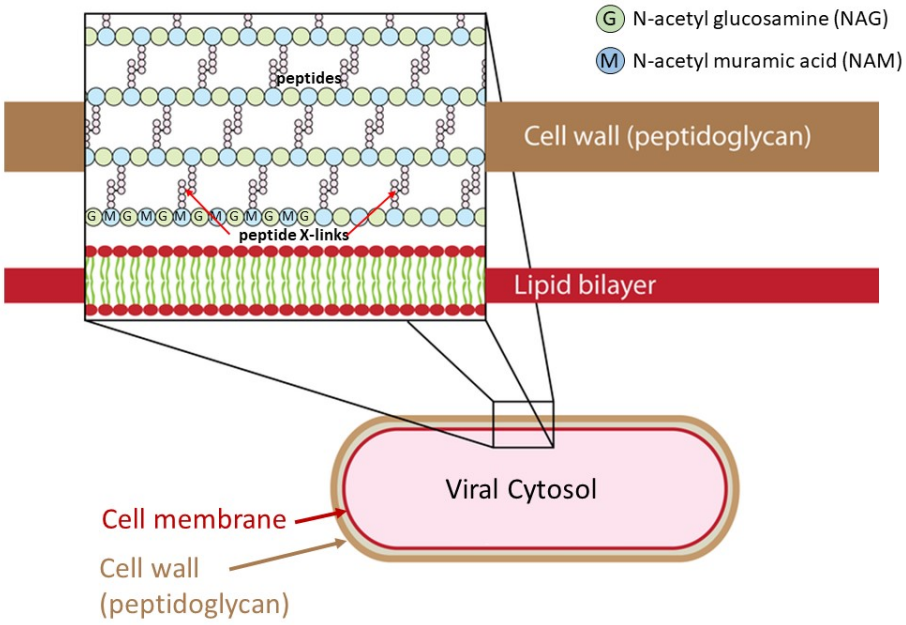


Figure **7.1.4**: Peptidoglycan structure. The cell wall of a bacterium is made of peptidoglycan, an alternating series of sugars called N-acetyl glucosamine or NAG (G, green circles) and N-acetyl muramic acid or NAM (M, blue circles). The NAM sugar is modified through the attachment of several peptides (small circles). Cross-linking between the peptides (peptide X-links, shown with red arrows) produces a rigid mesh-like structure that protects the bacteria. (Peptidoglycan Structure by Patricia Zuk, CC BY 4.0; adapted from Peptidoglycan membrane by Bradleyhintze, CC0 1.0)

The bacterial cell wall can be used to classify bacteria as either **gram-positive** or **gram-negative**, a classification technique that is named after the Gram staining method. Gram-positive cell walls consist of a thick peptidoglycan layer that is readily stained through Gram staining. Gram-positive bacteria will be dark purple in color (Figure **7.1.5**). In contrast, the gram-negative cell wall consists of a thin peptidoglycan layer sandwiched between the plasma membrane of the bacterial cell and an **outer membrane**. This outer membrane prevents the staining of the cell wall and gram-negative bacteria are pink in color (the color of the Safranin counterstain) (Figure **7.1.5**). The outer membrane is a phospholipid bilayer studded with fat and sugar components, called **lipopolysaccharides (LPS) (**Figure **7.1.5**). The lipopolysaccharides can be released from the cell wall and contribute to the pathogenicity of the bacteria.

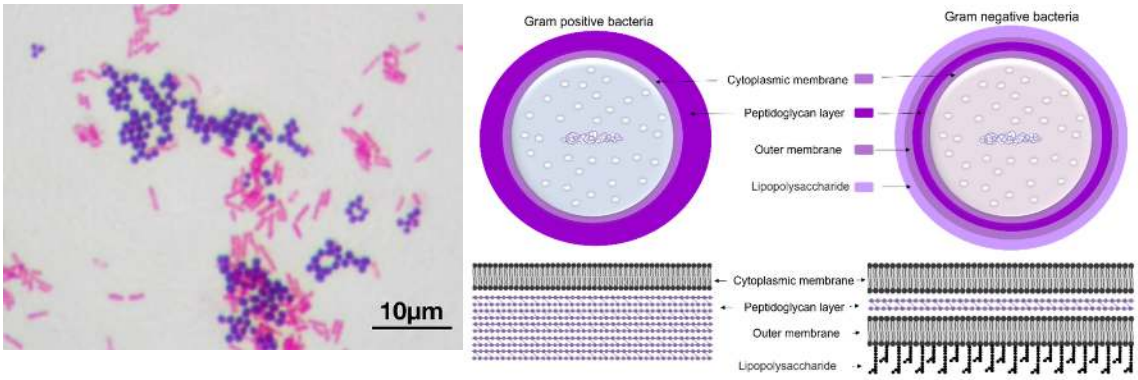


Figure **7.1.5**: Gram-positive and gram-negative bacteria. Left image: A micrograph of gram-positive cocci (purple circles) and gram-negative bacilli (pink rods). (Gram stain by Y tambe, CC BY-SA 3.0)
Right image: The cell wall composition of gram-positive bacteria (left) is a thick peptidoglycan layer over the plasma membrane (i.e. cytoplasmic membrane). Gram-negative bacteria (right) have a thinner peptidoglycan layer found in between the cytoplasmic membrane and an outer membrane. The outer membrane is a phospholipid bilayer studded with lipopolysaccharides. (Cell wall composition by Malihe Mehdizadeh Allaf and Hassan Peerhossaini, CC BY-SA 4.0)

Bacteria divide rapidly, mainly through binary fission. In binary fission, the bacteria will duplicate its genetic material and partition it into two daughter cells. Many bacteria divide every 20 minutes. Hence, with the population doubling every 20 minutes, millions of identical cells can be produced in a short period of time. Compare this with the 24 hours doubling time of eukaryotic cells, and it is obvious why bacteria are a favorite organisms in biotechnology.

Archaea

Archaea are a group of single-celled microorganisms that make up the domain, Archaea. They are similar in appearance to bacteria but are genetically and biochemically distinct, with unique evolutionary histories. Archaea thrive in a wide range of environments, including some of the most extreme on Earth, such as hot springs, deep-sea hydrothermal vents, and highly acidic or saline habitats. This ability to survive in extreme conditions is due to their specialized cell membranes and enzymes, which allow them to maintain stability and function. Unlike bacteria, archaea have distinct metabolic pathways and can utilize a variety of energy sources, including methane, sulfur, and hydrogen, making them key players in global nutrient cycles.

Despite their microscopic size, archaea are critical to many ecological processes and have potential applications in biotechnology due to their resilience and unique biochemistry. Their enzymes, known as **extremozymes**, are particularly valuable in industrial processes that require extreme conditions, such as high temperatures, high salinity, or extreme pH levels. For instance, thermostable DNA polymerases from archaea are widely used in PCR (polymerase chain reaction) which requires using cycles with very high temperatures. Archaea are also used in bioremediation, where they help break down pollutants in harsh environments, and in biofuel production, where their metabolic pathways can be harnessed to produce methane or hydrogen. The resilience and adaptability of archaea make them promising candidates for innovations in biotechnology, including drug development, waste management, and sustainable energy solutions.

Yeast

Yeast are single-celled eukaryotic organisms belonging to Domain Eukarya, Kingdom Fungi. Yeast thrive in a variety of environments, including those that are moist and warm. They grow rapidly in aqueous environments and have been extensively studied for their metabolic processes. Yeast is widely used in biotechnology due to its versatility and ease of manipulation. Dating back several millennia., yeast has been employed in the production of bread, beer, and wine through **fermentation** - a biological process that use microbes, like yeast, to convert sugars (e.g. glucose) into energy (i.e. ATP). Nowadays, yeast is also used in many different applications. They are used to produce renewable fuels, like bioethanol, through fermentation. Yeast are used to produce enzymes for industrial applications. The ability of yeast to efficiently express foreign genes and its well-characterized genetics make it a powerful tool in the development of pharmaceuticals, like insulin and vaccines.

Virus

Viruses are microscopic infectious agents that can infect all life forms; bacteria, archaea, animals, plants, and fungi. Even though a virus has both genetic material and protein components, *it is not a living organism*. It is unable to self-replicate and relies on the cellular biochemistry of the host cell it has infected. This makes a virus an **obligate parasite,** or an organism that is required to infect a living cell.

The key features of viruses are (Figure **7.1.6**):

1. **Genetic material:** The genetic information of a virus is either DNA or RNA, in either single stranded or double stranded forms. The nucleic acid content of a virus determines how they replication and can be used to classify them. The viruses that carry DNA are called **DNA viruses** (e.g. Herpesvirus) whereas those carrying RNA are called **RNA viruses** (Coronavirus, HIV). The size of viral genome depends on the type of virus. Some viruses contain enough genetic material to make only a few proteins; others code up to 100 proteins. The smaller the genome, the more viral copies can be made by a host cell. In order to replicate, a virus needs to infect a cell and so its genome can be copied. Once in the host cell, it will "hijack" the host's cellular machinery. What aspects of the host cell is used depends on whether the genome is single-stranded or doubled-stranded DNA or RNA. For example, a DNA virus will use the host's DNA polymerase to replicate its genome, followed by host cell transcription and translation. In contrast, RNA viruses have no need of DNA polymerases but will use host cell ribosomes for protein translation.
2. **Capsid:** The capsid is a protein shell that surround the viral genome. The capsid protects the genome from the environment, plays a role in binding the host cell, permits its entry into the host cell, and assists in viral assembly following replication. The protein subunits of the capsid are known as **capsomeres**. The way these capsomeres assemble determines the shape of the virus. For example, many viruses are **icosahedral**, a shape made of 20 identical triangular faces. Others viruses have a helical capsid, such as the Ebola virus.
3. **Envelope**: The envelope is not found in all viruses. When it is present, the envelope is composed of a phospholipid bilayer that is derived from the host cell. The envelope forms around the virus, when it exists the host cell. As a result of this process, some of the proteins of the host cell membrane will be found embedded within the envelope. These proteins aid in recognition by host cells and facilitate their entry. Viruses with an envelope are called "enveloped" viruses; those lacking an envelope are often called "naked" viruses.
4. **Surface proteins:** Surface proteins can be found embedded in either the capsid or the envelope (if present). Those embedded in the envelope are **glycoproteins** - proteins that have been modified through the addition of short sugar monomers. Most of these proteins are spike-like in shape and are called **spike proteins**. Spike proteins help in viral attachment and entry by binding to receptors at the surface of a cell. They also play a role in evading the host's immune system. Surface proteins found embedded in the capsid are known as **capsid proteins**. Capsid proteins serve as attachment proteins that interact directly with receptors on the host cell's surface. Viral surface proteins are critical for the specificity of the virus, allowing certain viruses to bind and enter only a particular type of cell found in a certain species. While most viruses are highly specific, some viruses may cross the barrier species in a process called "spillover". These viruses are called **zoonotic viruses**. Zoonotic viruses originate in other animals (e.g. bats, birds, pigs) but adapt to infect humans, sometimes leading to endemics or pandemics. The SARS outbreak of 2002-2003 is an example of a zoonotic virus that originated in bats and cats and made the jump to humans.

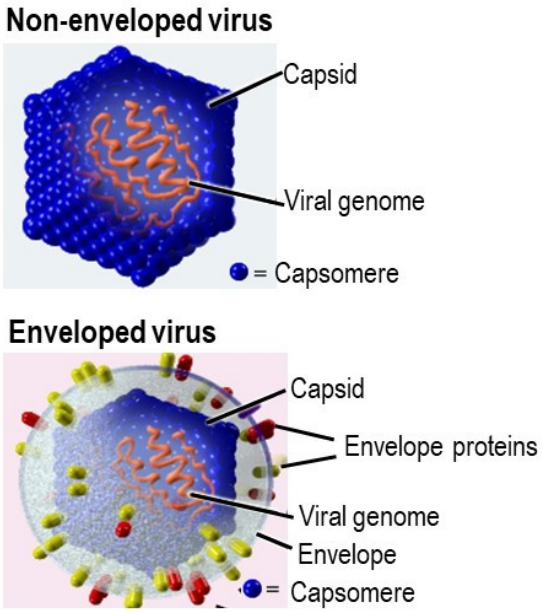


Figure **7.1.6**: Enveloped and non-enveloped viruses. Top image: the non-enveloped virus is surrounded by a capsid made of individual capsomeres (blue spheres) that assemble to give the capsid its shape. The capsid surrounds the viral genome (shown in orange). Surface proteins can be found embedded in the capsid (not shown). Bottom image: the enveloped virus has a viral genome and a capsid like the non-enveloped virus. However, the capsid is surrounded by an phospholipid bilayer called the envelope. Envelope proteins (shown in red and yellow) can be found embedded in the envelope. (Enveloped and Non-enveloped Viruses by Patricia Zuk, CC BY 4.0)

Viruses have made major contributions to biotechnology. Viruses are critical to the development of vaccines that train the immune system to recognize and fight infections. They can be used as cloning vectors to introduce foreign genes into host organisms, like bacteria. They have been used in gene therapy to introduce functional genes to specific cell types in order to treat diseases, such as sickle-cell anemia. Viruses called oncolytic viruses have been engineered to infect and destroy cancer cells, while leaving normal cells unaffected. Modified plant viruses can be used as vectors to introduce foreign genes into plant cells in order to create genetically-engineered plants with beneficial traits, such as increased resistance to pests, diseases, or environmental stresses. The future of viral biotechnology is likely to include personalized medicine with custom-designed viruses or vaccines, synthetic viruses to target specific diseases, and improved gene editing tools using viral systems.

Key Concepts

Microbial biotechnology is the use of microbes for industrial, environmental, and pharmaceutical applications. Some important concepts to remember are:

- all living things are made of cells
- there are two types of cells: prokaryotic and eukaryotic
- prokaryotic and eukaryotic cells have DNA in the form of chromosomes, a cytoplasm, ribosomes, and a plasma membrane
- additional features like the presence of a nucleus distinguish eukaryotic cells from prokaryotic cells
- the microbes used in microbial biotechnology include prokaryotic cells (e.g. bacteria and archaea), eukaryotic cells (e.g. yeast), and viruses
- bacteria and archaea are single-celled prokaryotic cells found in Domain Bacteria and Domain Archaea, respectively
- bacteria can be classified using several approaches, including cell shape, cell arrangement and gram staining
- the cell wall of bacteria is made of peptidoglycan, long chains of NAG and NAM sugars that crosslink for rigidity and strength
- gram-positive bacteria have a thick layer of peptidoglycan in their cell wall; the peptidoglycan is stained by the gram stain

- gram-negative bacteria have a thin peptidoglycan layer found in between the cell membrane and an outer membrane; the outer membrane prevents staining by the gram stain
- archaea are found in extreme environments
- yeast are single-celled eukaryotic cells found in Domain Eukarya
- yeast have several uses in biotechnology, including fermentation and genetic engineering
- viruses are non-living, infectious agents that require host cells for their replication
- viruses are made of a nucleic acid genome (DNA or RNA), a capsid, and surface proteins; some viruses may also have a envelope

Glossary

Archaea – a domain of single-celled microorganisms that are genetically distinct from bacteria and eukaryotes, often found in extreme environments

Asexual reproduction – a mode of reproduction in microbes where offspring arise from a single parent, without genetic recombination

Bacillus – a rod-shaped bacterium

Bacteria – single-celled prokaryotic microbes that can be found in diverse environments and classified on parameters like cell shape and gram stain

Binary fission – a common asexual reproduction method in bacteria where a single "parent" cell splits into two identical "daughter" cells

Budding - an asexual reproduction strategy where a small "bud" detaches itself from the "parent" cell, followed by maturation

Capsid – a structure that surrounds and protect the viral genome

Coccus – a spherical-shaped bacterium

Domain - the largest group used to classify organisms; all life can be classified into three domains (Bacteria, Archaea, Eukarya)

Diplo - a pair of bacterial cells joined together; e.g. "diplococcus" - a pair of spherical bacteria joined together

Eukaryote – an organism whose cells contain a nucleus and membrane-bound organelles, including fungi, plants and animals

Extremophile – a microorganism that thrives in extreme conditions, such as high temperatures, acidity, or salinity

Fungi – a kingdom of eukaryotic microorganisms, including yeasts and molds, that obtain nutrients through decomposition

Genophore - the bacterial chromosome

Gram staining – a differential staining technique used to classify bacteria as Gram-positive (purple) or Gram-negative (pink) based on cell wall composition

Host – an organism that provides a habitat or nutrients for a microbe, including pathogens and symbionts

Lipopolysaccharide (LPS) - a component of the outer membrane of gram-negative bacteria; plays a role in immune response and toxicity

Outer membrane - a structure made of phospholipids and lipopolysacchardies found on the outside of the cell wall of Gram-negative bacteria

Peptidoglycan – a polymer found in bacterial cell walls, providing structural strength; used to distinguish gram-positive from gram-negative bacteria

Plasmid – a small, circular DNA molecule found in bacteria that can carry antibiotic resistance genes and other traits

Spirillum – a spiral-shaped bacterium

Staphylo - a cluster of bacterial cells joined together; "staphylococcus" - a chain of spherical bacteria joined together

Strepto - a chain of bacterial cells joined together; "streptobacilli" - a chain of rod-shaped bacteria joined together

Vaccine – a biological preparation that stimulates an immune response against a microbe

Virus – a non-living infectious agent composed of nucleic acids contained in a protein coat; requires a host cell to replicate

---

7.1: Microbe classification is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

7.2: Microbial Identification

Biotech Focus

Artificial Intelligence (AI) is increasingly being used in healthcare to identify bacteria. AI can process a large amount of data faster than traditional lab methods, allowing for faster diagnosis and treatment. AI-drive automated systems utilize databases, algorithms, and artificial intelligence to interpret test results rapidly, providing reliable identification within hours. Using these systems, AI-driven image analysis is also being developed for recognizing colony morphology and analyzing microscopic images, offering new avenues for rapid and accurate identification. It can be used to analyze medical images, such as microscope slides, to detect bacterial presence and classify the species involved. AI can process vast amounts of data more quickly than traditional lab methods. One of the applications of AI in microbiology is to test whether bacteria isolated from a patient are resistant or not to an antibiotic, which helps in determining which antibiotic treatment could be used.

Watch the video below to learn more about AI is being used detect antibiotic resistance.

Introduction

Microbes are everywhere—from the human body to the depths of the oceans, from sterile medical devices to harsh environments like volcanic springs. Identifying microbes is fundamental to microbiology, biotechnology, medicine, environmental science, and many other fields. Understanding which microorganisms are present in a sample helps researchers make important decisions—whether diagnosing infections, understanding ecosystem dynamics, or developing new biotechnologies. Microbe identification involves isolating and characterizing them in order to determine their identity, using a variety of traditional and modern methods.

Microbe identification is critical for various purposes:

- **Healthcare**: Identifying pathogens causing diseases is crucial for appropriate treatment. Knowing whether an infection is bacterial, fungal, or viral helps determine the best therapeutic approach.
- **Biotechnology**: Identification helps in selecting the right microbe for industrial applications, such as enzyme production or waste treatment
- **Ecology**: Identifying microbes in soil or aquatic environments helps scientists understand nutrient cycling, plant-microbe interactions, and environmental changes.
- **Food Safety**: Identifying spoilage organisms and pathogens is essential for food safety and quality control.

Microbial identification involves understanding not only the taxonomy of organisms but also their physiology and capabilities. Advances in technology have transformed how we approach this task, making identification faster, more precise, and accessible.

Learning Objectives

Being able to correctly identify a microbe is essential to fields like microbiology, medicine, and biotechnology. By the end of this section, you will be able to:

- Describe some of the stains used in identifying microbial morphology, including bacteria and yeast

- Describe the major types of media used culturing microbes
- Explain the difference between a broth, a plate, a slant, and a deep
- Describe some of the metabolic tests used in identifying microbes like bacteria and yeast
- Describe some of the molecular tests used in studying microbes
- Describe some of the chemotaxic and spectrometry tests used in studying microbes

Traditional Methods of Identification

Traditional methods of identification have formed the foundation of microbiology. These methods include morphological, cultural, biochemical, and immunological approaches.

Morphological Identification

Morphology is the study of the form, structure, and shape of organisms, cells, or biological components. By observing the morphology of bacteria (e.g., rod, cocci, spirilla), fungi (e.g., molds, yeasts), and other microbes, scientists can classify them into broad categories. For identification using morphology, **microscopy** is often the first step. Microbes like bacteria and yeast can be visualized using bright-field, dark-field, phase-contrast, or electron microscopy. For more information on microscopy, go to Chapter 1.4 Advanced Biotechnology Equipment.

Morphology can be studied and identified under the microscope using specific staining techniques. Depending on the objective, different staining protocols can be used. Some common stains used are:

1. **Simple stains**
2. **Gram stain (differential stain)**
3. **Acid-fast stain (differential stain)**
4. **Spore stains (differential stain)**

Simple stains can be used to determine the cellular shape and arrangement. They are used in a **simple staining protocol**. Simple staining involves staining a heat-fixed sample *with only one dye* in order to increase contrast and allow visualization of cell shape, size, and arrangement under a microscope. Basic dyes, such as crystal violet (purple), methylene blue (blue), malachite green (green), or safranin (red/pink), work well for simple staining of bacteria and yeast. These positively charged dyes bind to the negatively charged components in the cell walls and cytoplasm. Go to Chapter 13.15: Bacterial Identification Methods to learn how to perform a simple stain.

Concept in Action

Video: Simple Staining with Methylene Blue

A differential stain, like a **Gram stain**, uses a minimum of two stains. Each stain is specific to a structure or an organism in the sample. The first stain used is called the **primary stain**. Following staining with the primary stain, a **mordant** may then be used. A mordant is not a stain but is used to fix the primary stain and enhance its staining ability. A **decolorizer** is then used to selectively

remove the primary stain from certain structures or organisms before a second stain, called a **counterstain**, is applied. Common decolorizing solutions are ethanol, acetone, or a combination of both.

The Gram stain stains the peptidoglycan component of bacterial cell walls (Figure **7.2.1**). For more information about the bacterial cell wall, go to Chapter 7.1 Microbial Classification. Based on how well they retain this Gram stain, bacteria are classified into **Gram-positive** and **Gram-negative** groups. The identification of bacteria as either Gram-positive or Gram-negative is critical to determining what antibiotics are chosen to treat a bacterial infection. The Gram stain uses a primary stain called crystal violet, combined with an iodine treatment as the mordant. The iodine forms a complex with the crystal violet stain which then allows it to bind the peptidoglycan layer of the cell wall more efficiently. An acetone or alcohol wash step "decolorizes" the bacteria, washing away the Gram stain. The bacteria are then counterstained with the counterstain, safranin O, which will stain the bacteria pink. Gram-positive bacteria, with their thick peptidoglycan layer, will efficiently retain the Gram stain and cannot be decolorized. As a result, they take on a purple color that masks the pink counter-stain. In contrast, Gram-negative bacteria are easily decolorized because the outer membrane prevents Gram staining of the peptidoglycan layer. The counter-stain is easily visualized in Gram-negative bacteria and these bacteria appear pink under the microscope. Go to Chapter 13.15: Bacterial Identification Methods to learn how to perform a Gram stain.

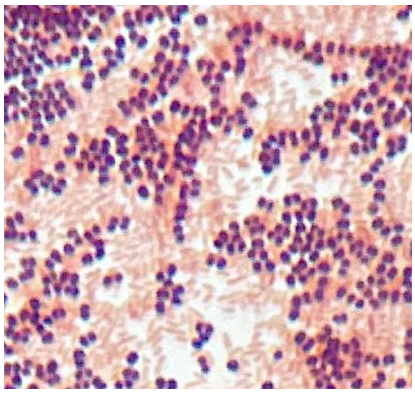


Figure **7.2.1**: Gram stained bacteria. Gram staining of a bacterial smear containing *Pseudomona aeroginosa* and *Staphylococcus epidermidis* is shown in this figure. The pink rods are *Pseudomona aeroginosa*, a Gram-negative bacteria. The purple cocci are *Staphylococcus epidermidis*, a Gram-positive bacteria. (Yaritza Castellanos, CC BY-NC 4.0)

Acid-fast staining is differential staining technique used to identify bacteria with cell walls rich in mycolic acid, a "waxy" lipid that makes the cell wall impermeable to the Gram stain. Bacteria stained using this technique are *Mycobacterium*, a genus that contain pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* that cause tuberculosis and leprae, respectively. In acid-fast staining bacteria are stained with a primary stain, called carbol fuschin, that binds to the mycolic acid and stains the bacteria reddish red or dark pink. Heat is used to help this stain penetrate the cell wall. The bacteria are decolorized to wash away the stain and then counter-stained with methylene blue. Acid-fast bacteria will retain their red color, while non acid-fast bacteria will lose the stain and appear blue as a result of the counter-stain. Go to Chapter 13.15: Bacterial Identification Methods to learn how to perform an acid-fast stain.

Spore staining is a differential staining technique used to view **endospores.** Endospores, also referred to as **spores**, are dormant structures that help bacteria survive unfavorable conditions. Endospores can germinate back into active bacterial cells when conditions improve. Endospore staining can be used to identify bacterial species and understand their ability to survive extreme conditions like high heat or desiccation, or to assess their pathogenicity. Due to their resistance to extreme conditions, it is important to take the presence of endospores into consideration when choosing conditions used to sterilize equipment as inadequate conditions will allow the endospores to survive. Common endospore forming bacteria include *Bacillus anthracis*, the bacteria that causes anthrax, and many bacteria in the *Clostridium* genus. The presence of high levels of **keratin** in the outer coat of the endospore makes conventional stains, like Gram stain, inadequate. Spore staining uses a primary stain, called malachite green, to stain spores and mature bacterial cells green. A decolorizing step is followed by counter-staining with safranin (Figure **7.2.2**). Spores are resistant to decolorizing and will remain green, while mature bacteria will display the pink color of the counter-stain. Go to Chapter 13.15: Bacterial Identification Methods to learn how to perform an endospore stain.

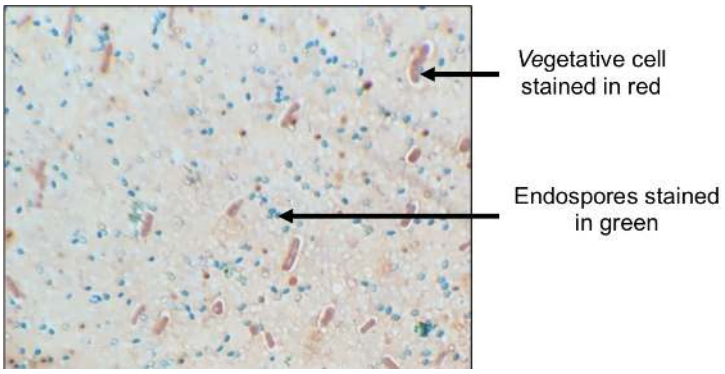


Figure **7.2.2**: Endospore stained bacteria. Endospore staining of a bacterial smear containing *Bacillus megaterium* is shown in this figure. The red rods are vegetative cells of *Bacillus megaterium*. The green structures are its endospores. (Kareen Martin, CC BY-NC 4.0)

Yeast can be stained using several stains. India Ink/Nigrosin detects the polysaccharide capsule surrounding specific yeast species. Yeast cells appear to have a clear halo around them. The presence of polysaccharides in the yeast cell wall can be detected using a Periodic Acid-Schiff/PAS (bright pink or red). Chitin within the fungal cell wall can be stained using a Lactophenol Cotton Blue (LPCB) stain (blue). Several fluorescent stains are also available, with acridine orange staining live yeast cells green and dead cells orange. The fluorescent stain Calcofluor white is highly sensitive and specific to yeast, binding to the chitin in their cell walls and fluorescing blue/green under UV light. In addition, yeast cells can also be stained using many bacterial stains. Simple stains like methylene blue can be used to distinguish between live yeast cells (colorless) and dead cells (blue). Gram staining is also used to identify yeast cells because their thick cell wall structure efficiently retains the Gram stain. As a result, yeast cells will stain dark purple. Distinguishing Gram-positive bacteria from stained yeast cells in a mixed sample is relatively straightforward owing to the larger size of the yeast cell.

Identification Using Culture Techniques

Culturing microbes, like bacteria and yeast, is essential for isolating and identifying individual species from a sample and also for the maintenance of stock cultures. A **culture medium** is a specially formulated mixture of nutrients and salts used for this purpose. Some culture media are chemically defined, meaning their exact chemical composition is known. Others are more complex, with their chemical composition is unknown. These undefined media usually consist of extracts and digests of yeasts, meat or plants. Different types of media are used depending on the need of the microbiologist. Table **7.2.1** below gives a few examples of common bacterial media.

Table **7.2.1**: Culture Media Types

Media Type	Purpose	Examples
Non-selective media	Provides essential nutrients to allow for a wide variety of **non-fastidious bacteria** to grow (i.e., those bacteria that do not require special nutrients)	LB (Luria-Bertani) medium, Nutrient agar and Tryptic Soy Agar (TSA)
Selective media	Used for the growth of specific bacteria (i.e., **fastidious bacteria**). Includes compounds that inhibit the growth of unwanted species	MacConkey Agar - selects for Gram-negative bacteria Mannitol Salt Agar - selects for *Staphylococcus* species Eosin Methylene Blue (EMB) Agar - selects for Gram-negative gut bacteria
Differential media	Used to produce specific reactions (i.e. color changes) that distinguish between species based on metabolic characteristics.	Blood Agar
Enriched media	Contain extra nutrients for difficult to grow organisms	Chocolate Agar (contains lysed red blood cells)

Culture media can be prepared as a liquid (**broth**), as a solid (**plate** or **slant**) or as a semi-solid (**deep tube**) (Figure **7.2.3**). A broth is used to establish a **liquid culture**. Liquid cultures can be used to produce a desired volume of cells that can then be used in the laboratory in downstream applications, such as DNA isolation and metabolic assays. In a liquid culture, an individual cell, like a bacterium, is added to the broth and then incubated for a specific amount of time in order to allow them to "grow" (i.e. increase in number). The cloudier the broth, the more cellular growth. Hence, the amount of growth can be assessed by measuring the turbidity of the broth. To learn how to start a liquid culture of bacteria using a broth, go to Chapter 13.6: Bacterial Transformation.

Culture plates are prepared using a liquid medium that contains **agar**, a polysaccharide that polymerizes to a gel-like solid. When added to a broth, agar will cause the the liquid medium to solidify when a specific temperature is reached. To make a plate, the agar-containing liquid medium is poured into a petri dish and then the plate is allowed to cool. Culture plates are used to create individual colonies, using an **inoculation loop** and a technique called "streaking". A **bacterial colony** is a visible mass of bacteria that has grown from a *single bacterial cell*. Culture plates are used to observe the characteristics of colonies, such as size, color, shape, and texture. When the culture plates are stored properly, they can be used for short-term culture maintenance. The colonies that form on the plate can also be chosen to start liquid cultures in a broth. To learn how to streak a culture plate, go to Chapter 13.16: Basics of Bacterial Culture.

A slant is prepared in a test tube using an agar-containing medium. The test tube is tilted at an angle while the medium solidifies, leading to a slanted surface. The slant provides a larger surface area for cell growth. Slants are used to generate stocks for long-term maintenance. The cells (e.g. bacteria or yeast) can be streaked onto the slanted surface for observation of morphology or the inoculation loop can be "stabbed" into the agar to test for anaerobic growth. Cells grown in or on slants can survive for weeks to months and can be easily and safely transported.

A deep is prepared in a test tube that remains upright during solidification. The inoculation loop is stabbed into the column of agar. Depending on the media used, the deep can be used for biochemical analysis, along with an assessment of cell motility through the deep and their oxygen requirements. Those bacteria and yeast cells that can only grow on the surface of the deep require oxygen and are considered to be **obligate aerobes**. Cells that can survive throughout the deep are known as **facultative aerobes** (can growth with or without oxygen). Growth at the bottom of the deep classifies the cells as **obligate anaerobes**, cells that can grow without oxygen.

To learn how to make a culture plate, slant, or deep, in addition to learning about how to streak a plate and inoculate a bacterial culture, go to Chapter 13.16: Basics of Bacterial Culture.

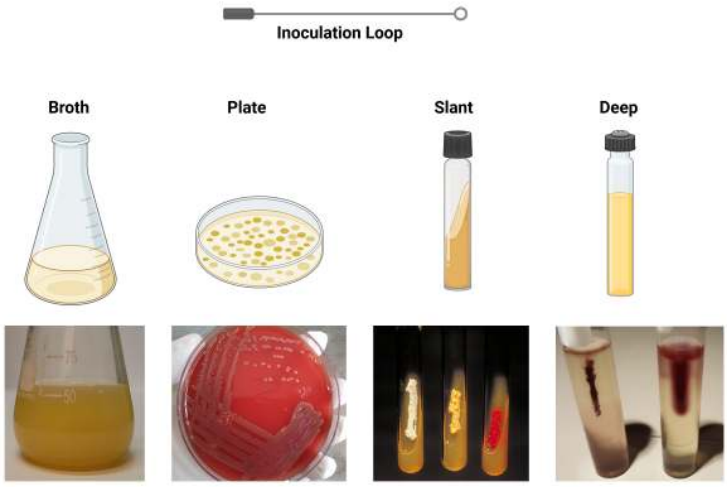


Figure **7.2.3**: Bacteria Culture Techniques. Techniques for culturing bacteria include, from left to right, liquid broth, plates, slants, and deep stabs. Each culture is created using tools such as an inoculation loop. (Bacteria Culture Techniques by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025); photo credits (left to right): K. rhizophila liquid culture by Alexandre.Cz, CC BY-SA 3.0; Bacterial growth on blood agar by Ajay Kumar Chaurasiya, CC BY-SA 4.0 ; Actinomycetes by CDC/Dr. David Berd, public domain; Stab culture by Todd Parker, public domain)

Identification Using Biochemical Tests

Biochemical tests help determine the metabolic and enzymatic properties of bacteria and other microbes. Common tests include:

1. **Catalase test**
2. **Oxidative test**
3. **Carbohydrate fermentation**
4. **Analytical Profile Index (API) strips**

The **catalase test** is used to determine whether a cell produces **catalase**, an enzyme that breaks down hydrogen peroxide into water and oxygen (O_2). Aerobic cellular respiration (biological reactions that require O_2) releases **hydrogen peroxide (H_2O_2)** as a toxic byproduct that can damage or kill cells. Some cells produce catalase to protect themselves against the damages caused by hydrogen peroxide. The catalase test is a simple test that involves transferring cells (e.g. bacteria) into a drop of hydrogen peroxide. The presence of bubbles indicates a positive test - the production of oxygen, meaning that hydrogen peroxide has been split by catalase. The absence of bubbles means the test is negative and the cell doesn't produce catalase.

The **oxidase test**, also known as the oxidative test, is used to determine whether a cell produces the enzyme **cytochrome c oxidase,** the last enzyme within the electron transport chain, that catalyzes the transfer of electrons to oxygen. Microbes, like bacteria or yeast, are smeared onto filter paper and a few drops of an oxidase reagent are added. The presence of cytochrome c oxidase will cause the reagent to turn purple or blue. The oxidative test is particularly useful in the identification of oxidase-positive bacteria, including *Neisseria sp.* (which includes *Neisseria gonorrhoeae* and *Neisseria meningitis*), *Vibrio sp* (which includes *Vibrio cholera*), and *Pseudomonas sp*.

Carbohydrate fermentation tests are used to determine whether a microbe, like a bacteria or yeast cell, can ferment specific sugars. Because fermentation release acids and gases, the carbohydrate fermentation test detects the production of these components using a pH indicator for acid production and an inverted glass tube to capture gas production. In the test, a liquid medium containing nutrients, a pH indicator, and a specific sugar is dispensed into a sterile test tube. A small, thin Durham tube is inverted and submerged completely in the test tube. The tube is then inoculated with the organism of interest (Figure **7.2.4**).

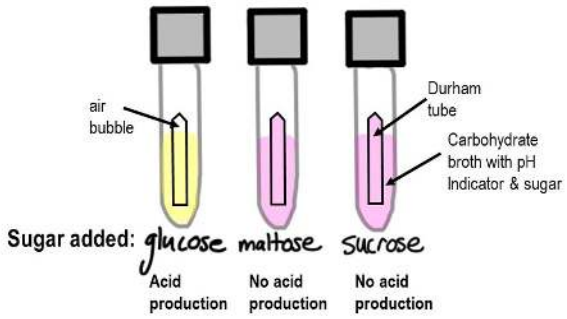


Figure **7.2.4**: The carbohydrate fermentation test. To test for sugar fermentation, bacteria are inoculated into test tubes containing a Carbohydrate broth composed of nutrients, a pH indicator, and a specific sugar (e.g. glucose, maltose, sucrose). A Durham tube is inverted and submerged in the broth so that it fills completely with broth. The tube is incubated at 37C for 24 to 48 hours to allow for growth and potential fermentation. The production of carbon dioxide will result in the presence of an air bubble in the Durham tube. The production of acids will be indicated by the pH indicator. The three tubes in this figure show positive fermentation (acids and gas) in the tube containing glucose and negative fermentation (no acids, no gas) in the tubes containing maltose and sucrose. (Carbohydrate Fermentation by Patricia Zuk, CC BY 4.0; adapted from Carbohydrate Fermentation by NewDancinShoes, CC BY-SA 4.0)

As the organism grows in the liquid medium, it will begin to ferment the added sugar and produce carbon dioxide gas and specific acids. The presence of a gas bubble in the Durham tube indicates the production of gas. The pH indicator will confirm the production of acids. Go to Chapter 13.15: Bacterial Identification Methods to learn how to perform a carbohydrate fermentation test.

Concept in Action

Video: Carbohydrate Fermentation Test

Analytical Profile Index (API) strips are composed of several biochemical tests on a single strip (Figure **7.2.5**). Each API strip is made of multiple "cups" or **cupules**. Each cupule serves as mini-testing chamber that contains a specific substrate. These substrates test for a specific metabolic function or for the production of specific enzyme. API testing strips can be used to analyze microbes, like bacteria or yeast. To use, a cellular suspension is added to each of the cupules and the strips are incubated. During incubation, reactions with each cupule produce color changes that are either spontaneous or revealed by the addition of reagents. The results are recorded as positive or negative depending on the color reaction on the strip. API strips allow for quick screening of multiple metabolic capabilities. For example, the API 20E test strip tests Gram-negative bacteria, like the *Enterobacteriaceae* family, for a variety of enzymatic reactions associated with fermentation. The use of API strips provide numerous advantages including fast reliable results for many clinical and environmental bacterial strains, standardized results, and no need for complex equipment. Furthermore, some API strips are compatible with automated readers that can compile the results and store them in a database. For the API strip protocol, go to Chapter 13.15: Bacterial Identification Methods.

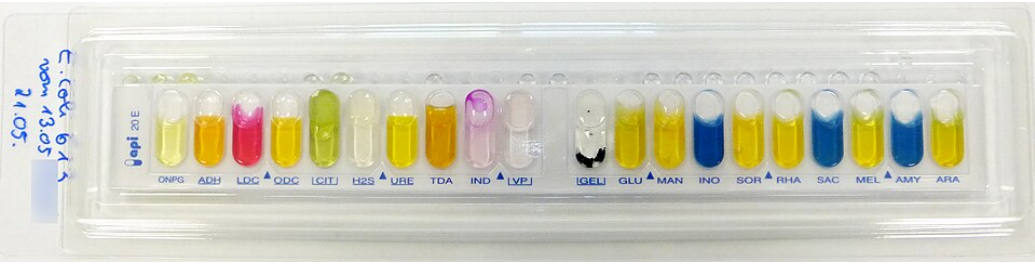


Figure **7.2.5**: An API strip. This figure shows results after incubating a suspension of *E.coli* cells in an API 20E test strip. The results in each well are classified as either positive or negative based on the colored product produced. (API 20E Escherichia coli by A doubt, CC BY-SA 4.0)

Molecular Methods for Identification

Advances in molecular biology have revolutionized microbial identification, providing fast, highly specific, and accurate methods for characterizing microorganisms, like bacteria, without needing extensive culture-based work.

Polymerase Chain Reaction (PCR) has become a cornerstone in microbial identification, allowing for the amplification of specific DNA sequences from small samples. By using species-specific primers, it is possible to amplify unique regions of bacterial DNA (and other microbes), confirming the presence of a particular organism. Variants such as **qPCR** (quantitative PCR) can be

used to not only identify but also quantify the presence of microbial DNA in a sample. For more about PCR, go to Chapter 3.2 Replication of DNA. To learn about the PCR protocol, go to Chapter 13.2: Polymerase Chain Reaction (PCR).

Gene sequencing is one of the most common molecular methods for microbe identification. Bacterial identification can be achieved through the sequencing of the 16S rRNA gene. The 16S rRNA gene codes for the 16S ribosomal protein found within the 30S small subunit. The 16S rRNA gene contains several variable regions that are unique to different bacterial taxa, making it a reliable marker for identification of bacteria at the genus or species level. In addition to 16S sequencing, **whole-genome sequencing (WGS)** has gained popularity. WGS provides comprehensive information about an organism's entire genetic makeup, offering insights not only into its taxonomy but also into its resistance genes, virulence factors, and potential metabolic capabilities. Identification of multiple microbial species in a sample through the sequencing of their genomes is being made possible through **Next-Generation Sequencing (NGS)**. This approach has been used to study complex microbiomes, such as those in soil, the human gut, or in environmental samples. By directly sequencing DNA from an entire community, researchers can characterize microbial diversity and understand the functional roles of different species. For more about DNA sequencing, including WGS and NGS, go to Chapter 5.3 Genome Sequencing. To learn about the DNA sequencing protocol, go to Chapter 13.9: DNA Sequencing.

Chemotaxonomic and Spectroscopy Methods

Another set of methods used in identification involves **chemotaxonomy (i.e., chemical taxonomy),** which consists on classifying organisms based on their biochemical composition, and **spectroscopic techniques**.

Chemotaxonomy can classify bacteria and other microbes based on the presence of proteins, lipids, carbohydrates and other metabolic compounds. Gram staining and its identification of the presence of peptidoglycans in Gram-positive bacteria could be considered a chemotaxonomic technique. **Fatty Acid Methyl Ester (FAME) analysis** and its identification of the unique fatty acid "signatures" of a cell is another. Profiling by FAME can help differentiate microbial species, including bacteria, by examining the types and proportions of fatty acids unique to each group. FAME is a cheap, reliable and easy technique.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry is a powerful tool for rapid identification of bacteria and other microbes. It works by analyzing the unique protein fingerprint of an organism, primarily focusing on **ribosomal proteins**, which are highly conserved across species. Results are then compared against a reference database to identify the microbe. MALDI-TOF has become popular in clinical and research laboratories for its speed, accuracy, and cost-effectiveness.

Immunological tests like **serological tests** use antibodies to detect specific antigens associated with an organism. In addition, the **ELISA (Enzyme-Linked Immunosorbent Assay)** is an example of an immunological technique used for microbial identification. These methods are particularly useful for detecting pathogenic microorganisms in clinical settings and are often used to identify bacteria, viruses, and fungi based on their specific surface markers.

Challenges and Future Directions

Despite the impressive advances in identification, challenges remain in identifying bacteria and other microbes. Identifying "unculturable" microorganisms, those that cannot be grown in a lab, is still a major obstacle. Another challenge is the presence of mixed microbial communities where multiple species are present in a single sample. Differentiating between closely related species requires precise methods, often involving multi-gene or whole-genome analysis. Next generation sequencing, together with **metagenomics**, the study of genetic material recovered directly from an environmental sample rather than from a cultured organism, and **single-cell genomics**, the sequencing of an entire genome from a single cell, are addressing these challenges, allowing researchers to sequence genomes directly from mixed populations, environmental samples or individual cells, respectively.

The future of microbial identification lies in continued advancements in sequencing technologies, machine learning, and bioinformatics. Developing more rapid, cost-effective, and high-throughput identification techniques will open doors to deeper insights into the microbial world and lead to new discoveries that impact health, biotechnology, and environmental management.

Lab Protocols

- Lab Technique: Bacterial Transformation
- Lab Technique: Polymerase Chain Reaction (PCR)

- Lab Technique: DNA Sequencing
- Lab Technique: Bacterial Identification Methods
- Lab Technique: Basics of Bacterial Culture

Key Concepts

Microbial identification is a cornerstone of many scientific and applied disciplines, providing critical insights into health, biotechnology, and ecology. From traditional microscopy and biochemical testing to cutting-edge molecular techniques and artificial intelligence, the journey of identifying microbes, such as bacteria allows us to explore microbial diversity in greater detail, understand complex communities, and harness the potential of microbes for various applications.

Some important concepts to remember are:

- Microbes like bacteria can be identified through morphology, the study of the form, structure, and shape of an organism
- Staining techniques enhance the morphological identification of microbes
- Simple stains use only one stain; differential stains uses multiple stains
- Media used to grow bacteria and other microbes can range from liquid broths to solid media like plates, slants, and deeps
- Non-selective media can be used to grow a wide variety of bacteria and other microbes
- Selective media compositions can be used to grow a specific type of microbe
- Molecular methods of microbe identification include PCR and DNA sequencing
- Chemical methods for identification include biochemical tests like catalase and oxidase tests
- More advanced techniques like FAME analysis and MALDI-TOF Mass Spectrometry identify microbes by examining their unique biochemical compositions

Glossary

Acid-fast staining - a staining technique used for staining bacteria with large amounts of mycolic acid in their cell walls

Aerobe – a microorganism that requires oxygen for growth

Agar - a polysaccharide isolated from red algae; when added to media it causes the solidification at a specific temperature

Anaerobe – a microorganism that does not require oxygen for growth and may even be harmed by it

Analytical Profile Index (API) test strip - a strip composed of several biochemical tests contained within small wells called "cupules"

Bacillus (plural = bacilli) – a rod-shaped bacterium

Bacterium (plural = bacteria) – a single-celled prokaryotic microbe that can be found in diverse environments and classified based on shape, Gram stain, and metabolism

Coccus (plural = cocci) – a spherical-shaped bacterium

Colony - a visible cluster of microorganisms, like bacteria, growing on a solid medium and originating from a single cell or a group of identical cells

Counter-stain - a secondary stain used in a staining technique; provides contrast to the primary stain

Classification – the organization of microorganisms into hierarchical groups based on characteristics such as genetics, morphology, and metabolism

Defined medium - a culture medium in which all chemical components are known and present in specific amounts

Differential medium - a type of culture medium that allows different types of microbes to be distinguished based on their biological characteristics

Differential staining - a staining technique that uses two or more contrasting stains to distinguish between different types of microbes or different structures within a single organism

Endospore – a highly resistant, dormant structure formed by some bacteria to survive harsh conditions

Endospore staining - a technique used to specifically stain endospores

Enriched medium - a microbial medium that provides additional nutrients to support the growth of fastidious organisms that require specific growth

Facultative anaerobe – a microorganism that can grow with or without oxygen

Fastidious - organisms that require special nutrients to grow

Genus – a taxonomic rank above species that groups related organisms based on common traits

Gram staining – a differential staining technique used to classify bacteria as Gram-positive (purple) or Gram-negative (pink) based on cell wall composition

Inoculation - the process of introducing microbes like bacteria, fungi, viruses, or other microbes into a culture medium

Medium - a mixture of nutrients used to support the growth of a cell; also called culture medium or growth medium

Microscopy - the technique of using a microscope to observe objects and structures that are too small to be seen with the naked eye

Mordant - a compound that enhances the staining effect of a dye by forming a complex with it

Morphology – the study of microbial shape, structure, and form

Non-fastidious - organisms that do not require special nutrients to grow

Obligate aerobe – a microorganism that requires oxygen for survival and metabolism

Obligate anaerobe – a microorganism that cannot survive in the presence of oxygen

Pathogen – a microorganism that causes disease in a host organism

Peptidoglycan – a polymer found in bacterial cell walls; providing structural strength and protection to the bacterial cell

Primary stain - the first dye applied in a staining procedure

Simple staining - a technique that uses a single stain to color microbes like bacteria

Stain - a chemical dye used to color cells, tissues, or cellular components in order to make them more visible under a microscope

Turbidity - the cloudiness of a liquid culture, indicating microbial growth

---

7.2: Microbial Identification is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

7.3: Microbial Cultivation and Manipulation

Biotech Focus

Are bacteria the next "hot" thing in fashion? The clothes people wear require a massive amount of energy and resources to produce. Only to have those clothes thrown away when next year's fashion trends appear. So, when it comes to sustainability, the fashion industry is pretty unfashionable. The solution? Bacteria, or rather, **bacterial cellulose (BC)**. BC is a network of carbohydrate molecules that can be excreted by some species of bacteria. BC is an exciting idea in the fashion industry because it can be crafted into everything from handbags to jackets and because BC is biodegradable, it is an attractive solution to an unattractive problem. But how is BC transformed into "fabulous fashion"? Microbiologists grow the bacteria in large **bioreactors** under carefully controlled conditions that promotes their growth and production of BC. The resulting BC fibers are extracted from the bioreactor, washed, and dried. Depending how the bacteria are cultured in the bioreactor, the BC can be similar to leather, thread, or paper-like. Despite how exciting BC is, it is still in the prototype phase and has only been shown at a few fashion shows. But it may only be a matter of time before BC-containing fashions become mainstream in the fashion world and out on the streets.

To read more about BC and the fashion industry, check out the article Are Bacteria the Next Big Thing in Fashion?

Introduction

The cultivation and manipulation of microbes, like bacteria and yeast, are foundational aspects of microbiology and biotechnology. The ability to grow, control, and genetically manipulate bacteria and yeast has revolutionized industries from medicine to agriculture, environmental management, and even energy production. This page explores the principles and techniques involved in microbial cultivation, with an emphasis on bacteria cultivation principles like aseptic techinque, and outlines some of the the challenges that arise in manipulating these organisms.

Learning Objectives

Microbiology and biotechnology rely upon the cultivation and manipulation of microbes like bacteria and yeast. At the end of this section, you will be able to:

- Describe the physical conditions necessary for microbial growth
- Describe the types of media used in growing microbes
- Explain the four phases of a microbial growth curve
- Describe microbial aseptic technique
- Describe bioreactors and fermenters and how they are used in microbial cultivation
- Describe how microbes can be manipulated genetically and metabolically
- Explain a microbial consortia and co-cultivation

Microbial Cultivation

Microbial cultivation in microbiology and biotechnology refers to the process of growing microbes, like bacteria or yeast, under controlled laboratory conditions in order to study their physiology, biochemistry, genetics, or for industrial production of valuable compounds. The ultimate goal of cultivation is to provide an environment that mimics the organism's natural habitat as closely as possible, allowing for growth and replication. In order to provide the optimal growth conditions, it is important to consider the species, the type of medium and the physical conditions for growth.

Physical Conditions for Growth

Microbes, like bacteria and yeast, require specific environmental conditions in order to optimize their growth. The primary factors that need to be controlled during cultivation include:

1. **Temperature**
2. **Ph**

3. **Oxygen levels**
4. **Nutrients**

Temperature

When culturing microbes, it's essential to consider the **incubation temperature** specific to each organism, as they have preferred growth temperatures. For instance, *E. coli*, commonly found in the human intestine, thrive at 37°C, aligning with body temperature. Similarly, the yeast *Saccharomyces cerevisiae*, used in brewing and baking, grows optimally between 28°C and 32°C. Based on their ideal growth temperatures, microbes can be classified into three main groups: **psychrophiles** (cold-loving, optimal growth between -20°C and 20°C), **mesophiles** (moderate temperature-loving, best growth between 20°C and 45°C), and **thermophiles** (heat-loving, growing above 50°C).

pH

The pH level of an environment, indicating its acidity or alkalinity, significantly influences enzyme activity and cellular processes. Like temperature, it is important to consider the pH when culturing microbes, as each type has an optimal pH for growth. **Acidophiles** thrive in acidic conditions, **neutrophiles** prefer a neutral pH, and **alkaliphiles** grow best in alkaline environments.

Oxygen Availability

Microbes have varying requirements for and responses to oxygen, so oxygen levels must be carefully controlled in culture conditions. Oxygen is essential for aerobic respiration as it provides an efficient way to generate ATP. However, oxygen can also produce **harmful reactive oxygen species (ROS)**, highly reactive molecules that can damage lipids, proteins, and DNA. ROS molecules are produced as normal by-products of cellular metabolism, but are also generated by environmental stresses. Some cells have enzymes that neutralize these toxic byproducts, while others lack this capability and therefore must be cultured in anaerobic conditions. Certain microbes require oxygen to grow, while others can switch to less efficient pathways, like fermentation, in its absence.

Microbes exhibit a spectrum of oxygen needs, including:

- **obligate aerobes:** cells that require oxygen to grow; use cellular respiration to generate ATP
- **facultative anaerobes:** cells that prefer to grow in the presence of oxygen but can survive in its absence; use aerobic respiration, fermentation and other anaerobic mechanisms to generate ATP
- **aerotolerant anaerobes:** cells that do not use oxygen but can survive in its presence; use only fermentation to generate ATP
- **obligate anaerobes:** cells that cannot survive in the presence of oxygen; use anaerobic metabolism like fermentation to generate ATP

Nutrient Availability

The availability of nutrients in microbial cultures is crucial to optimizing cellular growth rate and behavior. These nutrients serve as the building blocks for cell growth, development and reproduction, and provide essential elements for cellular processes like energy production and biosynthesis. Microbes need a carbon source (e.g., glucose, lactose), nitrogen, phosphorus, sulfur, trace elements, and vitamins for growth. Carbon sources, like glucose, provide essential energy, while nitrogen supports protein and nucleotide synthesis. Phosphorus is critical for nucleic acids and energy molecules like ATP, while sulfur is necessary for specific amino acids and vitamins. Minerals and trace elements, including potassium, magnesium, and iron, are crucial for enzyme function and cellular stability. Many microbes also need growth factors or vitamins as coenzymes for metabolic pathways, especially fastidious species requiring enriched media. By optimizing these nutrient sources, microbial growth and function can be effectively supported in culture. Therefore, specific types of media have been designed based on the metabolic profile of the microbe, in order to ensure that all nutrients are present in the correct forms and concentrations to support optimal microbial activity and research outcomes.

Types of Culture Media

Essential nutrients are supplied through a **culture medium** (plural = media). Different types of culture media serve different purposes. For additional information, go to Chapter 7.2 Microbial Identification.

- **Solid Medium:** Often containing **agar**, a gelatinous substance derived from seaweed, solid media supports surface growth, allowing for the formation of colonies. It is useful for isolating pure cultures and observing colony morphology. Solid media can be formed into plates, slants and stabs.
- **Liquid Medium:** Also known as broth, a liquid medium is used for growing large numbers of cells in a homogeneous suspension. Broth culture is ideal for studying growth kinetics and conducting biochemical tests.
- **Semi-solid Medium:** Has a lower concentration of agar, allowing it to be used for motility studies and the propagation of organisms that require low-levels of oxygen to grow (i.e., microaerophilic).
- **Selective Medium:** Contains compounds that suppress the growth of certain microorganisms while promoting the growth of others. For example, MacConkey agar selects for Gram-negative bacteria.
- **Differential Medium:** Designed to differentiate between cell types based on metabolic activity. Differential medium contains indicators that reveal specific biochemical reactions, such as the ability to ferment carbohydrates.
- **Enriched Medium**: Provides additional nutrients to support the growth of fastidious organisms that require specific growth factors.

Cultivation Growth Phases

When microbes, like bacteria and yeast, are grown in batch culture, they typically pass through four distinct phases of growth (Figure **7.3.1**):

1. **Lag Phase:** Cells are adapting to the new environment, synthesizing enzymes, and preparing for division. There is little to no cell division during this phase.
2. **Log Phase (Exponential Phase)**: Cells divide at a constant rate, and growth is exponential. This phase is ideal for studying metabolism and for industrial processes.
3. **Stationary Phase:** The rate of cell growth equals the rate of cell death, as nutrients become limited and waste products accumulate. The culture reaches a plateau in population size.
4. **Death Phase:** Nutrients in the culture medium are depleted, and waste products build up to toxic levels, causing the death of cells at an exponential rate.

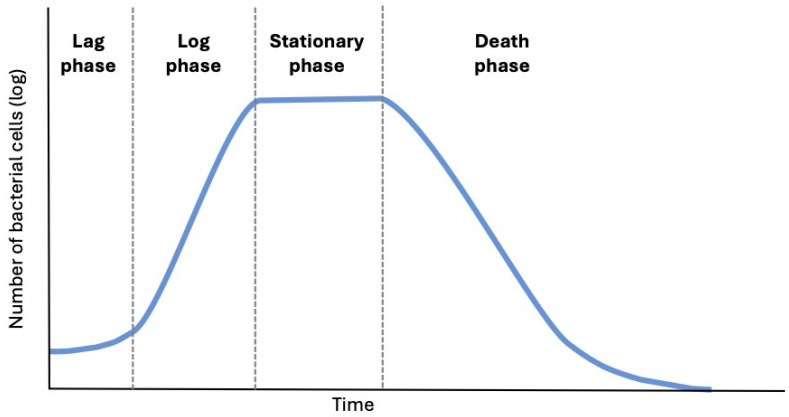


Figure **7.3.1**: Cell growth curve. The curve in this figure shows the number of bacterial cells plotted over time. The curve is divided into four distinct phases: the Lag phase with minimal increase in cell number, the Log phase marked by rapid cell multiplication, the Stationary phase where growth stabilizes, and the Death phase, reflecting a decrease in the number of live cells. (Cell Growth Curve by Kareen Martin; CC BY 4.0)

Aseptic Conditions for Microbial Cultivation

Aseptic conditions in microbial cultivation are essential to prevent contamination and the growth of unwanted microbes. Preparation of liquid and solid media, in addition to the establishment of bacterial cultures, are all done under aseptic conditions. Aseptic conditions for microbial cultivation include the use of sterile materials, the creation of a controlled environment, and the employment of specific aseptic techniques. Autoclaving to sterilize materials and equipment should be considered where appropriate. The purchase of pre-sterilized materials may also be an option. Work areas should be disinfected with 70% ethanol, a dilute bleach solution, or another suitable disinfectant. The work area should also be kept clutter free and should only contain the materials to be used. Hands should be washed thoroughly before and after working with microbes. A lab coat may be worn, along

with gloves, a face mask or goggles. Wearing a face mask or goggles is not required but will depend on the specific microbe being manipulated. As with face masks and goggles, the use of gloves is not mandatory but is generally encouraged when working with any microbe.

When establishing a microbial culture, specific steps are used in microbiology to prevent contamination. These steps are collectively referred to as **aseptic technique**. Key practices underlying aseptic technique include:

- Disinfect work surfaces before and after use.
- Work using a flame or in a laminar flow hood.
- Always sterilize inoculating loops and needles by flaming until red-hot.
- Do not place caps or lids on the bench; hold them in hand or keep upright.
- Minimize exposure time of media and cultures to air.

The most important tool used in microbial aseptic technique is the **Bunsen burner.** The Bunsen burner is used to "flame" tools like inoculating loops or needles until red hot before and after use. The burner is also used to quickly flame the opening of bottles, tubes, and flasks to eliminate potential contaminants. Wearing gloves when working with a Bunsen burner should also be carefully considered, as most disposable gloves (e.g., latex, nitrile) are flammable. Therefore, hands should be kept away from open flames.

Concept in Action

Aseptic Technique in Microbiology

While Chapter 6: Mammalian Cell Culture Fundamentals also describes aseptic technique, *this technique is specific to the culture of mammalian cells*. However, the principles of mammalian cell aseptic technique are much the same as bacterial aseptic technique in that sterile materials, a controlled environment, and specific techniques are used. There are some differences, such as the absence of a Bunsen burner and the use of a biosafety cabinet to ensure sterility.

Bioreactors Are Used in Microbial Cultivation

A **bioreactor** is a controlled vessel used to grow biological organisms under controlled conditions. The purpose of a bioreactors is the continuous culture of biological organisms, like microbes, for various applications. Bioreactors are widely used in industries like biotechnology, pharmaceuticals, and food production for the large-scale synthesis of products like antibiotics, enzymes, and pharmaceuticals. For example, insulin production uses genetically-modified bacteria or yeast grown in bioreactors to create human insulin for diabetic patients. The **microbial fermenter (i.e. fermenter)** is a type of bioreactor that provides the ideal environment for microbes, such as bacteria or yeast, to grow and perform fermentation processes under controlled conditions. Despite their name, fermenters can also grow organisms under aerobic conditions since aerobic fermentation is more efficient and can significantly increase cell biomass and their desired products. They can be used for batch culture or continuous culture.

Fermenters can range from small-scale laboratory reactors to large industrial fermenters. A typical industrial fermenter is a large stainless steel chamber (or multiple chambers) that can hold large volumes of culture medium (Figure **7.3.2**). The fermenter has a sterilization system to ensure the environment is free from contaminants before the microbe is added, an inoculation port into which the desired microbe can be added, and a nutrient feed system to provide a continuous supply of optimized culture medium.

The fermenter agitates the culture while it regulates key parameters such as temperature, pH, oxygen levels, and nutrient supply to optimize microbial activity and maximize yield. By-products of fermentation, like carbon dioxide, can be collected and released. The final products of fermentation can be harvested at the completion of the fermentation process.

Figure **7.3.2**: The bioreactor. Bioreactors for bacterial fermentation for production of vaccines. (The Bioreactor by Peter grotzinger, CC BY-SA 3.0)

Microbial Manipulation

Microbial manipulation involves modifying microbes to suit specific scientific, medical, or industrial purposes. This can include genetic engineering, metabolic pathway modifications, and environmental adaptations.

Genetic Manipulation of Microorganisms

Genetic manipulation, particularly with bacteria and yeast, is a crucial tool in biotechnology and synthetic biology. There are several techniques used to manipulate microbial genomes, including:

- **Recombinant DNA Technology:** This involves the insertion of foreign DNA into the microbial genome. The foreign DNA encodes for the production of a protein, allowing microbess to synthesize valuable products such as insulin, growth factors, or vaccines. Bacteria and yeast are used most often in this technology. For more about recombinant DNA technology, go to Chapter 4 Genetic Engineering and Recombinant DNA Technology.
- **CRISPR-Cas9 System:** This technology enables precise "gene editing" by utilizing the CRISPR-Cas9 system of bacteria to target specific genes in a genome for deletion, insertion, or modification. CRISPR is widely used to modify either prokaryotic and eukaryotic cells. For more about CRISPR, go to Chapter 8.1 Applications of Animal Biotechnology.

Metabolic Engineering

Microbial metabolic engineering is the practice of optimizing the metabolic pathways within microbes to increase the production of desired compounds or degrade specific environmental pollutants. Some examples of metabolic manipulation include:

- **Overproduction of Enzymes:** By enhancing the expression of genes involved in specific metabolic pathways, microbes, such as bacteria, can be engineered to produce large quantities of industrial enzymes, such as proteases, amylases, and cellulases.
- **Biosynthesis of Pharmaceuticals:** Yeast and bacteria are engineered to produce antibiotics, vaccines, and other therapeutics. For example, genetically-modified *Saccharomyces cerevisiae* can produce artemisinin, an important anti-malarial drug.
- **Biodegradation of Pollutants:** Bacteria such as *Pseudomonas* species have been engineered to degrade hydrocarbons and other pollutants, contributing to bioremediation efforts.

Microbial Consortia and Co-Cultivation

In many natural environments, microbes exist in complex communities where they interact with one another. This has led to the development of a **microbial consortia** in laboratory settings, where multiple species are cultivated together to perform complex tasks that single organisms cannot accomplish alone. Applications of such co-cultivation techniques include:

- **Biofuel Production:** Co-cultivation of photosynthetic algae and fermentative bacteria can enhance the production of biofuels like ethanol and biodiesel.

- **Bioremediation:** Microbial consortia are often more effective than single species in breaking down pollutants, as different species can metabolize different components of complex waste.

Key Concepts

Microbial cultivation and manipulation are at the heart of modern microbiology and biotechnology, offering powerful tools to address pressing global challenges. From advancing medical therapies to environmental sustainability, the ability to grow and engineer microorganisms holds tremendous potential for innovation.

Some important concepts to remember are:

- Microbes, like bacteria and yeast, require specific environmental conditions in order to optimize their growth
- Environmental conditions for growth include temperature, pH, oxygen, and nutrients
- Nutrients are provided using a medium; medium forms include liquid, solid, selective, and differential
- Bacterial growth curves track the growth of a bacterial culture over time
- Growth curves are made up of 4 phases: lag, log, stationary, and death
- Bioreactors are used for the large-scale growth of microbes, like bacteria and yeast
- Microbial manipulation of microbes can be achieved through genetic changes, metabolic engineering, and the creation of microbial consortia

Glossary

Agar: a gelatinous substance derived from seaweed; used as a solidifying agent in microbial culture media

Aerobic: a process, environment, or organism that requires oxygen

Anaerobic: a process, environment, or organism that does not require oxygen

Batch culture: closed system of microbial growth where nutrients are provided at the beginning, and no additional nutrients are added during cultivation

Broth: a liquid medium used to grow microorganisms in suspension

Colony: a visible cluster of microorganisms, like bacteria, growing on a solid medium and originating from a single cell or a group of identical cells

Continuous culture: a system in which fresh nutrients are continuously added, and waste products are removed to maintain microbial growth over time

Culture medium: a nutrient-rich solution or gel used to support microbial growth; it can be solid, liquid, or semi-solid

Facultative anaerobe: microorganism that can grow with or without oxygen but prefers oxygen for more efficient energy production

Fermentation: a biological process where microbes (like bacteria and yeast) convert sugars into other products like alcohol, gases, or acids

Inoculation: the introduction of microorganisms into a culture medium to promote growth

Incubator: laboratory equipment used to grow and maintain microbiological cultures or cell cultures under optimal temperature, humidity, and carbon dioxide levels

Lag Phase: the initial stage of microbial growth in which cells adjust to new conditions before active division begins

Log (Exponential) Phase: a phase of rapid microbial growth and division when conditions are optimal

Obligate Aerobe: a microorganism that requires oxygen for survival and metabolism

Obligate Anaerobe: a microorganism that cannot survive in the presence of oxygen

Selective medium: a culture medium designed to inhibit the growth of certain microbes while promoting the growth of others

7.3: Microbial Cultivation and Manipulation is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

https://bio.libretexts.org/@go/page/135683

7.4: Industrial Applications of Microbial Biotechnology

Biotech Focus

Modern Meadows Lab looks more like a brewery than a biotech company. But rather than making beer, this biotech company is using microbes to make a material that looks, feels, and performs like leather. Through a process called **biofabrication**, the company uses a specially designed DNA sequence inserted into yeast cells to produce collagen that becomes incorporated into their biofabricated leather. These yeast cells are grown in large fermenters that are no different then those found in beer and wine distilleries and the collagen extracted and processed. Because the product is biofabricated, the properties of the "leather" can be modified to be less flexible or more breathable. At the end of the process, Modern Meadow's "leather" can be used just like leather but without the environmental impact.

To read about Modern Meadow's biofabricated leather, check out The Future of Leather is Growing in a New Jersey Lab.

Introduction

The ability to cultivate and manipulate microbes, like bacteria and yeast, has wide-ranging implications across various industries, including pharmaceuticals, food, agriculture, and biofuels. This chapter explores the diverse applications of microbial biotechnology and highlights some of its key uses.

Learning Objectives

Microbial biotechnology can be applied to industry in diverse ways. At the end of this section, you will be able to:

- Define biofuel and list the common types
- Describe the four generations of biofuels being made today
- Explain the impact of microbial biotechnology on the food industry and the environment

Production of Biofuels by Microbes

As the global population increases, so does the importance of identifying renewable energy sources. Microbes can potentially provide a solution by producing biofuels. A **biofuel** is a type of fuel derived from *biological materials*. The two most common types are **bioethanol** and **biodiesel.** Bioethanol is ethanol produced through the fermentation of carbohydrates and it can be blended with gasoline to reduce carbon emissions and enhance fuel efficiency. For example, the bioethanol, E10, is 10% ethanol, 90% gasoline. Biodiesel is an alternative to petroleum-based diesel and is produced through a chemical process called **transesterification**, where fats or oils react with an alcohol (usually methanol or ethanol) in the presence of a catalyst (such as sodium hydroxide or potassium hydroxide). Both of these biofuels are considered to be renewable energy because they can be replenished. Many microbial species, such as microalgae (i.e. microscopic algae cells), yeast, and bacteria can convert organic matter (i.e. **biomass**) into bioethanol and biodiesel through natural metabolic pathways, resulting in environmentally friendly, sustainable, and cleaner alternatives to fossil fuels. The biomass used by these organisms include plant (e.g. wood, crops, and agricultural residues), animal (manure, fats, and livestock waste), microbial (e.g. bacteria, fungi, or their bioproducts), and wastes (e.g. food scraps, municipal solid waste, and industrial byproducts).

Biofuels can be classified into generations:

1. **First generation (food-based)** - made from food crops
    - source: edible crops (corn, sugarcane, soybean, and vegetable oils)
    - produce: bioethanol (from corn and sugarcane fermentation) and biodiesel (from vegetable oils and other fats)
    - example: the yeast *Saccharomyces cerevisiae* production of bioethanol
    - limitations: competes with the food industry
2. **Second generation (non-food biomass**) - made from non-food biomass
    - source: lignocellulosic biomass (i.e. wood, agricultural residues, grasses)
    - produce: bioethanol and synthetic biofuels

- example: the yeast *Saccharomyces cerevisiae* production of bioethanol
- advantages: no competition with food industry
- limitations: requires complex processing to break down cellulose and lignin

3. **Third generation (algae and microbial-based)** - made using microbes like algae and bacteria
   - source: algae and genetically-engineering microbes
   - produce: lipids, hydrocarbons, bioethanol, algal biodiesel
   - example: transesterification of lipids- and fermentation by microalgae
   - advantages: fast growth rate, ability to grow on water sources unsuitable for irrigation, and efficient use of carbon dioxide
   - limitations: high production costs and difficulty scaling up
4. **Fourth generation (advanced)** - made using synthetic biology and carbon capture
   - source: genetically-modified organisms in combination with carbon capture technologies
   - produce: carbon-neutral or carbon-negative biofuels; electrofuels (produced from CO2), solar biofuels (convert sunlight into fuel)
   - advantages: highly efficient, potentially carbon-negative
   - limitations: still in the development phase

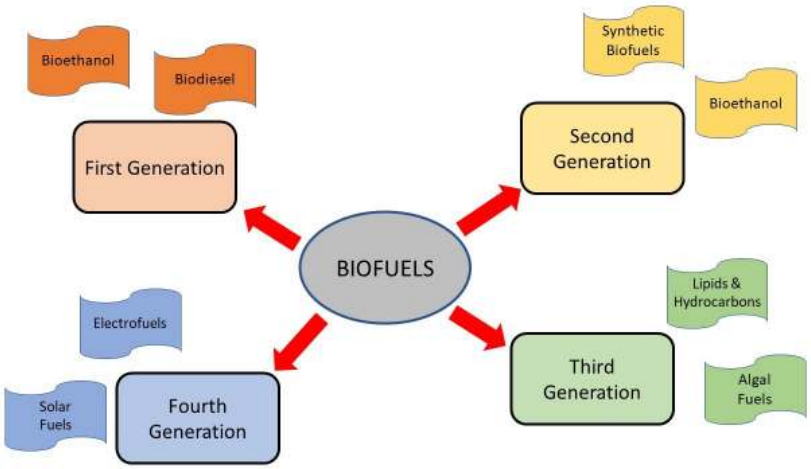


Figure 7.4.1: Biofuel generations. The development of biofuels has undergone several generations. First generation biofuels include bioethanol and biodiesel. Second generation biofuels also include bioethanol, but more advanced biofuels like synthetic biofuels. Third generation biofuels are biological in nature and include algal fuels and lipids and hydrocarbons. The fourth generation are the more advanced and include electrofuels and solar fuels. (Biofuel Generations by Patricia Zuk, CC BY 4.0)

Biotechnology can improve biofuel production by optimizing crop yields, engineering algae for higher lipid content, and developing enzymes that accelerate biomass breakdown. The use of microbes in biofuel production not only reduces dependence on non-renewable energy sources but also helps minimize carbon emissions, making it a key player in the transition toward cleaner energy solutions. However, while promising, many of these types of biofuels still need to be improved to meet the needs of the consumer.

Food Industry

The brewing and food industries rely heavily on fermentation to produce alcohol, bread, yogurt, cheese and various fermented foods. Bacteria like Lactobacillus and Streptococcus are used in the production of yogurt, cheese, pickles, sauerkraut, and kimchi. Yeast (e.g., Saccharomyces cerevisiae) is essential to bread making, beer, and wine production. Finally, molds like Penicillium and Aspergillus and used in blue cheese, soy sauce, and miso. Certain microbes, like Lactobacillus and Acetobacter produce acids, alcohols, and bacteriocins that inhibit spoilage and pathogenic bacteria, helping preserve food. As a result, the food industry has been greatly impacted by advancements in microbial biotechnology. Microbial engineering is being used in industrial molecule **bioproduction** - the use of biological systems, like those found in microbes, in large-scale production of value-added compounds as an alternative to isolating these compounds from natural sources. For example, genetically-modified bacteria (e.g. *Escherichia coli*, *Corynebacterium glutamicum*) and microalgae species are now being used to produce omega-3s at a commercial scale. Industries are using microbes' biochemical capabilities to sustainably bioconvert industrial by-products into aromas, colorants,

flavoring agents, and food additives to meet consumer demands for "natural" products. Addressing food poisoning, particularly from *Staphylococcus aureus*, is also a major focus in the food industry. Recent research explores the molecular characteristics, genotypes, resistance profiles, and enterotoxigenic status of *Staphylococcus aureus* in order understand its prevalence and transmission in food-borne infections.

Environmental Applications

Microbial biotechnology holds immense potential for fostering sustainable environmental and agricultural practices. Microbes are utilized in pollution control and toxic waste treatment, with bioengineered microbes facilitating the biodegradation of waste and oil spills. Genetically-modified microbes can degrade toxic compounds and convert hazardous substances into non-toxic forms. The discovery of new enzymes and molecules can further enhance these processes. In wastewater treatment, biotechnology improves efficiency by increasing plant capacity, replacing synthetic chemical additives, and removing harmful materials. Microalgae, for instance, absorb excess nutrients and organic pollutants from wastewater, improving water quality. The resulting biomass can be re-purposed for biofuel production, biofertilizers, and animal feed. In mining, advances in biotechnology can enhance the extraction of metals from ores by microbes like *Thiobacillus ferrooxidans* by increasing their tolerance to acidic and saline conditions and improving resistance to metals. In sustainable agriculture, microbial biotechnology offers innovative solutions such as biofertilizers that supplement chemical fertilizers, enhance crop yields, and improve soil fertility. Biopesticides present an environmentally friendly alternative to chemical pesticides, posing minimal risks to human health and ecosystems. Additionally, bioherbicides target the defense mechanisms of weeds, reducing farming costs while minimizing environmental impact. Fungal and virus-based insecticides further contribute to sustainable pest management. Despite ongoing challenges in research, development, regulation, and public acceptance, the benefits of microbial biotechnology warrant continued investment and exploration. Future research should focus on understanding microbial communities, optimizing biotechnological applications, and overcoming practical and regulatory hurdles to facilitate widespread implementation.

Key Concepts

Applications of microbial biotechnology has wide ranging impacts on many industries, including agriculture, wastewater treatment, the food industry, and the energy sector.

Some important concepts to remember are:

- Microbes are used in a wide variety of industries, including the generation of biofuels, the creation of food products, the treatment of wastewater, and other industrial applications
- A biofuel is a type of fuel derived from biological materials
- Biofuels, like bioethanol and biodiesel, are renewable because they can be replenished
- There are four generations of biofuels being made today, each with unique sources, advantages, and limitations
- Many types of biofuels still need to be improved to meet the needs of the consumer

Glossary

Biodiesel - a renewable and biodegradable fuel made from the transesterification of vegetable oils, animal fats, and microbial lipids

Bioethanol - ethanol produced through the fermentation of carbohydrates

Biofertilizer - a substance containing living microorganisms that, when applied to soil or plants, enhances nutrient availability—especially nitrogen and phosphorus—promoting healthy plant growth

Bioherbicide - a type of herbicide derived from bacteria, fungi, or viruses or their byproducts; help control unwanted weeds in agriculture

Biomass - organic matter derived from living or recently living organisms such as plants, agricultural waste, and microorganisms; used to produce renewable energy

Biopesticide - a type of pesticide derived from natural materials such as animals, plants, bacteria, or certain minerals, used to control pests in an environmentally friendly way

Bioproduct - any commercial or industrial product derived from a biological source

Bioproduction - the use of biological systems in the large-scale production of compounds

Fermentation - a biological process where microbes (like bacteria and yeast) convert sugars into other products like alcohol, gases, or acids

Lignocellulosic - biomass composed of lignin, cellulose, and hemicellulose; one of the most abundant natural resources; used in biofuel production

Microalgae - microscopic, photosynthetic organisms found in freshwater and marine environments

Transesterification - a chemical process in which fats or oils react with an alcohol in the presence of a catalyst to produce biodiesel

---

7.4: Industrial Applications of Microbial Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

7.5: Medical Applications of Microbial Biotechnology

Biotech Focus

In 1980, the World Health Organization (WHO) declared the disease **smallpox** officially eradicated. Smallpox is a highly contagious, deadly disease caused by the **variola virus**. Infected individuals become covered with pus-filled lesions, with the majority found around the head (Figure 7.5.1). Smallpox has no known cure, killing 1 in 3 infected people. Those surviving are left with deep scars across their body, with some people blinded by the disease. The first evidence of smallpox were found in Egyptian mummies dating back from 1350 BCE, whose skin was covered with lesions that resembled those of the smallpox rash. To combat this disease, early physicians in Asia and Africa used the practice of **variolation**, which consisted of transferring pus collected from smallpox sores to healthy individuals to induce a milder illness. Although variolation was mostly successful, it presented many risks, such as introducing an opportunistic infection. In the 18th century, **Edward Jenner** created the first smallpox vaccine using the cowpox virus, a closely relative to smallpox. Jenner collected pus from a cowpox blister from the hand of an infected milkmaid and use it to inoculate a cut in a boy's arm. When exposed to smallpox, the boy did not develop the disease. Subsequent advances in research and production has resulted in a safer and more stable vaccine. The current smallpox vaccine is made from a "live" virus related to smallpox. It does not contain the virus and cannot cause smallpox but provides immunity.

Figure 7.5.1: Left image: Smallpox lesions. (Obsolescent variolous lesions by Wellcome Collection Gallery, CC BY-SA 4.0) Right image: An unvaccinated boy surrounded by his two siblings who were vaccinated for smallpox. Note the absence of lesions in the unvaccinated siblings. (Smallpox in siblings by National Museum of Health and Medicine, CC BY 2.0).

Introduction

For centuries, natural products have been the cornerstones of traditional medicine. Today, microbial biotechnology is revolutionizing medicine by changing how we approach drug discovery and treatment, and offering new solutions to combat diseases, like infections and cancer. Microbes, like bacteria and fungi, are proving to be invaluable in the pharmaceutical industry. Bacteria and fungi are being used as a rich source of **bioactive compounds** with therapeutic potential. Bioactive compounds are naturally occurring chemicals found in plants, animals, microbes, or other biological sources, that have an effect on a living organism. These compounds include antibiotics, anti-fungals, and anti-cancer agents. Genetic engineering of these microbes, together with **synthetic biology** are being used to enhance microbial production of these compounds, discover new drug leads, and develop innovative therapies such as vaccines and targeted drug delivery systems.

Learning Objectives

Microbial biotechnology is a key part of the pharmaceutical industry, revolutionizing the development of drugs and the treatment of diseases. At the end of this page, you will be able to:

- Define key microbial biotechnology terms in relations to medicine
- Describe the three basic types of antibiotics
- Describe how microbial biotechnology is used in the production of antibiotics
- Describe the major types of vaccines that use microbial technology in their production

Microbes as drug factories

Antibiotic Production

An **antibiotic** is a type of antimicrobial agent specifically designed to fight bacterial infections by either killing the bacteria (i.e., bactericidal antibiotics) or by inhibiting their growth (i.e., bacteriostatic antibiotics). Antibiotics are considered to be **secondary metabolites** - compounds not essential for the growth of the organism. Many antibiotics, such as penicillin from *Penicillium* fungi or streptomycin from *Streptomyces* bacteria, are naturally synthesized by microbes to inhibit the growth of bacteria. These antibiotics are called **natural antibiotics.** In addition, there are two other types of antibiotics: semisynthetic, and synthetic (Figure 7.5.2). **Semi-synthetic antibiotics** are modified versions of natural antibiotics, with chemical alterations to enhance their effectiveness, stability, or resistance to bacterial degradation. One well-known example is amoxicillin, an antibiotic derived from penicillin G, and enzymatically modified to remove a side chain from the natural version. **Synthetic antibiotics**, on the other hand, are artificially designed and chemically synthesized compounds that are designed to target critical bacterial processes. Synthetic antibiotics are man-made and are often based on knowledge obtained from natural antibiotics. Synthetic antibiotics include novel compounds, such as the fluoroquinolones, which are engineered to inhibit bacterial DNA replication.

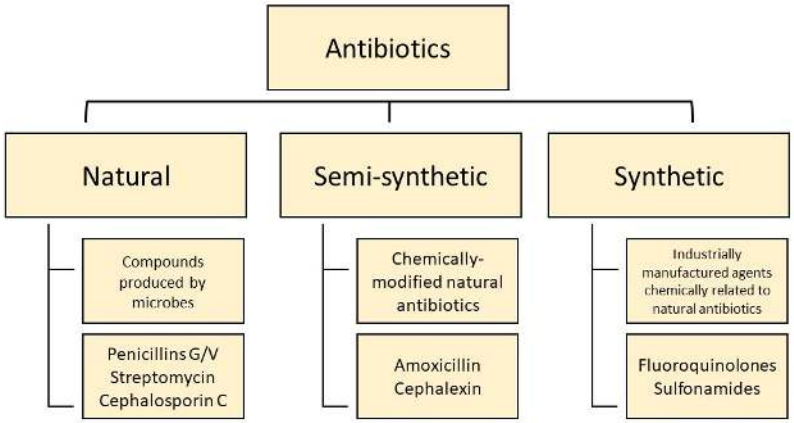


Figure 7.5.2: Types of antibiotics. Antibiotics are classified as natural, semi-synthetic, and synthetic. Natural antibiotics are compounds produced by microbes and include agents such as penicillins G/V, streptomycin, and cephalosporin C. Semi-synthetic antibiotics are chemically-modified natural antibiotics and include compounds like amoxicillin and cephalexin. Synthetic antibiotics are industrially manufactured agents chemically related to natural antibiotics. Synthetic antibiotics include the fluoroquinolone and sulfonamide families of agents. (Types of Antibiotics by Patricia Zuk, CC BY 4.0)

The production of natural and semi-synthetic antibiotics at an industrial scale, together with many synthetic versions, involves a **fermentation step** at some point in their production (Figure 7.5.3). In industrial fermentation, the microbe is grown in large bioreactors (100,000 – 150,000 liters or more), called **fermenters**, containing a liquid growth medium. The size of the microbe population must be controlled very carefully to ensure that maximum yield of the antibiotic is obtained before the cells die. Therefore, oxygen concentration, temperature, pH, and nutrient levels are optimized, closely monitored, and adjusted if necessary. For more information about fermenters, go to Chapter 7.3 Microbial Cultivation and Manipulation. Once the fermentation process is complete, the antibiotic is extracted and purified. For semisynthetic and synthetic antibiotics, continued chemical processing is required.

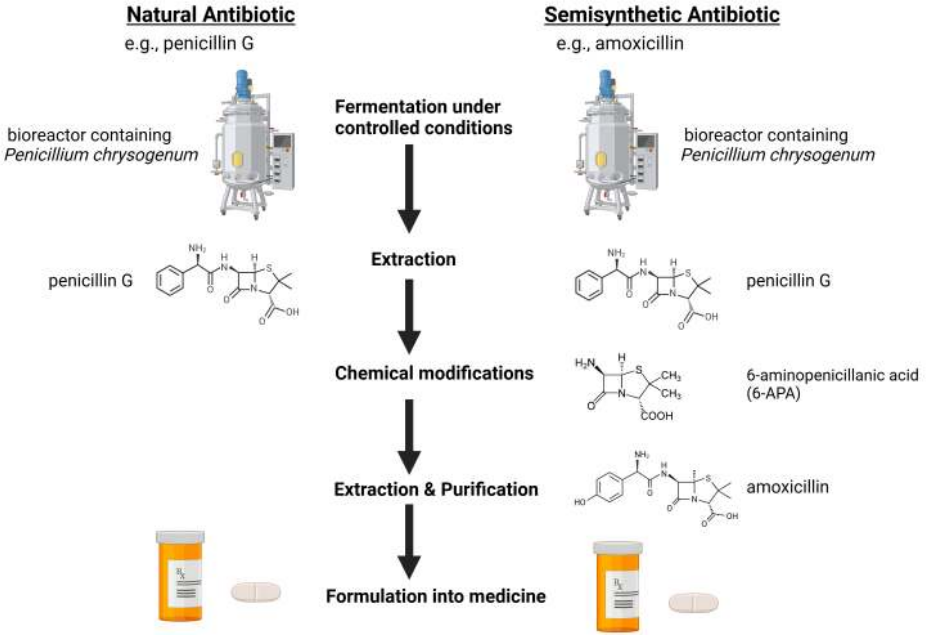


Figure **7.5.2**: Natural and synthetic antibiotic production. The steps involved in the production of the natural antibiotic, penicillin G, and the semisynthetic version, amoxicillin are shown in this figure, together with the chemical structures of the critical compounds. Both antibiotics begin with large-scale fermentation of the fungus, *Penicillium chrysogenum* under controlled conditions in a bioreactor. Penicillin G is extracted from the bioreactor. In amoxicillin production, penicillin is enzymatically converted to 6-APA, which is then chemically modified into amoxicillin. At the end of this process, both penicillin G and amoxicillin are formulated into medicines. (Antibiotic production by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Despite their variety, approximately 99% of antimicrobial agents, such as antibiotics, have little medical or commercial value. Many of these "low-value" antibiotics simply lack a significant advantage over those already in use or have no practical application. This, together with the rise of antibiotic resistance, requires continued exploration and optimization of antibiotic development and production. To promote this, pharmaceutical companies use microbial biotechnology to enhance antibiotic production, develop new antibiotics, or modify existing ones to make them more efficacious. The introduction of mutations (i.e. **mutagenesis**) into the microbial genome to boost antibiotic production is a common microbial biotechnology method. Mutagenesis is produced by exposing microbial cells to **mutagens** such as ultraviolet radiation, x-rays, or certain chemicals. Selection of microbial strains for high-yield antibiotic production is followed by propagation in bioreactors and purification (see Figure **7.5.2**). Mutated strains with antibiotic yields as high as 20-fold or more have been produced using this technique.

Biotechnologists can also boost antibiotic production through **gene amplification,** where copies of genes coding for enzymes involved in antibiotic production can be inserted back into the microbial cell, via vectors such as plasmids. Genetic engineering can create new antibiotics by modifying microbial pathways. Altering microbial DNA can be used to produce entirely new synthetic antibiotics. Genes from different bacteria can be combined to generate novel antibiotic structures. Gene editing through techniques like CRISPR are also being explored.

Vaccine Production

A **vaccine** is a biological preparation that stimulates the host's immune system to recognize and fight a specific pathogen (i.e., a virus, bacterium, or other microbe) without causing the actual disease. Vaccines help the body develop immunity by preventing future infections or reducing their severity. There are a wide variety of vaccines technologies used today, with some technologies produced for a specific pathogen (Table **7.5.1**). The two most common types of vaccines used today are the **live-attenuated vaccine**, which uses a weakened version of a microbe, and the **inactivated (killed) vaccine**, which uses a killed version. These two vaccines are relatively inexpensive to produce and generate a robust immune response.

Table **7.5.1**: Vaccine Types

Vaccine Type	Antigen used	Pathogen	First introduced
Live attenuated (weakened of inactivated)	• uses a weakened (attenuated) form of a virus or bacterium • the pathogen is modified so that it cannot cause disease in healthy individuals but still stimulates a strong and long-lasting immune response.	• measles • mumps • rubella • yellow fever • influenza • varicella (chicken pox) • typhoid	• 1798 (smallpox)
Killed	• uses a killed virus or bacterium	• pertussis • polio • influenza • hepatitis A • rabies	• 1896 (typhoid)
Toxoid	• uses an inactivated toxin produced by bacteria • the toxin is chemically treated to render it harmless but is still capable of stimulating an immune response • the immune response doesn't target the bacteria but creates antibodies that neutralize the toxin during an infection	• tetanus • diptheria	• 1923 (diptheria)
Subunit	• made from a fragment, like sugars and proteins, isolated from a pathogen • the fragments can be produced in large quantities by genetically-engineered bacteria (e.g., *E.coli*) or yeast (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*)	• hepatitis A & B • influenza • pertussis • meningococcal • pneumococcal	• 1970 (anthrax)
Viral vector	• uses harmless viruses or bacteria (i.e., the vector) to deliver genetic material into cells • the bacteria or virus is genetically modified to deliver pathogenic genes into host cell • protein production by host cells generates a strong immune responses • viral vector vaccines currently in use; bacteria	• Ebola	• 2019 (Ebola)

Vaccine Type	Antigen used	Pathogen	First introduced
	vector vaccines are experimental		
Outer membrane vesicle (OMV)	• uses membrane fragments, called vesicles, that are released from the outer membrane of certain gram-negative bacteria • the vesicles contain various components from the bacterium's outer membrane which are crucial for the immune reaction	• Group B meningococcal	• 1987 (meningococcal group B)
Protein-polysaccharide conjugate	• uses sugars from the outer capsule of a bacteria • the sugar is coupled (conjugated) to a viral protein to boost the immune response	• *Hemophilus influenzae* type B • pneumococcal • meningococcal • typhoid	• 1987 (*Hemophilus influenzae* type B)

In addition to these two types of vaccines, there are additional types of vaccines that use microbial biotechnology for their development. These more advanced vaccine technologies are capable of decreasing the cost of vaccine production and increasing production efficiency through their use of microbes to produce the antigens and adjuvants that are key to vaccine production, in addition to using microbes that act as vectors to stimulate the immune response.

Key Concepts

Microbial biotechnology is revolutionizing medicine by changing drug discovery and treatment, and offering new solutions to combat diseases, like infections and cancer.

Some important concepts to remember are:

- a bioactive compound is a naturally occurring chemical found in plants, animals, microbes, or other biological sources, that is used to affect an organism
- an antibiotic is a compound that is used to treat bacterial infections
- a natural antibiotic is naturally synthesized by microbes to inhibit the growth of bacteria
- a semisynthetic antibiotic is produced by chemically modifying a natural antibiotic
- a synthetic antibiotic is a man-made antibiotic
- antibiotic production involves a fermentation step
- mutagenesis and gene amplification in microbes are techniques used to boost antibiotic production
- vaccines stimulate the host's immune response to fight a specific pathogen
- microbial biotechnology is used in the production of several kinds of vaccines, such as subunit vaccines and vector-based vaccines

Glossary

Antibiotic - any substance that can destroy or inhibit the growth of bacteria and similar microbes

Antimicrobial agent - a substance that kills or inhibits the growth of microbes such as bacteria, fungi, viruses, and parasites

Bioactive molecule - a substance that interacts with living cells or tissues to influence their behavior, such as promoting growth, differentiation, or healing; examples include growth factors, cytokines, or peptides; also called a bioactive compound

Fermentation - any of many anaerobic biochemical reactions in which an enzyme (or several enzymes produced by a microorganism) catalyses the conversion of one substance into another; especially the conversion (using yeast) of sugars to alcohol or acetic acid with the evolution of carbon dioxide

Gene amplification - results from the insertion of multiple copies of a gene into the genome of an organism

Metabolite - any substance produced by, or taking part in, a metabolic reaction

Mutagen - any physical or chemical agent that causes changes or mutations in the DNA sequence; includes UV radiation and certain chemicals

Mutagenesis - the process by which mutations are induced or occur naturally within an organism's DNA

Plasmid - a small, circular piece of double-stranded DNA found outside of the bacterial chromosome; used in DNA cloning procedures; often used as a vector to introduce foreign DNA into cells

Secondary metabolite - a substance produced by an organisms that are not directly involved in growth, development and reproduction (these basic functions are carried out by primary metabolites)

Vaccine - a biological preparation that stimulates the immune system to protect against a specific disease

---

7.5: Medical Applications of Microbial Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

8: Animal Biotechnology

8.1: Applications of Animal Biotechnology
8.2: Genetic Engineering in Animals

8: Animal Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

8.1: Applications of Animal Biotechnology

Biotech Focus

What comes first, the chicken or the egg?

Well, thanks to biotechnology and **precision fermentation,** turns out its neither one of them. Precision fermentation is an emerging food technology that uses microbes, like yeast, to produce specific functional food ingredients, some of which traditionally require farm animals. One target of this technology is the egg. The "white" of a chicken egg is 88% water, 10% protein (mainly ovalbumin), and trace minerals and carbohydrates. At EVERY Company, scientists use genetically-modified yeast cells to produce the ovalbumin found in chicken egg whites. The yeast are genetically-modified with the gene for chicken ovalbumin and grown in large fermenting tanks. Today, the company uses this technology to produce egg white proteins without the hen. What results is called the EVERY Egg™ - the world's first "henless" egg. So similar is the EVERY Egg™ to the real thing, EVERY Egg™ debuted in 2023 at Eleven Madison Park in New York City, a three-star Michelin restaurant considered among the world's best of the best.

To learn more about precision fermentation, check out the article The Eggs of the Future will be from Precision Fermentation.

Introduction

Animal biotechnology is a branch of biotechnology in which recombinant DNA techniques are used to genetically engineer (i.e. modify the genome of) animals in order to improve their suitability for use in the livestock industry, medicine, or pharmaceuticals. The last decade has seen significant advances in animal biotechnology, with researcher making progress in sequencing animal genomes, gene expression, and the proteomic analysis of animal cells. Through their work, animal biotechnology has lead to increased food production from healthier, faster-growing livestock, increased disease resistance as engineered animals are less vulnerable to infections, improved breeding using assisted reproductive technologies, and medical breakthroughs through transgenic animals producing human medicines.

Learning Objectives

Animal biotechnology genetically engineer animals to improve their use in the livestock industry, medicine, and pharmaceuticals. At the end of this section, you will be able to

- Describe how genetic engineering (gene addition, gene silencing) is used to improve the health of animals and their productivity
- Describe how biopharming can be used to produce compounds beneficial to humans
- Describe how liposomes and nanoparticles are an alternative to viral vectors in gene delivery
- Describe how CRISPR works in gene editing
- Describe the types of cell cloning

Techniques & Applications of Animal Biotechnology

Animal biotechnology uses specific biological techniques and tools. Some of these techniques include **genetic engineering, gene editing,** and **cell cloning**. Their applications in animal biotechnology can be wide and varied.

Genetic Engineering

Genetic engineering uses recombinant DNA techniques to introduce, remove, or modify genes within an organism's genome. Several terms are used to describe genetically engineered animals, including genetically-modified, genetically-altered, genetically-manipulated, and transgenic. For an introduction to genetic engineering and the tools used for this field, see Chapter 4: Genetic Engineering & Recombinant DNA Technology.

The two principle methods in genetic engineering are gene introduction/addition and gene silencing. The goal of **gene addition** is to introduce a functional copy of a desired gene into the genome of an organism. In humans, this gene is usually added in order to replace a defective or missing one. However in animals, gene addition is most often used to introduce a new trait to an animal or

improve upon an existing one. The new or modified trait(s) can improve the health of a species, enhance its nutritional profile or its productivity, or enable the use of animal products for use by humans. Examples include the addition of genes that make pigs resistant to Porcine Reproductive and Respiratory Syndrome (PRRS), or genes to cows to protect them from Bovine Spongiform Encephalopathy (BSE) (i.e., mad cow disease). Increasing disease resistance using gene addition in endangered animals is seen as a promising way of improving the health and survival of these animals. Gene addition has also been suggested as a means of re-introducing extinct animals, such as the woolly mammoth, by inserting mammoth genes into the elephant genome. Commercially, the addition of the Pacific Chinook salmon growth hormone gene, in combination with a promoter from ocean pout (an eel-like fish) into Atlantic salmon has resulted in the production of AquAdvantage® salmon, a genetically engineered salmon with a two-fold increase in growth rate when compared to their unmodified counterparts. Regulatory genes have also been added to the genomes of cattle, pigs, and sheep in order to increase their muscle mass, thus decreasing the time it take for these animals to come to market. Outside of its commercial applications, gene addition has be used to insert genes for the production of therapeutic compounds by a wide variety of animals for their use in medicine. For more information, go to Chapter 8.2 Genetic Engineering in Animals.

Viral vectors, such as adeno-associated virus (AAV) or lentivirus, can be modified to introduce genes into animal and human cells without causing disease. Viral vectors have the advantage of being able to deliver the functional gene to a significant number of cells. However, the potential for a deleterious immune reaction can be high with the use of viral vectors. In humans, modified viral vectors are currently being using in the treatment of sickle cell anemia, blindness and spinal muscular atrophy (SMA). While the results are promising, the cost of viral-based gene therapy is very high. As an alternative, genes can be introduced using synthetic delivery methods like **liposomes and nanoparticles**. Liposomes and nanoparticles can be used to deliver genes to cells owing to their ability to encapsulate DNA or RNA and protect them from degradation in the bloodstream. The attachment of specific ligands to nanoparticles so that the delivery of genes can be targeted to a specific cell type has shown promise in enhancing treatment efficacy and minimizing side-effects. Encouraging results have been observed using targeted nanoparticles for the delivery of apoptotic genes to lung cancer cells. However, like viral vectors, the immune system can decrease the effectiveness of nanoparticle delivery. In addition, the percentage of cells receiving these nanoparticles is significantly lower versus viral vectors.

In **gene silencing**, a defective gene is "turned off" inside of a cell. The most common way of doing this is through the use of **RNA interference (RNAi)** technology. RNAi mechanisms within a cell target specific mRNA molecules for degradation or inhibit their expression into proteins. RNAi molecules, including **small interfering RNA (siRNA)** and **microRNA (miRNA)** can be delivered and introduced into cells using nanoparticles, antibodies or modified viruses. RNAi molecules can also be attached to cell surface molecules for internalization. The first commercial RNAi-based therapeutic, approved by the FDA in 2018 is sold as ONPATTRO® for the treatment of hereditary amyloidogenic transthyretin (hATTR) amyloidosis. Today, the gene therapy using RNAi is being explored in the treatment of Huntington's disease and certain cancers. For more about gene silencing and RNAi, read Chapter 5.5: Functional Genomics.

Gene Editing using CRISPR

Gene editing allows for the precise and direct modification of a genome by adding, removing, or altering DNA at a specific location. Like genetic engineering, gene editing modifies an organism's genome to change a specific trait or characteristic. These modifications can include gene addition or gene silencing. However, gene editing typically does not introduce foreign DNA into a host's genome but uses DNA from the same species. One powerful method for gene editing is the technique known as **CRISPR**, or Clustered Regularly Interspaced Short Palindromic Repeats. CRISPR is derived from a bacterial defense mechanism that protects bacteria from the integration of viral DNA (from bacteriophage) into the bacterial genome. For this mechanism, bacteria evolved a location in the genome called a **CRISPR locus.** The CRISPR locus is made of short regions of repetitive bacterial DNA known as **direct repeats** (Figure 8.1.1). In between these repeats, the bacteria inserts short sequences of bacteriophage DNA (approximately 20-50 nucleotides each) known as **"spacers"**. These spacers serve as a "memory" of previous bacteriophage infections and act as future defense against another invasion by the same bacteriophage. Transcription of the CRISPR locus followed by RNA processing produces several pieces of CRISPR RNA or **crRNA**. Each crRNA will contain one of the bacteriophage spacers. The bacteria also produces an additional piece of RNA called **tracrRNA**, which becomes associated with a type of bacterial nuclease called a **CRIPSR-associated nuclease (Cas)**. Bacteria express several Cas isoforms. However, the most commonly studied one is **Cas9.** Upon infection of the bacteria with a bacteriophage, the tracrRNA matches up the crRNA with its corresponding piece of bacteriophage DNA. When this match occurs the Cas9 enzyme cuts the bacteriophage DNA, rendering it inactive.

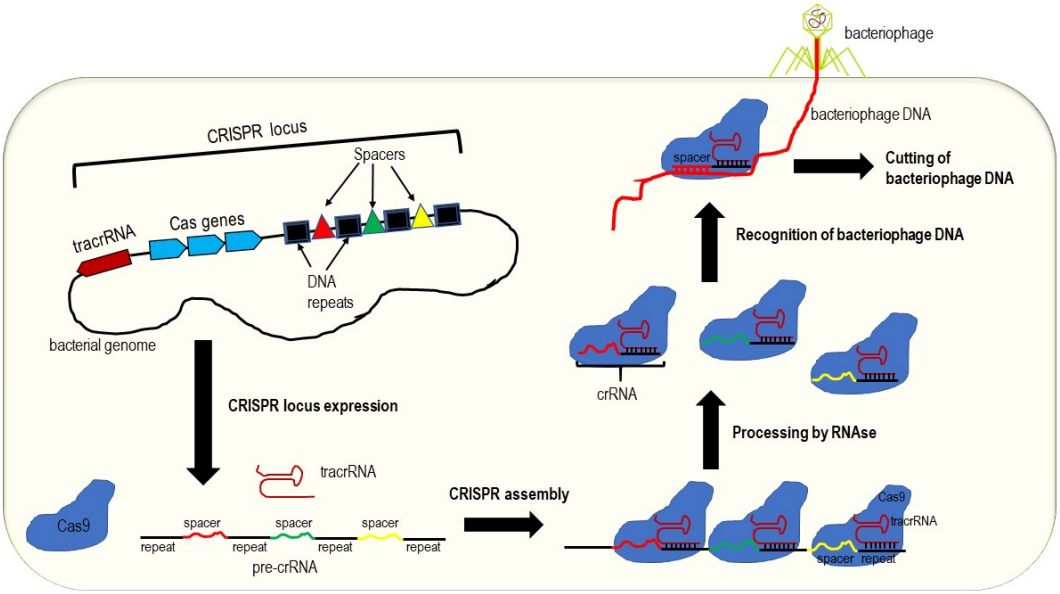


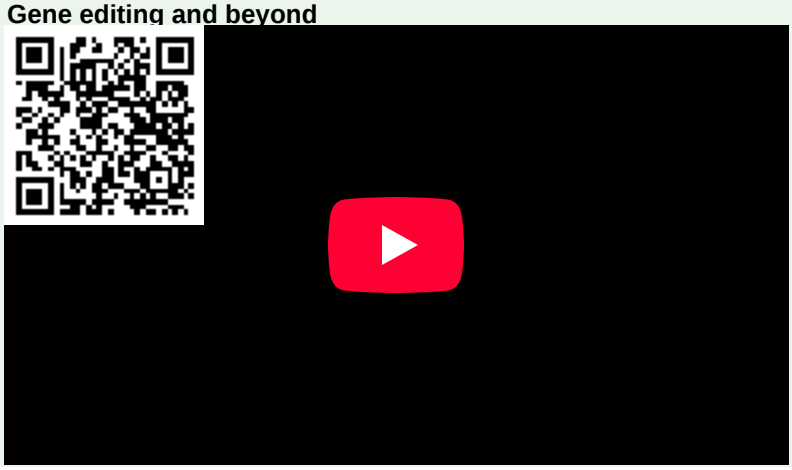
Figure 8.1.1: The CRISPR system of bacterial immunity. Adaptive immunity against bacteriophage infection is achieved in bacteria through the CRISPR system. The CRIPSR locus is comprised of several palindromic repeats of DNA (DNA repeats, black squares) found in between regions of integrated bacteriophage DNA known as spacers (red, green and yellow triangles). The CRISPR locus also contains genes for several isoforms of the Cas enzyme (Cas gene, blue arrows), the gene for tracrRNA (red arrow), and a leader sequence (not shown) that plays an essential role in transcription of the spacers and DNA repeats. CRISPR locus gene expression produces several Cas isoforms, of which Cas9 plays an essential role in immunity. Transcription from the locus also produces tracrRNA (red line), and a piece of "pre-crRNA" comprised of the spacers and DNA repeats. CRISPR assembly results in the hybridization of the tracrRNA with its complementary sequence found in the pre-cRNA and the binding of the Cas9 to the pre-cRNA as well. Processing by an RNAse, cuts the pre-cRNA into several pieces of crRNA (i.e., a spacer and a DNA repeat), each associated with a piece of tracrRNA, and a Cas9 protein. Upon infection by a bacteriophage, the spacer in the crRNA binds to its complementary region in the bacteriophage DNA. The Cas9 enzyme cuts the bacteriophage DNA at that region, rendering it inactive. (CRISPR by Patricia Zuk, CC BY 4.0)

Since its discovery, the CRISPR system has been modified to edit the genome of mammalian cells in the laboratory. For this, researchers combined the tracrRNA and crRNA into a **single guide RNA** (**sgRNA**) that retains the functions of both its crRNA and tracrRNA components. This sgRNA can be designed in the lab to match up with a specific sequence within the mammalian cell's genome where DNA changes are desired. The Cas enzyme is provided to the cell via a DNA plasmid for gene expression or directly as purified protein. The sgRNA directs the Cas9 enzyme to the targeted DNA sequence where it will unwind a short stretch of host DNA. The RNA in the crRNA component can then bind to its complementary DNA sequence. The Cas9 enzyme cuts both strands of the host's DNA helix at that site. Sensing damage to its genome, the cell will repair the cut. However, because mammalian DNA repair is error-prone, the cell will often introduce mutations to the repaired site. If this area of the genome is a gene, the gene with the error will likely be inactivated. As such, CRISPR is often used to "knock-out" gene function.

CRISPR can also be used to excise a mutated section of a gene and replace it with the correct sequence. This can be done if an exogenous piece of DNA is provided to the cell during the repair process. If an entire gene sequence is provided to the cell during repair, the cell can insert this gene. In more advanced applications, Cas9 can be fused to other enzymes for delivery to a DNA sequence. Once there, these enzymes can modify the DNA sequence directly, producing mutations like stop codons to inhibit gene expression or changing the sequence of a mutated gene back to the correct sequence. While a powerful piece of technology, the efficiency of CRISPR gene editing is not 100% so correctly edited cells must be identified. Additionally, scientists are continuing to study the incidence of non-intended edits to the genome when using CRISPR-based systems.

Concept in Action

Animation: CRISPR - Gene editing and beyond



Cloning and Somatic Cell Nuclear Transfer (SCNT)

Somatic Cell Nuclear Transfer (SCNT), otherwise known as "cell cloning", transfers a diploid (2n) nucleus from a donor cell into a recipient egg in which the nucleus has been removed (i.e., enucleated)(Figure **8.1.2**). The genetically-modified egg is programmed by the egg's cytoplasm to begin its development into an embryo that is genetically identical to the donor cell. As such, this early stage embryo is often referred to as a **clone**. In **reproductive cloning**, the clone is implanted, through embryonic transfer, into a surrogate mother for continued development and delivery. Reproductive cloning has been employed to produce genetically identical copies of animals that have exceptional traits, such as enhanced milk production. Despite a high failure rate, numerous animals have been cloned and delivered using this method, including Dolly the sheep in 1996. Reproductive cloning holds promise for the conservation of extremely endangered animals and researchers have also proposed that Reproductive cloning could be used to "resurrect" extinct animals using frozen cells and suitable surrogate mothers (e.g. the woolly mammoth). In **therapeutic cloning**, the clone is cultured and allowed to develop to the blastomere stage (or blastocyst stage for human embryos). Embryonic stem cell (ES) cells are retrieved from this stage. The ES cells are used in regenerative medicine for the development of new tissues and organs.

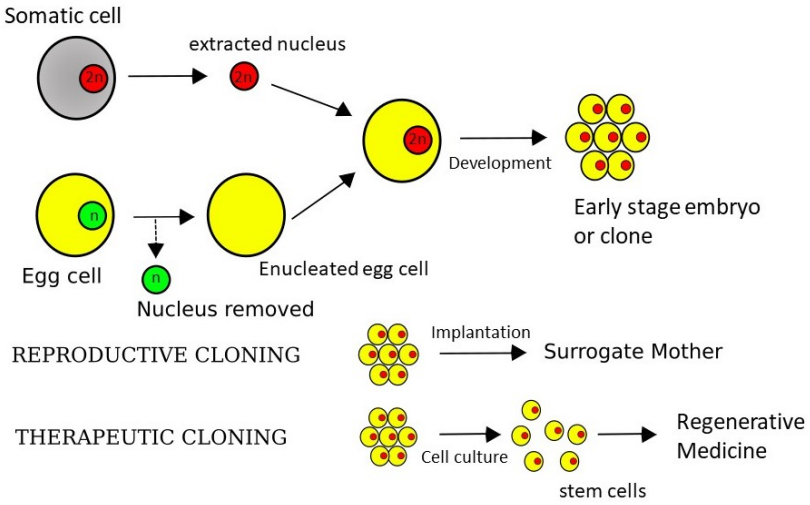


Figure **8.1.2**: Somatic Cell Nuclear Transfer (SCNT). The diploid nucleus from a somatic cell with the desired genes is removed and inserted into a recipient egg cell in which the haploid nucleus has been removed. The genetically modified egg cell is then allowed to undergo development to form an early stage embryo that is known as a clone. In reproductive cloning, the clone is implanted into a surrogate mother for continued development and delivery. In therapeutic cloning, the clone is placed into cell culture in order to produce stem cells that can be used in downstream applications, such as regenerative medicine. (SCNT by Patricia Zuk, CC BY 4.0; adapted from Somatic Cell Nuclear Transfer by Wikibob, CC BY-SA 3.0)

Concept in Action

Video: Microinjection of Nuclei for SCNT

Both reproductive and therapeutic cloning have raised concerns about cloning humans and the destruction of human embryos for the isolation of human ES cells. Today, most countries have stringent legal and ethical rules surrounding SCNT and cloning.

Key Concepts

The techniques used in animal biotechnology are wide and varied and include advanced techniques like CRISPR for gene editing and SCNT for cell cloning.

Some important concepts to remember are:

- genetic engineering and gene editing use recombinant DNA techniques to introduce, remove, or modify genes within an organism's genome
- gene editing allows for the precise and direct modification of a genome by adding, removing, or altering DNA at a specific location
- foreign DNA is usually added through genetic engineering; non-foreign DNA is added through gene editing
- genetically engineered organisms are referred to a transgenic organisms
- gene addition introduces a functional copy of a desired gene into the genome of an organism
- gene silencing inhibits the expression of a defective gene inside a cell

Glossary

Animal biotechnology – the application of genetic engineering, cloning, and molecular biology to modify animals for agriculture, medicine, and industry

Biopharming – the use of genetically-engineered plants or animals to produce valuable medical or therapeutic compounds such as drugs, vaccines and antibodies; also called pharmaceutical farming or "pharming"

Cloning – the process of creating a genetically identical copy of an organism, often using somatic cell nuclear transfer (SCNT)

Cell cloning - also known as somatic cell nuclear transfer (SCNT)

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) - a gene-editing tool that uses a guide RNA and a Cas9 enzyme to find and cut specific DNA sequences

Disease-resistant animals – genetically modified or selectively bred animals that are less susceptible to infections

Embryo Transfer (ET) – a reproductive technology where fertilized embryos are transferred into a surrogate mother to improve breeding efficiency

Gene editing – the modification of an cell's DNA to introduce beneficial traits; uses technologies like CRISPR

Genetic engineering - the direct modification of an organism's DNA using biotechnology; often done to introduce new traits, to improve resistance to disease, or to produce beneficial products

Genetically Modified Organism (GMO) - any organism whose DNA has been altered using genetic engineering techniques

Gene silencing - the inhibition of a gene's expression through natural or artificial means; can be achieved in the laboratory using siRNA or miRNA techniques

Genome - the entirety of the genetic information in a cell

Enucleated - a cell in which the nucleus has been removed; used in cell cloning

Liposome - a spherical vesicle made of phospholipids that mimics the structure of a cell membrane; used in transfection of mammalian cells

Recombinant DNA (rDNA) technology – techniques that combine DNA from different sources, used to create genetically modified animals

Reproductive cloning – cloning that uses SCNT technology to create identical animals for research, agriculture, or conservation

RNA interference - a lab technique used to target specific mRNA molecules for degredation; uses wither siRNA or miRNA molecules to affect mRNA stability and expression

Somatic cell nuclear transfer (SCNT) - a process in which a diploid (2n) nucleus from a donor cell is transferred into an enucleated recipient egg

Transgenic animal – an animal that carries genes from another species, typically used for research, medicine, or agriculture

Therapeutic cloning - cloning that uses SCNT technology for the isolation of embryonic stem cells

Xenotransplantation – the process of transplanting cells, tissues, or organs from one species to another, typically from animals to humans

---

8.1: Applications of Animal Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

8.2: Genetic Engineering in Animals

Biotech Focus

In the late 1990s, researchers at the National University of Singapore added the gene encoding the green fluorescent protein from jellyfish to the genome of wild zebrafish (*Danio rerio*). Under natural white and UV light, these fish glowed a bright green. Recognizing a commercial application, Alan Blake and Richard Crockett founded the **GloFish** brand in 2001 to market and sell these genetically modified fish. After two and a half years of research and consultation with experts, GloFish were introduced to the US market in late 2003 to be sold as tropical aquarium fish. Because aquarium fish are not used as a food source, the US Food and Drug Administration (FDA) determined that GloFish did not pose a greater environmental risk than unmodified zebrafish and opted not to regulate the sale of these fish. However, some environmentalists became concerned about the possibility of GloFish escaping into the wild and thriving. In 2014, a single GloFish was spotted in Florida, and in 2015, groups of GloFish were spotted in Brazil. These fish likely escaped from ornamental fish farms in both locations. How their presence is affecting natural ecosystems remains unknown.

Introduction

Genetic engineering in animals involves modifying an animal's DNA in order to introduce desirable traits that improve the animals health, or enhances its productivity. By using tools like CRISPR-Cas9, recombinant DNA technology, and cloning, scientists can insert, delete, or modify specific genes in animals for various applications. Genetic engineering of animals has revolutionized research, agriculture (i.e., livestock management), the treatment of diseases, medicine and the pharmaceutical industry. Animals have been genetically modified for disease resistance, faster growth, and improved nutritional value. Transgenic animals are now used routinely to produce pharmaceutical proteins, and genetically-engineered pigs are being developed for the transplantation of organs into humans (i.e., **xenotransplantation**). Research on engineering animals also helps scientists study genetic diseases and drug development. Despite its benefits, genetic engineering in animals raises ethical concerns, including animal welfare, ecological risks, and regulatory challenges. As this technology advances, careful evaluation will be needed to balance innovation with ethical responsibility.

Learning Objectives

Genetically-modified animals have found use in a wide variety of industries, including agriculture and medicine. Transgenic animals form the core of "biopharming", disease management, and xenotransplantation. At the end of this page, you will be able to:

- Define appropriate terms like GMO and transgenic
- Outline the basic steps in making a GMO
- Describe how genetically-modified animals can be used a bioreactors to produce pharmaceuticals
- Describe how genetically-modified animals are used to manage diseases
- Describe how genetically-modified animals are used in xenotransplantation

Genetically modified organisms (GMOs)

Genetically-modified organisms (GMOs) are organisms whose genetic material has been altered through genetic engineering techniques. This alteration has been performed to express desirable traits or improve resilience of an organism. When the gene being introduced comes from a different organism, the GMO is often referred to as a **transgenic organism**. This means that all transgenic organisms are GMOs, but not all GMOs are transgenic.

The history of GMOs dates back to the early 1980s, when the human insulin gene was introduced into the genome of the bacteria *E.coli*. These genetically-engineered bacteria were quickly approved for medical use, revolutionizing diabetes treatment. In agriculture, the "Flavr Savr" tomato became the first genetically modified crop to be approved for commercial sale in 1994. Engineered to delay ripening, it sparked the rapid development of other GMO crops, such as pest-resistant corn and herbicide-tolerant soybeans. Since then, GMO applications have expanded into numerous fields, including medicine, biofuels, and environmental management, becoming an integral part of biotechnology.

- 1982: FDA approves the first consumer GMO product developed through genetic engineering: human insulin to treat diabetes.
- 1986: The federal government establishes the Coordinated Framework for the Regulation of Biotechnology. This policy describes how the U.S. Food and Drug Administration (FDA), U.S. Environmental Protection Agency (EPA), and U.S. Department of Agriculture (USDA) work together to regulate the safety of GMOs.
- 1990s: The first wave of GMO produce created through genetic engineering becomes available to consumers: summer squash, soybeans, cotton, corn, papayas, tomatoes, potatoes, and canola.
- 2003: The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations develop international guidelines and standards to determine the safety of GMO foods.
- 2005: GMO alfalfa and sugar beets are available for sale in the United States.
- 2015: FDA approves an application for the first genetic modification in an animal for use as food, a genetically engineered salmon.
- 2016: Congress passes a law requiring labeling for some foods produced through genetic engineering and uses the term “bioengineered,” which will start to appear on some foods.

To create a GMO, researchers study and identify a gene responsible for giving an organism a desired trait. This in itself can be difficult because the degree to which a single gene affects a trait depends on the desired trait. Once a gene has been identified, there are several steps that are followed in the laboratory:

- the gene is copied from an organism through polymerase chain reaction (PCR)
- the copied gene is ligated into a cloning plasmid to create a recombinant plasmid
- the recombinant plasmid is introduced into another organism (i.e., the "host" organism)
- the host organism is allowed to develop under laboratory conditions
- the resulting GMO is studied extensively in the laboratory to ensure that it exhibits the desired trait and does not pose a threat to the unmodified organism found in nature.

The "host" organism whose genome will be altered can range from something as simple as unicellular bacteria and yeast, to multicellular, complex organisms such as animals and plants. The way in which the gene is introduced into the host organism will depend on the host organism. For animals, recombinant plasmids are typically introduced into cultured embryonic stem (ES) cells through a process called **transfection**. In transfection, the DNA is incorporated into small particles, called **liposomes**, in order to facilitate the entrance of the DNA into the cell. Once expression of the introduced gene is confirmed, the modified ES cells are then microinjected into an early stage embryo (i.e. the blastocyst) for incorporation into its inner cell mass. Alternatively, recombinant plasmids can be directly microinjected into into a fertilized egg (i.e. the zygote), or the gene can be inserted into the zygote's genome using CRISPR. Following implantation of the genetically altered embryo into a surrogate female, the offspring are assessed for their expression of the new gene (Figure 8.2.1). For more information about transfection, go to Chapter 13.10: Liposomal transfection into mammalian cells.

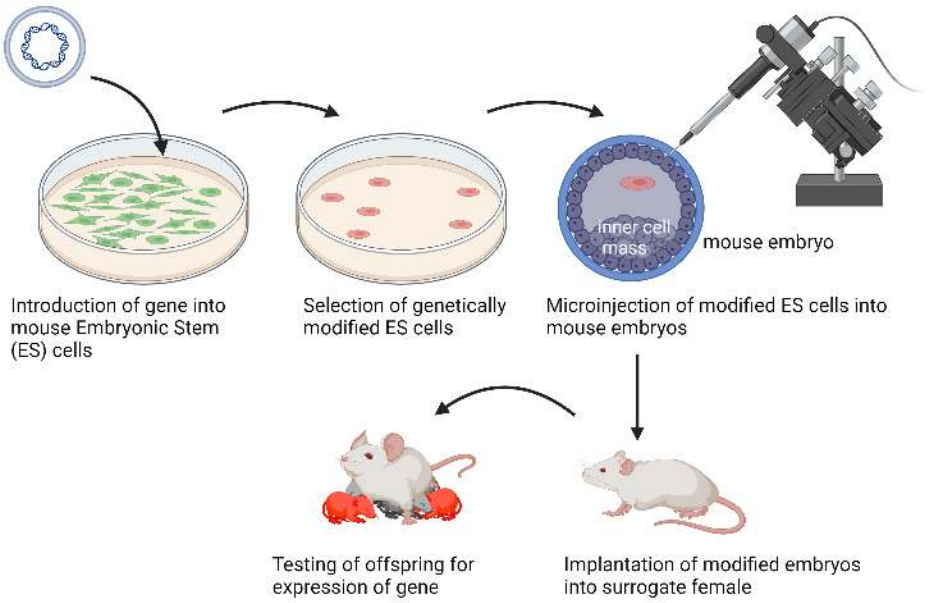


Figure 8.2.1: GMO animal production. One method of producing a genetically-modified organism (GMO) is shown in this figure. The gene is introduced into embryonic stem cells (ES cells) that have been isolated from the host organism, such as a mouse. The selection of genetically modified mouse ES cells are is performed to ensure a purified population. Microinjection of these modified ES cells into a mouse embryo results in the incorporation of the cells into the inner cell mass of the embryo. Incorporation is followed by implantation of the modified embryo into a surrogate female for continued development. Testing of the offspring for expression of the gene (red mice) identifies the GMO. (GMO animal production; Patricia Zuk CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

Genetically-Engineered Animals in Medicine

Animals as Bioreactors

Drug production, regenerative therapies, xenotransplantation, and disease modeling all use genetically-engineered animals. For example, spider silk genes have been inserted into goats, allowing them to produce silk proteins in their milk, which can be used for medical sutures and bulletproof materials. This technology, known as **biopharming** or "pharming", has also been used for the insertion of human proteins into animal genomes, essentially turning these modified animals into "living bioreactors". For example, insertion of the gene encoding anti-thrombin, a natural blood protein that prevents excessive blood-clotting, into the genome of goats has enabled them to produce this therapeutic protein in their milk (Figure 8.2.2). The resulting product, known as ATryn® is currently used to treat conditions like deep vein thrombosis, hereditary anti-thrombin deficiency or for preventing clots during surgery or childbirth.

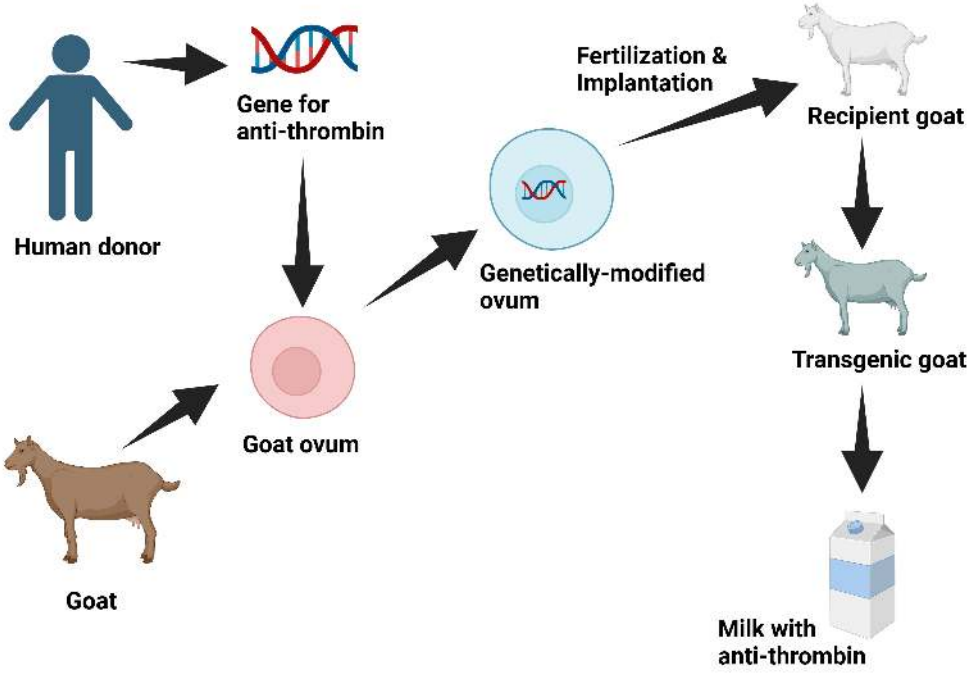


Figure **8.2.2**: Biopharming of human anti-thrombin. The human anti-thrombin gene is isolated from a human donor and a goat is used for the donation of an ovum. The gene is microinjected into a goat ovum (pink cell) to created a genetically-modified ovum (blue cell). The ovum is then fertilized and implanted into a recipient goat (shown in white). The resulting transgenic goat (shown in gray) is bred for milk production. The milk, containing the anti-thrombin, can then be processed and the clotting agent extracted and purified. (Biopharming by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Additional examples of biopharming and the use of animals as bioreactors include:

- Transgenic cows for the production of human insulin and human growth hormone in their milk
- Transgenic cows modified with human immunoglobulin genes and challenged with specific pathogens to produce human antibodies
- Transgenic goats engineered to produce spider silk proteins in their milk for medical sutures and biomaterials
- Transgenic rabbits and the production of the hormone erythropoietin (EPO)
- Transgenic sheep production of alpha-1 anti-trypsin for the treatment of cystic fibrosis and emphysema
- Transgenic sheep and pigs in the production of clotting factor IX

Such applications, while exciting, require the breeding of large amounts of animals and the isolation of specific tissues like blood. As a result, many have called into question the ethics of using transgenic animals.

Disease Management

The use of animals as bioreactors for pharmaceutical production is an established method for the treatment and/or management of a disease. However, there are additional approaches like the production of disease-resistant animals through genetic engineering. For example, the genetic manipulation of cattle is being used to protect herds from deadly infections like foot-and-mouth disease. Genetic engineering of mosquitoes is revolutionizing how the spread of mosquito-borne diseases like malaria, dengue, Zika, and chikungunya is limited in affected populations. Genetically-modified mosquitoes have been created that prevent their reproduction (e.g. Oxitec's Friendly™ Aedes Mosquitoes). The males contain a "self-limiting gene" that, when expressed in their offspring, causes them to die before they reach adulthood. The release of these males into the wild population eventually leads to population collapse. Since their approval and introduction, decreases of over 90% have been measured *Aedes aegypti* mosquito populations in Brazil, Florida, and the Cayman Islands. Disease management through vaccine production has also seen significant advancements using genetic engineering. Chickens can be genetically modified to express a specific pathogenic protein in their eggs. The protein can then be isolated and purified from the egg and used for immunization, offering a faster, more efficient, and cost-effective way to manufacture vaccines for human and animal diseases. Since a single chicken can lay over 300 eggs per year, large amounts of

vaccines can be made for a lower cost versus traditional vaccine culture techniques. These vaccines can also be quickly produced for viruses that show a rapid rate of mutation (e.g. influenza, COVID-19).

Xenotransplantation

Xenotransplantation is the process of transplanting cells, tissues, or organs from one species to another, typically from animals to humans (Figure 8.2.3). This technology is being explored as a solution to the shortage of human organ donors for patients in need of transplants. To increase success rates, animal organs can be genetically-modified through somatic cell nuclear transfer (SCNT) to knock out immunoreactive porcine genes and to express human immune proteins, in the hope of decreasing the risk of immune rejection. Pigs have become the primary choice for xenotransplantation because their organs are similar in size and function to human organs. Prior to use, those pig genes that would illicit an immune reaction are silenced. In addition, the presence of the PERV (Porcine Endogenous Retrovirus) genome within the genome of the pig is removed to prevent infection of human recipients. For this, scientists are exploring the use of CRISPR to silence or edit out these genes. Human genes are then inserted to help prevent immune rejection and blood clotting.

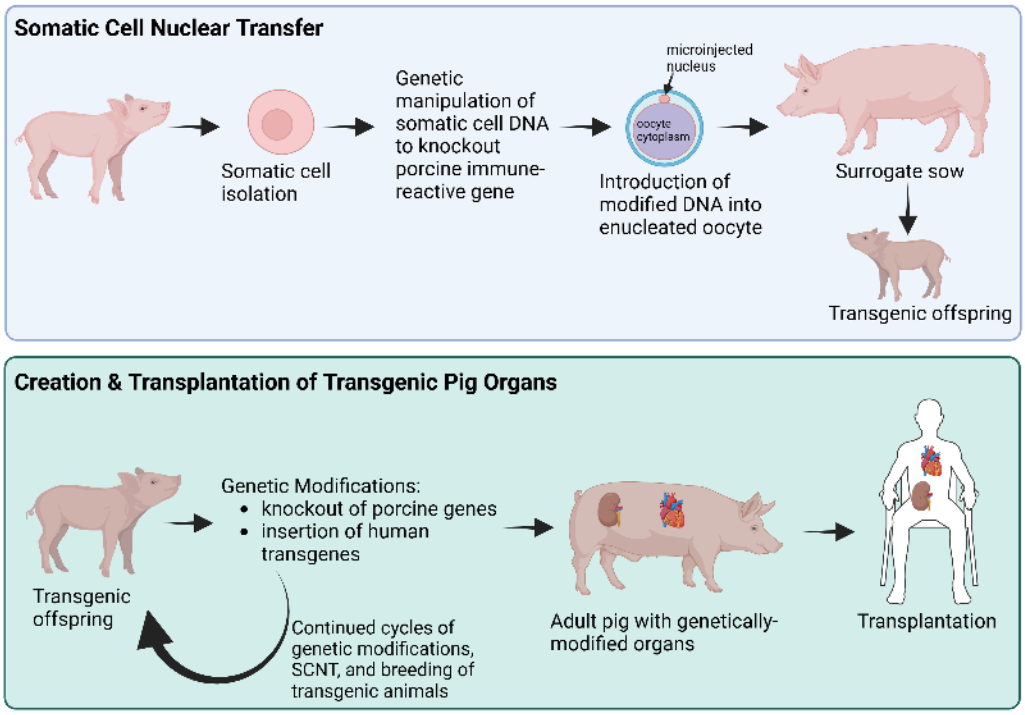


Figure 8.2.3: Xenotransplantation. The modification of pig organs for human transplantation is done in two phases: Somatic Cell Nuclear Transfer (top box in blue) and the creation and transplantation of transgenic pig organs (bottom box in green). In SCNT, a somatic cell (pink) is isolated from a pig. Genetic manipulation of the somatic cell's DNA is performed to knock-out genes that could cause immune rejection in humans. Introduction of the modified DNA into an enucleated oocyte is accomplished by microinjection of the nucleus taken from the modified somatic cell. The oocyte is activated to initiate embryonic development (not shown) and then implanted into a surrogate sow. The resulting transgenic offspring is then used in the creation and transplantation of transgenic pig organs. In this process, the transgenic offspring undergo repeated cycles of additional genetic manipulation (e.g. knock-out of porcine genes, insertion of transgenes), SCNT, and breeding to produce transgenic pigs that express human immune antigens. The adult transgenic pigs are used for the harvesting of genetically-modified organs, such as the heart and kidneys. These organs are used for transplantation into humans. (Xenotransplantation by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

In 2021, the first genetically-modified pig heart was successfully transplanted. In 2024, a modified pig kidney was transplanted and functioned in a brain-dead patient for over a month. In 2024, physicians at Massachusetts General Hospital transplanted a genetically-edited pig kidney into a 62 year old living patient. Genetically-modified pig hearts were first transplanted into living patients in 2022 and 2023. Most recently, in 2025, a genetically-modified pig liver was transplanted into a brain-dead human recipient in order to monitor its function and potential rejection. As the technology improves, pig organs are likely to see increased use in transplant facilities.

While xenotransplantation provides hope for alleviating organ shortages, there are several problems associated with this technology. Even with careful gene editing, rejection of the modified organ is still a risk. The presence of PERV in the pig genome presents concerns about unanticipated cross-species infections. In response, researchers have begun to explore the use of modified primate organs in place of pig organs. However, primate use is likely to increase the opposition people feel about using animals for human organ transplants.

Molecular Lab Protocols

- Lab Technique: Liposomal transfection into mammalian cells

Key Concepts

Genetically-modified animals form the core of areas like "biopharming", disease management, and xenotransplantation, offering advancements in medicine, agriculture, and research.

Some important concepts to remember are:

- Genetically-modified organisms (GMOs) are organisms whose genetic material has been altered through genetic engineering techniques
- The introduction of foreign genes into an organism produces a GMO known as transgenic
- The method used to introduce genetic information into an organism depends on the organism itself
- In medicine, transgenic animals such as goats, cows, and chickens are modified to produce therapeutic proteins in a process known as biopharming
- Transgenic animals can also be used in the production of vaccines, and in the management of diseases
- Transgenic animals can also be engineered for the production of disease-resistant animals
- Genetically-modified pig organs with reduced potential for immune rejection are being developed for xenotransplantation
- Innovations using transgenic animals raise important ethical considerations

Glossary

Biopharming - the use of genetically engineered plants or animals to produce valuable medical or therapeutic compounds such as drugs, vaccines, and antibodies; also called pharmaceutical farming or "pharming"

Blastocyst - an embryonic stage comprised of a hollow, fluid-filled ball of cells; develops about 5–6 days after fertilization in humans and consists of a thin outer layer of cells and an inner cell mass made of embryonic stem cells (ES cells)

ES cells (Embryonic Stem Cells) - totipotent stem cells derived from the inner cell mass of a blastocyst, an early-stage embryo

GMO (Genetically-Modified Organism) - an organism whose genetic material has been altered through genetic engineering techniques

Inner cell mass - a group of cells found within the blastocyst stage of embryonic development; a source of ES cells

Transfection - the process of introducing foreign DNA (or RNA) into eukaryotic cells using chemicals, like liposomes or calcium phosphate

Transgenic organism - an alternate term used for a GMO; often used to refer to genetically-modified animals

Xenotransplantation - the process of transplanting cells, tissues, or organs from one species to another, typically from animals to humans

8.2: Genetic Engineering in Animals is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

9: Agricultural and Environmental Biotechnology – “Green” and "Gray" Biotechnology

9.1: "Green" vs. "Gray" Biotechnology

9.2: Applications of "Green" Biotechnology

9.3: Applications of "Gray" Biotechnology

9.4: Benefits of "Green" Biotechnology

9: Agricultural and Environmental Biotechnology – “Green” and "Gray" Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

9.1: "Green" vs. "Gray" Biotechnology

Biotech Focus

Brassica juncea is a type of Indian mustard, a fast-growing plant species in the mustard family (*Brassicaceae*). Indian mustard is native to South Asia (India, China). It grows quickly and easily in a variety of soils, even under poor or stressful conditions. Upon harvesting, it is used as a leafy vegetable and produces mustard seeds for cooking oil and spices. Industrial processes in south Asia has resulted in the deposition of many types of heavy metals, like selenium, into the soil. Using both "green" and "gray" biotechnology, scientists have engineered Indian mustard to absorb more selenium from contaminated soils through a process called **phytoremediation**. In phytoremediation, native plants or plants that have been genetically-engineered can be used to clean up heavy metal contamination from selenium, cadmium, lead, and arsenic. Through phytoremediation, plants can improve the environment (gray biotech) and can be harvested for safe disposal or metal recovery, making the land usable for agriculture again (green biotech).

Introduction

"Green" biotechnology, often called agricultural biotechnology, is the use of science and engineering on agriculture and the environment. As such, it overlaps with **"gray" biotechnology**, which is also known as environmental biotechnology. Gray biotechnology is a branch of biotechnology that focuses on solving environmental problems using living organisms. While these two areas share numerous areas in common, they can have distinct approaches and focuses. Table \(\PageIndex{1}\) compares these two areas of biotechnology.

Table \(\PageIndex{1}\): Green vs. Gray Biotechnology

	Green Biotechnology	Gray Biotechnology
Purpose	Improve farming and crop production	Clean and restore the environment
Main Tools	Genetic engineering of plants, biofertilizers, biopesticides	Bioremediation, engineered microbes and plants
Focus Areas	Agriculture, food safety	Pollution control, waste management

Learning Objectives

"Green" biotechnology uses science and engineering to improve agriculture and the environment. "Gray" biotechnology focuses on the environment. As such, these two biotech areas overlap significantly in their goals and techniques. At the end of this page, you will be able to:

- Define "green" biotechnology
- Define "gray" biotechnology
- Compare these two areas of biotechnology

Green vs. Gray

"Green" biotechnology focuses on agriculture and food production. Its main goal is to improve crops, farming, and the environment using plant-based biotechnology techniques. "Gray" biotechnology uses plants, microbes, or enzymes to clean up pollution and waster. Its main goal is to restore ecosystems, reduce pollution, and make waste management more sustainable and eco-friendly. A quick way to distinguish these two biotech fields is to remember that green biotechnology grows better crops, while gray biotechnology cleans up a polluted world. While they may focus on distinct areas, both fields can use genetically-modified organisms created through biotechnology techniques to minimize environmental harms (Figure \(\PageIndex{1}\)).

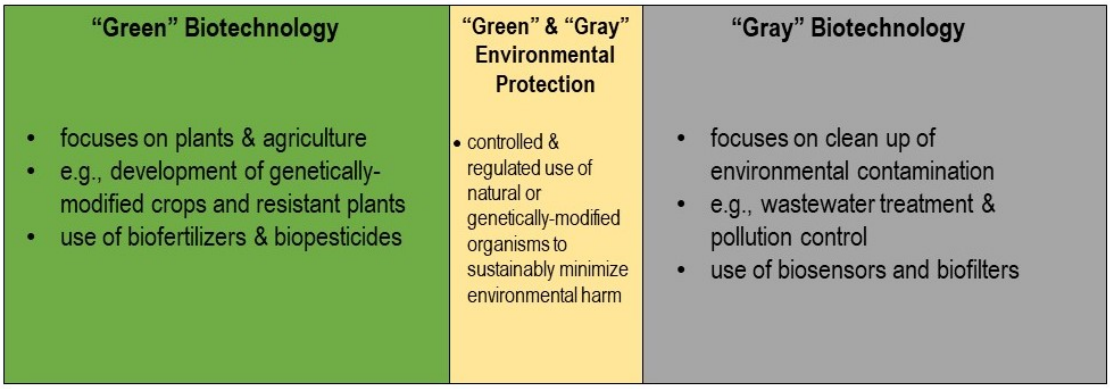


Figure \(\PageIndex{1}\): "Green" vs. "Gray" biotechnology. "Green" biotechnology focuses on plants and agriculture practices and can involve the development of genetically-modified crops and plants resistant to disease and pests. "Green" biotechnology uses biofertilizers and biopesticides to minimize environmental impact. "Gray" biotechnology focuses on the clean-up of environmental contamination. "Gray" biotechnology uses biosensors and biofilters in areas such as wastewater treatment and pollution control. Both biotechnology areas involved the controlled and regulated use of natural (i.e., unmodified) organisms or their genetically-modified counterparts to sustainably minimize harm to the environment. ("Green" vs. "Gray" Biotechnology by Patricia Zuk; CC BY 4.0)

Key Concepts

"Green" and "gray biotechnology, through their focus on the environment, can overlap significantly. Some important concepts to remember are:

- green biotechnology grows better crops and minimizes the impact of agriculture on the environment
- green biotechnology tries to improve farming practices and crop yields
- green biotechnology improves crop yields without damaging the environment through its use of biopesticides and biofertilizers
- gray biotechnology cleans up a polluted world
- gray biotechnology aims to clean and restore ecosystems through bioremediation
- gray biotechnology areas include pollution control, waste management

Glossary

Agriculture - the science and practice of growing crops, raising animals, and producing food, fiber, and other products to support human life

Biofertilizer - a substance containing living microorganisms that, when applied to soil or plants, enhances nutrient availability—especially nitrogen and phosphorus—promoting healthy plant growth

Biopesticide - a type of pesticide derived from natural materials such as animals, plants, bacteria, or certain minerals, used to control pests in an environmentally friendly way

Bioremediation - the process of cleaning up a polluted environment using living organisms, such as microbes or plants, that break down harmful substances into less toxic or harmless forms

Fertilizer - a substance that help plants grow by increasing the availability of nutrients (such as nitrogen or phosphorus)

Food safety - practices and conditions that preserve the quality of food to prevent contamination and food-borne illnesses; ensured that food is safe to eat

Genetic engineering - the direct modification of an organism's DNA using biotechnology; often done to introduce new traits, to improve resistance to disease, or to produce beneficial products

"Green" Biotechnology - a branch of biotechnology focused on developing genetically modified crops, biofertilizers, and biopesticides to promote sustainable farming

"Gray" Biotechnology - a branch of biotechnology that uses using biological systems to protect and restore the environment; includes activities like bioremediation, pollution control, and wastewater treatment

Pesticide - a synthetic or natural substance used to control pests

Pollution control - efforts and technologies aimed at reducing or eliminating the release of harmful pollutants into the environment

Wastewater treatment - the process of removing contaminants from used water (from homes, businesses, or industries) so that it can be safely returned to the environment or reused

---

9.1: "Green" vs. "Gray" Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

9.2: Applications of "Green" Biotechnology

Biotech Focus

Most crops depend on nitrogen fertilizers to grow well. However, producing and applying those fertilizers can be expensive and harmful to the environment. With the advent of the "green" biotechnology field, researchers have become interested on whether crop production can be improved without the use of synthetic fertilizers.

Enter the humble legume.

Legumes, including peas, beans, lentils, and soybeans, can naturally remove nitrogen (N_2) from the atmosphere and convert it into nitrogenous compounds through a process called **nitrogen fixation**. These nitrogenous compounds are used by the legumes to make proteins and nucleotides. This results in enhanced growth even in poor nutrient soils. However, it isn't the legume doing the nitrogen fixation. Rather, the nitrogen fixation is being performed thanks to a symbiotic relationship with certain bacteria. Scientists in the green biotechnology field are now exploring whether this capability can be transferred to other crops like corn, wheat, and rice. One way may be to genetically engineer crops to form similar symbiotic relationships with nitrogen-fixing bacteria. Another way is to engineer the crops themselves to express the necessary bacterial genes from nitrogen fixation.

Whatever method is used, giving plants the ability to fix nitrogen will reduce their reliance on synthetic fertilizers, lower farming costs, and minimize environmental damage. Turning plants into self-feeding powerhouses will certainly have a dramatic impact on the agricultural field.

Introduction

Green biotechnology, often called **agricultural biotechnology**, is the use of science and engineering on agriculture and the environment. It combines biology with technology to solve problems related to food production, sustainability, and environmental challenges. To do this, green biotechnology uses many of the same techniques and tools as other areas of biotechnology. These techniques include genetic engineering, genetically-modified organisms (GMOs), and genome editing (e.g., CRISPR-Cas9). In agriculture, these tools are used to:

- Increase crop yields
- Enhance resistance to pests, diseases, and harsh climates
- Improve nutritional content
- Reduce the need for chemical fertilizers and pesticides

In botany, green biotechnology reduces the need for chemical fertilizers and pesticides, helps crops survive climate stress like drought or extreme temperatures, improves soil and water quality, and increases food security while being more eco-friendly. Some of the key applications of this field of biotechnology include:

- **Genetically Modified (GM) Crops:** Crops engineered to resist pests, tolerate drought, or grow in poor soil
- **Biofertilizers:** Natural microbes used to boost plant growth by fixing nitrogen or breaking down nutrients in the soil
- **Biopesticides:** Natural organisms or compounds that control pests without harmful chemicals

Learning Objectives

"Green" biotechnology improves farming practices to increase crop yields, enhance resistance, improve nutritional content, and reduce the need for chemical treatments. At the end of this page, you will be able to:

- Define green biotechnology
- List some key features of green biotechnology
- Discuss some applications of green biotechnology, such as the development of genetically-modified crops

Applications of Green Biotechnology

Throughout history, farmers have selectively bred plants and animals to achieve desirable traits, such as sweeter apples or larger corn cobs. However, this traditional method can be time-consuming and may inadvertently introduce unwanted characteristics. Biotechnology offers a more precise approach to developing desirable traits. These enhancements can include improved drought resistance, increased tolerance to pests and diseases, enhanced nutritional content in food, or the production of pharmaceuticals and industrial products. The applications described below are only a few examples of where "green" biotechnology can be applied.

Genetically Modified Crops & Foods

Genetically-modified (GM) crops are classified as **Genetically-Modified Organisms (GMOs)** - organisms whose genetic material has been altered using biotechnology. In botany, genetically-modified crops are used for multiple purposes, including to resist herbicides (e.g., "Roundup Ready" soybeans), prevent spoilage (e.g., Flavr Savr® tomatoes), and enhance nutrition (e.g., Golden Rice with added vitamin A).

When making a GMO, the "host" organism whose genome will be altered can range from something as simple as unicellular bacteria and yeast, to multicellular, complex organisms such as animals and plants. To read about how genetically-modified animals are created and used, go to Chapter 8: Animal Biotechnology. For plants, the introduction of a new gene is often done using a bacteria called *Agrobacterium tumefaciens*, a rod-shaped Gram-negative soil bacterium. In plants, *A. tumefaciens* causes "crown gall" disease, in which tumors, called "galls" form within the infected plant. These galls, often at the base of the stem or roots, can disrupt nutrient and water transport, weakening the plant and potentially leading to its death. As such, *A. tumefaciens* is a serious pathogen of many commercial crops like walnuts, grape vines, stone fruits, nut trees, sugar beets, and rhubarb. The persistent nature of the tumors caused by the disease make it particularly harmful for these crops.

Figure \(\PageIndex{1}\): *Agrobacterium tumefaciens* and crown gall disease. Left image: *Agrobacterium tumefaciens* is a rod-shaped, Gram-negative bacteria that infects plant cells. (*Agrobacterium tumefaciens* by A. G. Matthysse, K. V. Holmes, R. H. G. Gurlitz; Public domain) Right image: Infection with *Agrobacterium tumefaciens* leads to "crown gall" disease - the production of several small tumors (called "galls") on the plant. (Crown Gall Disease by author unknown; CC BY-SA 3.0)

The tumors caused by *A. tumefaciens* are due to the presence of a tumor-inducing plasmid, known as the **Ti plasmid (or T-plasmid).** This plasmid contains all of the genes required for the bacteria to be virulent and efficiently infect plants cells. Once within the cell, the Ti plasmid incorporates a segment of its DNA, called **T-DNA**, into the genome of the host cell. This makes T-DNA an efficient gene delivery method that can be exploited in the laboratory. Today, Ti-based plasmids are used by researchers for the transfer and insertion of their desired gene into a plant genome (Figure \(\PageIndex{2}\)). *A. tumefaciens*-infected cells with the Ti plasmid can be selected and used to regenerate new plant tissue. Because plant cells are "totipotent", a single plant cells can be used to recreate an entirely new plant. This means that the genetically-modified cells created using *Agrobacterium tumefaciens* can easily be grown into genetically-modified plants that are clones of the originating cell.

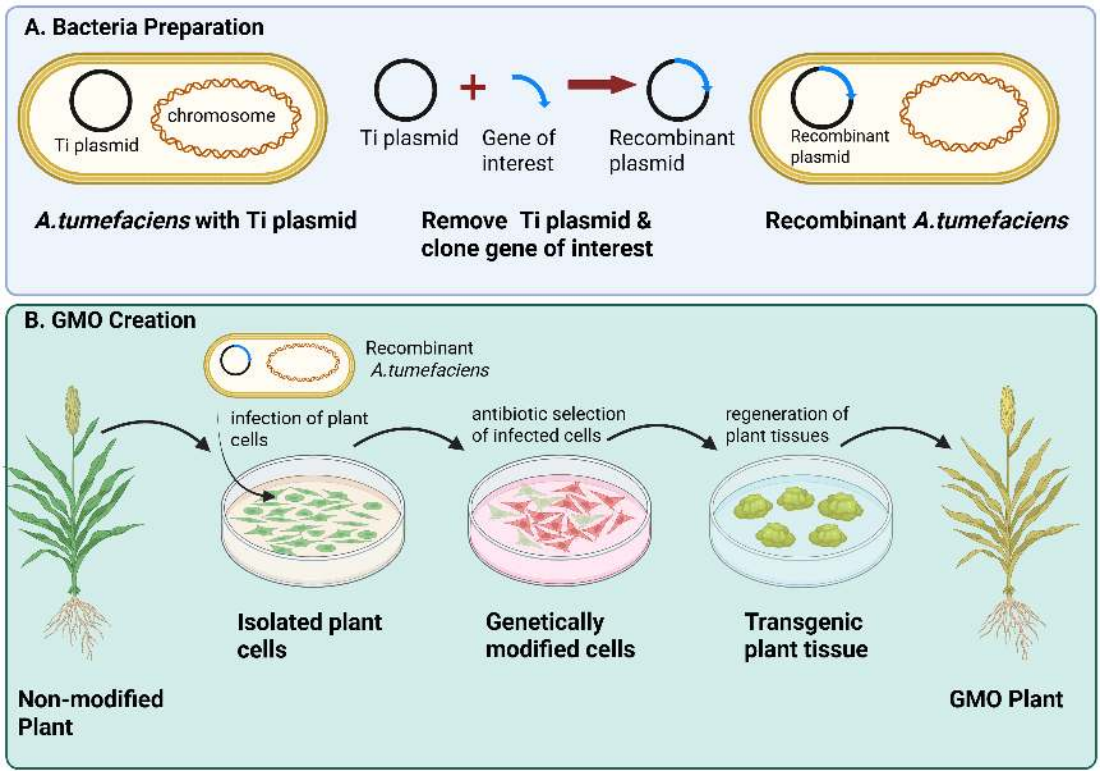


Figure \(\PageIndex{2}\): Production of a genetically-modified plant. (A) Bacteria Preparation (blue box at top of figure): *Agrobacterium tumefaciens* bacteria contain a plasmid called the Ti plasmid. The Ti plasmid is removed from *A.tumefaciens* bacterial cells (black circle) and the gene of interest (blue arrow) is inserted to make a recombinant Ti plasmid (black and blue circle). *A. tumefaciens* bacteria are transformed with this plasmid to produce recombinant *A. tumefaciens* cells. (B) GMO Creation (green box at bottom of figure): Non-modified plants are used for the isolation of plant cells. These plant cells are infected with recombinant *A. tumefaciens* cells. Antibiotic selection of infected cells selects for those genetically-modified cells in the culture (red cells). Expansion of these cells in culture produces transgenic plant tissue that can be developed into the GMO plant. (GMO Plant Production by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

Certain plants, like wheat and corn, do not respond well to Agrobacterium-mediated transformation. As an alternative, botanists can use a **"gene gun"** to introduce their gene of interest into these plants. A gene gun, also known as a biolistic particle delivery system, can be used to deliver genetic material (like DNA or RNA) into prokaryotic or eukaryotic cells, but is typically used on plant cells (Figure \(\PageIndex{3}\)). For this, the plant cells are plated onto a surface and placed into the gene gun. Tiny metal particles (usually gold or tungsten) are coated with DNA, containing the desired gene. The particles are loaded onto a plastic membrane or cartridge and placed inside the gene gun. The gene gun is aimed at the cells. A burst of compressed gas (like helium) or a high-voltage electric discharge is applied to the membrane, propelling the particles at high speed into the cells. The particles shoot through the cell walls and membranes, carrying the DNA into the cell interior. Some of the cells will then integrate the DNA into their genome for gene expression. Antibiotic resistance is used to identify those cells containing the integrated DNA. Following development under highly controlled conditions and genetic testing, the organism may be considered a GMO.

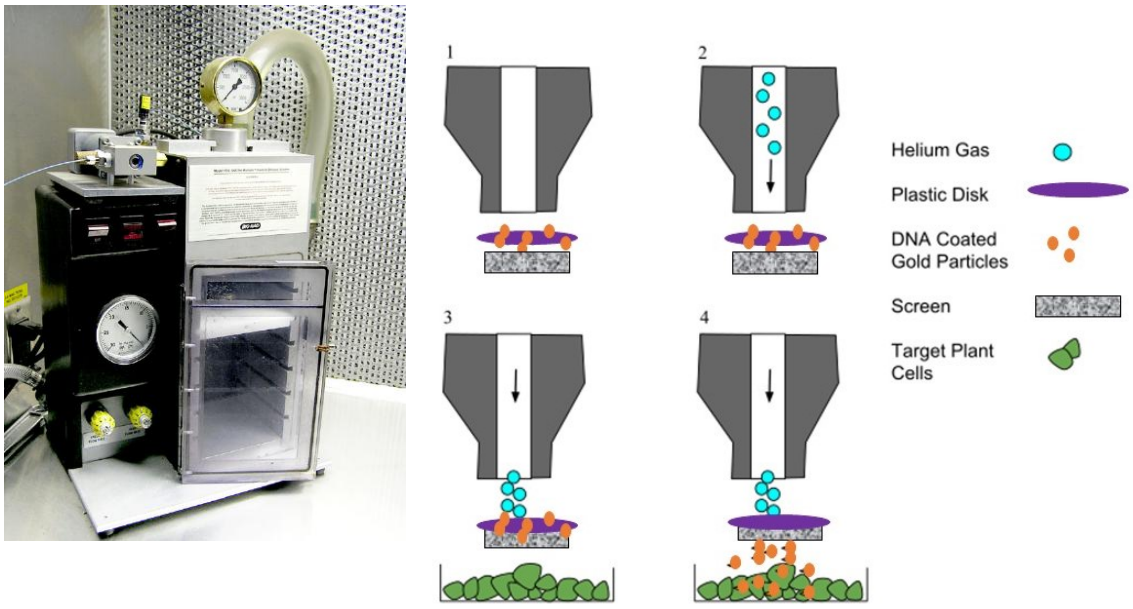


Figure \(\PageIndex{3}\): Gene delivery using a "gene gun". Left image: A picture of a gene gun with the chamber open to receive the cell sample. (Genegun by xmort; Public domain) Right image: 1. DNA-coated gold particles are loaded onto a plastic disk and placed in the gene gun on top of a screen. Cells are plated onto a surface and placed into the gun (not shown). 2. Helium gas fills the chamber and pressure builds against the gold-coated disk. 3. The pressure pushes the disk into the screen. 4. The DNA-coated gold particles are propelled through the screen and into the targeted plant cells. (How the Gene Gun Works by Patricia Zuk CC BY-SA 4.0; adapted from Gene Gun by RachelBrooks15, CC BY-SA 4.0)

While the introduction of genetic material into cells is typically done using **liposomal-mediation transfection** (see Lab Protocol 17.9 Liposomal transfection into mammalian cells), plant cells, with their rigid cell walls made of cellulose, make transfection with liposomes difficult. As such, botanists will use a gene gun to improve their transformation efficiency. This is because the gene gun is capable of "firing" genetic information through the cell wall and plasma membrane. In addition, the gene gun can introduce genetic material into a few plant cells, a single plant tissue or a specific plant organ (e.g. a leaf) in order to observe the effects of genetic manipulation without creating an entire genetically-modified plant.

GMOs like genetically-modified crops have helped address food security and resource sustainability. However, they also raise ethical and environmental concerns. Some have expressed concerns about the potential impacts of GMOs on biodiversity, long-term human health effects, and the monopolization of food resources by biotech companies, making GMOs a complex and often controversial area of modern science.

Biofertilizers and Biopesticides

Once of the focuses of green biotechnology is in the creation and use of biofertilizers and biopesticides that are eco-friendly and sustainable. A **biofertilizer** is a natural fertilizer that contains living microbes that help plants grow by increasing the supply or availability of nutrients in the soil. The types of microbes found will depend on the biofertilizer but can contain beneficial bacteria, fungi, or algae. One important function of biofertilizers is in **nitrogen fixation** - the process by which atmospheric nitrogen gas (N_2) is converted into ammonia (NH_3) or other nitrogenous compounds (Figure \(\PageIndex{4}\)). These nitrogenous compounds are used by the plants to synthesize proteins.

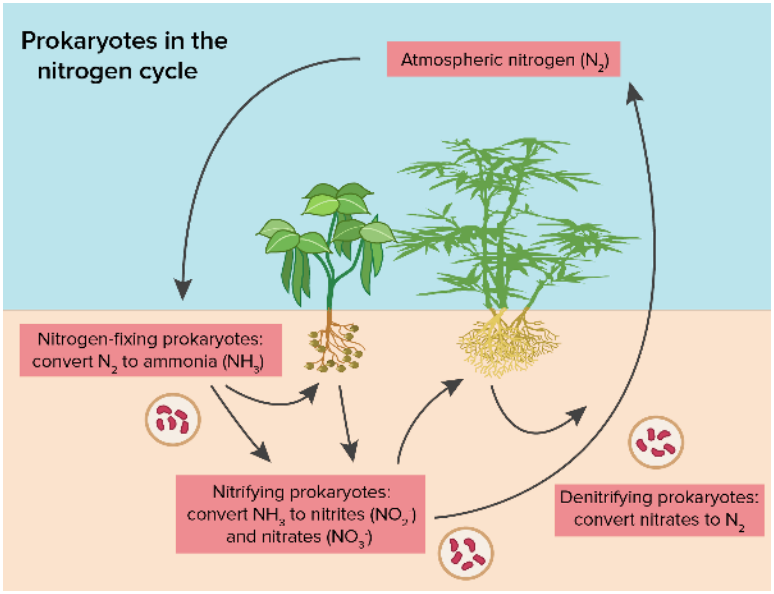


Figure \(\PageIndex{4}\): The nitrogen cycle. Nitrogen-fixing prokaryotes that live in soil and convert atmospheric N_2 intro ammonia (NH_3). In addition, nitrifying prokaryotics convert NH_3 into nitrites (NO_2^-) and nitrates (NO_3^-). Each of these compounds are used by plants. Denitrifying prokaryotes can convert nitrates back into N_2 which returns to the atmosphere. (Prokaryotes in the nitrogen cycle modified from Nitrogen cycle by Johann Dréo; CC BY-SA 3.0)

Biofertilizers are easily applied to seeds, soil, or to plant roots. The seeds can be coated with the biofertilizer prior to planting. This is common for legumes like beans and peas. Alternatively, the biofertilizer can be mixed with compost materials or carrier material, like peat, and the spread into the soil. Other techniques include root "dipping" in which the roots of the plants are dipped into a solution containing the biofertilizer and the spraying of a biofertilizer solution onto leaves. Table \(\PageIndex{1}\) lists some commonly used biofertilizers.

Table \(\PageIndex{1}\): Biofertilizers

Microbe	What They Do	Commonly Used In
Rhizobium (Gram-negative bacteria)	fix nitrogen into the nodules of roots	legumes (beans, peas, lentils)
Azospirillum (Gram-negative bacteria)	fix nitrogen into the soil around the root zone	corn, wheat, rice
Azotobacter (bacteria)	fix nitrogen into soil	vegetable, cereal crops
Cyanobacteria: *Anabaena sp.*, *Nostoc sp.*	fix nitrogen into waterlogged soils	rice fields
Phosphate-solubilizing bacteria (PSB): *Bacillus* and *Pseudomonas* species	make soil phosphorus more available	many commercial crops
Mycorrhizal (fungi)	improve phosphate uptake and water absorption by plant roots	trees, vegetables, flowers

Biopesticides are biologically-based substances that control pests, such as insects, weeds, fungi, and bacteria. Biopesticides tend to be safer for humans, animals, and the environment. They don't pollute the soil or water and break down quickly in the environment. Biopesticides are target-specific as they usually affect only the pest and not beneficial organisms. Moreover, the targeted pests are less likely to develop resistance to the biopesticide. However, biopesticides can be slower-acting than chemical pesticides and may need specific conditions in order to work effectively.

There are several types of biopesticides, including:

- **Microbial biopesticides:** contain living organisms, like bacteria, fungi, viruses
  - e.g. *Bacillus thuringiensis (Bt)*

- Bt produces toxins that kill insect larvae

- **Botanical pesticides:** pesticides extracted from plants
  - e.g. neem oil
  - disrupts insect hormones and feeding
- **Biochemical pesticides:** natural substances that affect pest behavior
  - e.g. insect sex pheromones
  - interfere with mating to reduce pest populations
- **Entomopathogenic fungi:** fungi that infect and kill insects
  - e.g. Beauveria spores infect and grow inside insect hosts

One of the most widely used biopesticides in both organic and conventional farming is the soil bacteria, *Bacillus thuringiensis* (Bt). Bt bacteria are used to control the populations of several moth and butterfly species, in addition to beetles, flies and mosquitoes. During sporulation, Bt cells produces **crystal (Cry) proteins,** which are also known as δ-endotoxins (Figure \(\PageIndex{5}\)). When ingested by certain insects, the alkaline environment of the gut processes and activates the toxin. The activated form of the toxin binds to specific receptors expressed by the epithelial cells lining the mid-gut portion of the digestive system. Receptor activation results in the formation of pores in the epithelial lining. The insect stops feeding and dies within days to a few weeks. Because the receptors to the activated Cry proteins are found only in certain insect species, Bt is safe to beneficial insects, plant cells, and animals. In organic farming, Bt spores and toxins can be directly sprayed onto crops. In addition, genetically-modified crops, such as corn, cotton, and soybeans, have been engineered to produce the Bt toxins themselves. This genetic engineering involved use of a gene gun to introduce the relevant Bt genes.

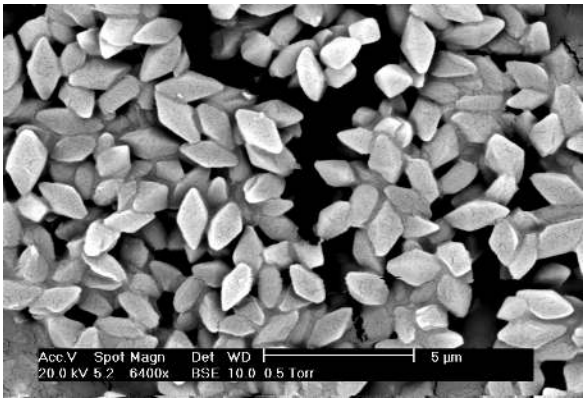


Figure \(\PageIndex{5}\): Bt-produced cry proteins are insecticidal towards certain insect pests. (Crystals of Bt Toxin by Jim Buckman; public domain)

Key Concepts

Applications of green and gray biotechnology can range from the creation of genetically-modified crops to the use of plants to trap heavy metals in soils. Some important concepts to remember are:

- some of the key applications of green biotechnology include genetically-modified crops, biopesticides, biofertilizers, and phytoremediation
- genetic modification of plant cells can be accomplished using a gene gun or transformation using the Ti plasmid of the soil bacteria, *Bacillus thuringiensis*
- biofertilizers contains living microbes that help plants grow by increasing the supply or availability of nutrients in the soil
- biofertilizers can be easily applied to seeds, soil, or to plant roots
- one important role of biofertilizers is nitrogen fixation
- biopesticides are biologically-based substances that control pests, such as insects, weeds, fungi, and bacteria
- there are several types of biopesticides including microbial, botanical, and biochemical
- the bacteria, *Bacillus thuringiensis*, is one of the most commonly used biopesticides in farming
- plants, and their associated microbes, can be used to clean up environmental pollutants through a process called phytoremediation

Glossary

Agriculture - the science and practice of growing crops and raising animals for food, fiber, fuel, and other products used to sustain and enhance human life

Agricultural biotechnology - a branch of biotechnology focused on using scientific tools and techniques, like genetic engineering and molecular markers, to improve plants, animals, and microorganisms used in farming; known as "green" biotechnology

Biofertilizer - a substance containing living microorganisms that, when applied to soil or plants, enhances nutrient availability—especially nitrogen and phosphorus—promoting healthy plant growth

Biopesticide - a type of pesticide derived from natural materials such as animals, plants, bacteria, or certain minerals, used to control pests in an environmentally friendly way

Botany - the scientific study of plants, including their structure, growth, reproduction, metabolism, development, diseases, and interactions with their environment

Crop - a plant that is cultivated and harvested for food, fiber, fuel, or other economic purposes

Genetically-modified (GM) crop - a crop whose DNA has been altered using genetic engineering techniques to introduce traits like pest resistance, herbicide tolerance, or improved nutritional content

Genetically-modified organism (GMO) - any organism whose DNA has been altered using genetic engineering techniques

Pest - any organism that damages or interferes with crops, livestock, food, or property—commonly includes insects, weeds, fungi, and rodents

Pesticide - a chemical or biological agent used to kill or control pests, including insects (insecticides), weeds (herbicides), fungi (fungicides), and others

9.2: Applications of "Green" Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

9.3: Applications of "Gray" Biotechnology

Biotech Focus

The textile industry produces copious amount of wastewater that is full of toxic dyes. These dyes are resistant to breakdown by "normal" bacteria. In response, scientists genetically-modified bacteria to enhance their degradative capacity. To do this, genes to enzymatically process textile dyes, like **azoreductase**, were inserted intro bacterial strains like *Pseudomonas putida*. Introduction of these bacteria into the wastewater effluent resulted in the breakdown of these dyes into harmless byproducts. The modified bacteria cleaned the wastewater much faster than "traditional" methods. They worked even in low-oxygen environments, which is important for deep water treatment.

Introduction

"Gray", or **environmental biotechnology**, uses science, technology, and engineering to study the natural world and solve a variety of environmental problems. Because environmental biotechnology could also imply the harnessing of biological processes for commercial use, the International Society for Environmental Biotechnology now defines environmental biotechnology as "the development, use and regulation of biological systems for remediation of contaminated environments (land, air, water), and for environment-friendly processes (green manufacturing technologies and sustainable development)".

Some of the key applications of gray biotechnology include:

- **Bioremediation:** Using bacteria to clean up oil spills or toxic waste
- **Phytoremediation:** Using plants to clean up polluted soil, water, or air
- **Waste management:** Using engineered bacteria to break down plastics and other difficult to degrade materials
- **Air pollution control:** Using bacteria to trap and break down pollutants from industrial gases
- **Environmental monitoring:** Using bacteria in the development of biosensors that can detect pollution early on in the process

Learning Objectives

"Gray" biotechnology restores ecosystems, reduces pollution, and improves waste management practices to make them eco-friendly. At the end of this page, you will be able to:

- Define gray biotechnology
- Discuss some applications of gray biotechnology, such as phytoremediation and bioremediation

Applications of Gray Biotechnology

The use of living organisms in the reduction of environmental waste and pollutants has become a very popular area of biotechnology. As such, environmental biotechnology integrates extensively with industrial biotechnology. Because agriculture practices can produce significant amounts of waste, environmental biotechnology also intersects with agricultural biotechnology. This means that the applications described below could also be considered to be part of other biotechnology fields.

Phytoremediation

Phytoremediation is a natural and eco-friendly method of cleaning up environmental pollutants using plants and their associated microbes. Phytoremediation is environmentally friendly, aesthetically pleasing, cost-effective compared to conventional cleanup methods and can improve soil health and biodiversity. However, this natural process is slower than conventional methods and is only effect to the depth of the plant roots. In addition, phytoremediation may not work well in areas with high contamination levels.

Types of phytoremediation include:

1. **Phytoextraction:** the plants absorb contaminants, like heavy metals from soil or water through their roots and store them in the tissues of their stems or leaves; the contaminated plants can then be harvested and safely disposed of
    - e.g. sunflowers planted at Chernobyl (Ukraine) absorb cesium-137 and strontium-90
    - e.g. Indian mustard planted at mining sites for the absorption of heavy metals like lead, mercury, selenium, and cadmium.

2. **Phytostabilization:** the plants reduce the movement of heavy metal contaminants by immobilizing them in the soil through root systems, preventing them from spreading to water sources
    - e.g. Buffalo grass, Vetivar grass, willow and poplar trees
3. **Phytodegradation:** the plants use enzymes to break down organic pollutants, like pesticides or petroleum products, into less harmful substances
    - e.g. use of poplar trees at landfills and hazardous waste sites
    - e.g. Vetivar grass planted in oil-contaminated soils
4. **Phytovolatilization:** the plants absorb contaminants, like heavy metals and solvents, and release them into the atmosphere in a less toxic form through transpiration from the leaves
    - e.g. ferns and wetland species
5. **Rhizofiltration:** plant roots, especially those in water, absorb or filter pollutants from contaminated water
    - e.g. water hyacinths use in ponds and canals
6. **Phytodesalination:** uses halophytes (e.g., plants adapted to saline soil) to extract salt from the soil to improve its fertility

Genetic engineering has become a powerful method for enhancing natural phytoremediation capabilities, or for introducing new capabilities into plants. Genes from metal-tolerant organisms may be introduced into other plants to turn them into "hyperaccumulators" capable of absorbing significant amounts of toxic metals and store them safely in their tissues. Plants can be engineered to express degradative enzymes like cytochrome P450 enzymes. Genetic engineering can also be used to turn the native plants that are better adapted to the environmental conditions at a specific cleanup site into plants capable of accelerated phytoremediation.

Bioremediation

The natural ability of organisms to adsorb, accumulate, and degrade pollutants has attracted the use of biotechnology in the treatment of contaminated environments. **Bioremediation** refers to any process that uses a biological system (e.g., bacteria, algae, fungi, and plants), or its products (e.g., enzymes), for the removal of environmental pollutants from air, water, soil, and industrial effluents (Figure \(\PageIndex{1}\). New bioremediation approaches involve the use of genetically-modified bacteria but with strict controls to ensure the bacteria will not harm other ecosystems. Genetically-modified bacteria can produce new enzymes to digest pollutants, can be modified to improve the efficiency of natural pathways, or to make bacteria more resistant to the environmental stresses at the clean up location. *Pseudomonas putida* has been engineered to degrade hydrocarbons and resist toxic metals at clean-up sites. *Deinococcus radiodurans* has been modified to survive elevated radiation levels and clean up nuclear wastes. Engineering bacteria to treat multiple types of pollution at once (e.g., oil and heavy metals) is also being explored. Whatever the approach, the end goal of bioremediation is to remove harmful compounds to improve soil and water quality.

Bioremediation techniques can be classified as *in situ* techniques, which treat polluted sites directly or *ex situ* techniques which are applied to materials that have been removed from a contaminated site.

In situ bioremediation can involve the addition of microbes for the treatment of the pollutant or the addition of specific nutrients to encourage the growth of naturally-occurring microbes at the site. Examples of *in situ* techniques include:

1. **Bioventing:** a method where oxygen is pumped into the soil to help natural bacteria break down pollutants
    - used for the clean up of oil, gasoline, and solvent spills in soil
2. **Biostimulation:** a method where nutrients, like nitrogen or phosphorus, are added to "feed" microbes and speed up their breakdown of the pollutant
    - used for soil, groundwater, or oil spill cleanup
    - does not involve the use of genetically-modified microbes
3. **Bioattenuation:** a method of letting natural processes slowly break down pollutants without the addition of microbes or nutrients
    - scientists monitor the process rather than direct the process

Figure \(\PageIndex{1}\): Biostimulation at the Snake River Plain Aquifer in Idaho. Whey powder is added to the site to promote the utilization of naturally present bacteria. The bacteria at this sitebreak down the carcinogenic compound trichloroethylene (TCE). (Biostimulation by bomazi; Public domain)

Examples of *ex situ* techniques include:

1. **Biopiles:** soil is removed and piled into mounds with an aeration system to encourage bacterial growth
   - microbe use is similar to bioventing
   - used to remove petroleum pollutants
2. **Windrows:** contaminated soil is laid out in long, narrow rows and then regularly turned over to mix in air
   - also used in composting
3. **Land farming:** contaminated soil is spread over a large, flat field and then tilled to give microbes access to air and sunlight
   - treats soils contaminated with petroleum or pesticides

Wastewater Treatment

Wastewater is any water that has been used and is now dirty because it picked up pollutants like chemicals, food scraps, oil and grease, human waste, and industrial toxins. There are two types of wastewater: domestic (toilets, showers, kitchens) and industrial (factories and businesses). Wastewater needs to be cleaned (i.e.,treated) before it can safely go back into the environment or be reused. The process of cleaning wastewater is known as **wastewater treatment**.

Wastewater is treated is 4 steps:

1. **Preliminary treatment:** the filtering of water of particulate matter
2. **Primary treatment:** the separation of wastewater into phases using large tanks
   - heavy solids sink to the bottom of the tank; lighter materials (oil and grease) rise to the top
   - both phases can be removed
3. **Secondary treatment:** the addition of bacteria to the water phase
   - aeration is added to improve bacterial efficiency
   - the water is transferred to a tank to allow for the settling and removal of bacteria
4. **Advanced (tertiary) treatment:** special filters, chemicals, or UV light is used to kill any remaining pathogens and chemicals
   - artificial "swamps" can be constructed to filter water and eliminate the need for harsh chemicals or UV light

Scientists can engineer bacteria to improve the secondary treatment phase of wastewater by making them break down pollutants faster, remove heavy metals, dyes, oils, and pharmaceuticals, and work in harsh environments. Strains of *Pseudomonas* bacteria are one example of a bacterial strain used in wastewater treatment. These bacteria can be engineered to break down oil, heavy metals, or organic pollutants in wastewater. *E. coli* strains are also engineered for wastewater treatment. Some *E. coli* strains can be even be engineered to glow when pollutants are present, turning them into biosensors. As with bioremediation, the introduction of genetically-modified bacteria to wastewater treatment plants are heavily regulated and must be carefully monitored to ensure they do not escape into natural water systems.

Pollution Control

Biotechnology can control pollution in many ways, including phytoremediation and bioremediation. In addition, scientists are now creating **biofilters** that use microbial films to clean dirty air (and water). A biofilter is a moist bed of soil, compost, or wood chips that contains beneficial microbes, such as natural bacteria or genetically-modified bacteria. The dirty air is pushed through the

biofilter, giving the bacteria access to the pollutants. Wastewater treatment plants use such biofilters to control the bad odors and harmful gases that result from the treatment process. Bacteria or fungi designed to break down specific pollutants can also be added to a system in a process called **bioaugmentation**. As with biofilters, these microbes are chosen for their degradative ability or they can genetically engineered. Bioaugmentation is also a critical part of bioremediation and wastewater treatment.

Environmental Monitoring

Environmental monitoring is the process of regularly checking air, soil, and water to measure pollution levels and track changes in the environment. When monitoring, scientists collect samples and analyze them. By monitoring, scientists can detect problems early in order to protect ecosystems and human health. One technique being developed for use in environmental monitoring is the creation of biosensors. A **biosensor** is a special device that can detect pollution using biological components, like bacteria, enzymes, or antibodies. The typical biosensor produces some type of "signal" like a color change, light, or an electrical signal. By measuring the signal, scientists can determine how much pollution is present.

A basic biosensor contains the following parts:

- **Bioreceptor:** what detects the pollutant
  - can be an enzyme, antibody, gene, or an entire organism (e.g. microbe)
- **Transducer:** converts the detection into a measurable signal that can be measured
  - light (i.e., fluorescence, luminescence), color change, electrical signal
- **Display:** shows the signal
  - the signal could be displayed used a computer screen, a camera, or something simple as film
  - the display can be coupled to devices that measure the signal

One example is the use of genetically-engineered bacteria like *E.coli*, *Bacillis subtilis*, or strains of *Pseudomonas* in the detection of arsenic in water. For this biosensor, a gene for a **reporter protein** is inserted into the bacterial genome. This reporter protein is under the control of a promoter capable of being bound by arsenic. The amount of reporter protein expressed, as a result of arsenic binding, can then be detected and measured through a simple bioassay, indicating the presence and concentration of arsenic (Figure \(\PageIndex{2}\)).

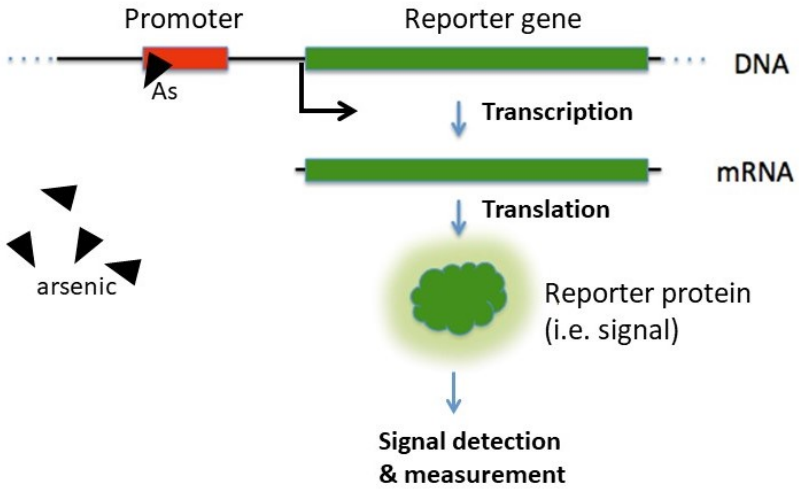


Figure \(\PageIndex{2}\): Biosensors can be built using reporter systems. A typical reporter system has a promoter (red rectangle) capable of being bound by the compound to be detected and measured (e.g., arsenic, As). Binding of this compound results in transcription of the reporter gene into mRNA and then translation into the reporter protein, also called the "signal". This signal could be fluorescent, luminescent, or colorimetric. The signal is detected and then measured as an indication of the amount of compound. (Reporter Systems by Patricia Zuk, CC BY 4.0; modified from Reporter Gene by Transcontrol, CC BY-SA 3.0)

Several types of reporter protein systems can be used in biosensors, including fluorescent (e.g. Green Fluorescent Protein or GFP), luminescent (e.g. firefly luciferase), or colorimetric systems. Each have their own advantages and disadvantages. For example, the fluorescent signal produced by reporter systems using GFP can be measured directly, but the signal can be weak. Reporter systems

using luminescent or colorimetric proteins require additional steps to detect the signal but this signal can be amplified, making these systems more sensitive. Ultimately, this type of biosensor is inexpensive to create, and robust. It can be highly sensitive. Moreover, this biosensor is portable and can be used easily for field testing.

Key Concepts

Applications of gray biotechnology range from the use of plants to trap heavy metals in soils and the engineering of bacteria to breakdown hydrocarbons in oil. Some important concepts to remember are:

- plants, and their associated microbes, can be used to clean up environmental pollutants through a process called phytoremediation
- bioremediation uses biological systems, or its products, to remove environmental pollutants
- environmental monitoring can involve the use of biosensors which utilize reporter systems for the detection of a pollutant
- the binding of an environmental pollutant, like a heavy metal, to a reporter system promoter leads to the expression of the reporter protein
- quantitation of a reporter protein can be used to measure the amount of environmental pollutant
- bioremediation, wastewater treatment, and pollution control can use "natural" bacteria or those that have been genetically-engineered
- genetically-engineered bacteria use in environmental biotechnology must be carefully used to prevent their introduction into natural ecosystems

Glossary

Bioassay - a scientific test that uses living organisms to measure the effect or concentration of a chemical substance, such as pollutants or toxins

Bioaugmentation - the process of adding specially selected or genetically-modified microbes to a contaminated environment in order to help break down pollutants faster and more efficiently

Biofilter - a system that uses living materials, like microorganisms on a filter medium (like soil, compost, or synthetic materials), to remove contaminants from air, water, or industrial waste streams

Biosensor - a device that uses a biological component, like an enzyme, antibody, cell, or DNA sequence) to detect and measure specific chemicals, substances, or conditions and convert them into a signal (like light, electricity, or color change)

Effluent - a liquid waste or sewage that flows out of a factory, treatment plant, or other facility; often requires treatment before it can be safely released into the environment

Ex situ - Latin for "off site," referring to treatments that are performed away from the original location of a process or problem

In situ - Latin for "on site"; referring to performing treatments directly at the location where the problem or process naturally occurs

Phytoremediation - the use of plants to clean up contaminants from soil, water, or air by absorbing, containing, degrading, or volatilizing pollutants

Promoter sequence (promoter) - a DNA sequence where the RNA polymerase binds; determines the site of transcription initiation

Remediation - the process of cleaning up, removing, or neutralizing pollutants from soil, water, or air to restore the environment to a healthier state

Reporter system - a tool used to visually or easily measure the activity of a gene, promoter, or cellular process

Wastewater - used or contaminated water that comes from homes, businesses, or industries, often containing pollutants, and requiring treatment before it can be reused or released into natural water bodies

9.3: Applications of "Gray" Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

9.4: Benefits of "Green" Biotechnology

Biotech Focus

India has one of the highest rates of iron-deficient anemia globally, especially in women and children. In response, a public health initiative was undertaken in India to provide school-aged children in rural schools with biofortified "pearl millet" that was high in iron for 6 months. Children were served meals (like chapatis or porridge) made from either biofortified pearl millet or standard pearl millet with its normal iron level. Pearl millet is a staple food in many dry regions of India. It is a drought-resistant crop that is easy to improve using conventional breeding or biotechnology. Hemoglobin levels and iron stores in the students were measured before and after the study. At the end of the study, the children who ate the iron-biofortified pearl millet showed a significant improvement in iron status and a reduction in iron-deficiency anemia. Even small daily servings of pearl millet made a noticeable impact on the student's health. The iron-enriched pearl millet study in school children in India is a real-world example of **biofortification in action**—where science and nutrition come together to fight a nutritional deficiency in a vulnerable population.

Introduction

Throughout history, farmers have selectively bred plants to achieve desirable traits, such as sweeter apples or larger corn cobs. However, this traditional method can be time-consuming and may inadvertently introduce unwanted characteristics. Green biotechnology offers a more precise approach to developing desirable traits in plants. These enhancements can include improved drought resistance, increased tolerance to pests and diseases, enhanced nutritional content in food, or the production of pharmaceuticals and industrial products.

Learning Objectives

"Green" biotechnology improves farming practices to increase crop yields, enhance resistance, improve nutritional content, and reduce the need for chemical treatments. At the end of this page, you will be able to:

- Explain some of the benefits of green biotechnology

Benefits of "Green" Biotechnology

Plant biotechnology is part of the **"Green" biotechnology** field. Green biotechnology, often called agricultural biotechnology, is the use of science and engineering on agriculture and the environment. It combines biology with technology to solve problems related to food production, sustainability, and environmental challenges. It is used to modify products, improve plants (or animals), or develop or engineer microbes for specific agricultural uses. Green biotechnology, in combination with traditional breeding techniques, can provide essential tools to farmers that can make agricultural production cheaper and more manageable.

There are many benefits of "Green" biotechnology with regards to plants and farming.

Improved crop production: Biotech crops can make farming more profitable by increasing crop quality and may in some cases increase yield. In addition, biotechnology can improve the health of crops. Some crops are engineered to tolerate specific herbicides without compromising their yields, making weed control simpler and more efficient. Other crops have been engineered to be resistant to specific plant diseases caused by certain microbes and insect pests, which can make pest control more reliable and effective, and/or can decrease the use of synthetic pesticides. Resistance to viruses, bacteria, fungi, insects or herbicides is known as **biotic stress tolerance**.

Biofortification: Biofortification is the process of increasing the nutritional value of crops through breeding, genetic engineering, or biotechnology. Biofortification focuses on the generation of **micronutrient-dense crops** through green biotechnology in order to improve the nutritional content in staple crops. Micronutrient-dense crops can effectively reduce vitamin and mineral deficiencies in populations who have limited access to diverse diets. Studies have shown its effectiveness, with iron-biofortified beans improving iron levels in Rwandan women and iron-biofortified pearl millet reversing iron deficiency in Indian schoolchildren. **Golden rice**, modified through biotechnology techniques to produce beta-carotene, a precursor to vitamin A (Figure \(\PageIndex{1}\)), was developed in 2000 to help combat vitamin A deficiency, a major cause of childhood blindness. Crops like

canola, soybean, and corn have been improved with healthier oil compositions. Along with enhanced nutritional value, crops can also be generated to be resilient to environmental stresses, such as drought or pests. This dual benefit offers a promising strategy to address global malnutrition, improving the well-being of communities worldwide. However, biofortification comes with its challenges. Biofortified crops may be produced with heightened nutrient levels, but they need to be in a form the body can digest and absorb. These nutrients should also remain stable and retained throughout cooking, processing, and storage to ensure their full nutritional benefits are delivered. Another challenge lies in gaining acceptance and adoption of biofortified crops among both farmers and consumers, especially when traditional options are already well-established.

Figure \(\PageIndex{1}\): White rice and golden rice. Thanks to green biotechnology, golden rice (shown at the right) has significantly more vitamin A compared to white rice (shown at the left). The increased vitamin A content has been credited with preventing blindness in millions of people (Golden Rice by the International Rice Research Institute; CC BY 2.0)

Environmental benefits: Insect-resistant cotton has significantly reduced the use of persistent, synthetic pesticides that may contaminate groundwater and the environment. Herbicide-tolerant crops are compatible with no-till or reduced tillage agriculture systems that help preserve topsoil from erosion.

Pharmaceuticals: Green biotechnology has emerged has a promising tool for vaccine production through its development of an **edible vaccine**. In this approach, genes encoding bacterial or viral **antigens** - the compounds that trigger an immune response - are introduced into plants. One of the most common ways of introduction is the bacteria *Agrobacterium tumefaciens* (for more information see Chapter 9.1 Applications of Green Biotechnology). The plant then produces these antigens in its edible parts (e.g. seeds, leaves, roots). When a person eats the plant, their immune system will recognize the antigen as foreign and launch a small immune response, thus building immunity to the antigen. Bananas, potatoes, tomatoes, rice, and lettuce are all being explored as edible vaccine carriers. Pathogens being targeted include those that cause widespread diseases, like hepatitis B, cholera, measles, and even COVID-19. Although edible vaccines provide the advantage of convenient and needleless administration, they are not currently as effective as normal vaccines as they are consumed and not injected into the blood. This is because in order to elicit an significant enough immune response, the antigens must survive the digestive process and be absorbed into the blood.

Production of phytochemicals: Green biotechnology is being used to genetically-modify plants to enhance their production of **phytochemicals** or to allow them to make new phytochemicals not normally found in the plant. Phytochemicals (Figure \(\PageIndex{2}\)) are naturally-occurring compounds found in plants that are not essential nutrients, like vitamins or minerals, but can have health-promoting properties. Phytochemicals are made by plants to protect themselves from pests, diseases, and environmental stress. In humans, phytochemicals act as **antioxidants**, **anti-inflammatory agents** and immune boosters. Common phytochemicals include the **flavonoids** and **carotenoids**. Using green biotechnology, tomatoes have been engineered to make higher levels of anthocyanins, a type of flavonoid, that can act as an antioxidant. The insertion of the stilbene synthase (STS) gene into the genomes of tomatoes and rice has resulted in the production of the antioxidant, resveratrol, in these plants. Outside of the farm, plant cells grown in tissue culture facilities can be used to produce specific phytochemicals in highly controlled environments, allowing for year-round production and avoidance of environmental variability.

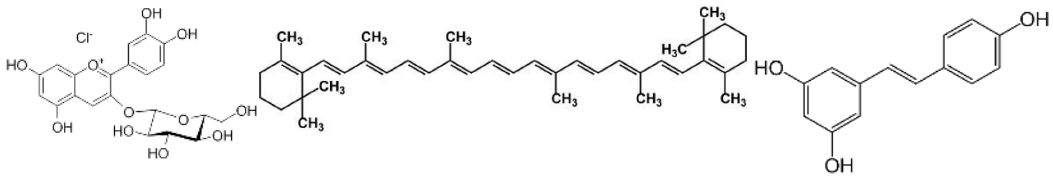


Figure \(\PageIndex{2}\): Phytochemicals. Left image: the chemical structure of the anthocyanin flavonoid, chrysanthemin chloride. (Chrysanthemin chloride by NiCrMo, CC BY-SA 4.0)
Center image: the chemical structure of the carotenoid, beta-carotene (Beta-carotene by Polimerek, CC BY-SA 3.0)
Right image: the chemical structure of resveratrol (Resveratrol by Fvasconcellos, Public Domain)

Green Biotechnology Concerns

One key concern of green biotechnology is the potential transfer of novel traits to wild relatives. However, this potential transfer is carefully evaluated during the review and approval of genetically-engineered crops. Environmental impacts of engineered plants are also assessed, particularly regarding their effects on birds, mammals, insects, and other organisms, especially in the case of engineering insect or disease resistance traits. For example, studies have been conducted to ensure that bees are not adversely affected by the crops engineering to be resistant to pests. Food safety is another critical focus; newly introduced traits in green biotechnology plants undergo rigorous testing to assess potential toxicity and the risk of allergic reactions. These comprehensive evaluations help ensure that genetically engineered crops are both safe and effective for consumers and the environment.

To provide regulatory oversight of organisms derived through genetic engineering, the federal government developed a **Coordinated Framework for the Regulation of Biotechnology** in 1986. This framework spells out the basic federal policy for regulating the development and introduction of products derived from biotechnology. The policy has three tenets:

1. U.S. policy focuses on the *product of genetic modification* (GM) techniques, not the process of genetic modification itself
2. only regulation grounded in verifiable scientific risks is tolerated
3. genetically-modified products are on a continuum with existing products and, therefore, existing statutes are sufficient to review these GM products

Products made through biotechnology are regulated according to their intended use, with some products being regulated under more than one agency. The three principal agencies that have provided primary guidance on the experimental testing, approval, and eventual commercial release of modified organisms to date are the:

1. **Animal and Plant Health Inspection Service (APHIS)** - under the USDA
2. **Environmental Protection Agency (EPA)**
3. **Food and Drug Administration (FDA)** - under the Department of Human and Health Services (HHS)

APHIS protects US agriculture from pests and diseases. APHIS regulations concentrate on genetically-modified plants in order to ensure that they don't harm other plants or the environment. The EPA protects human health and the environment. In the biotechnology field, the EPA regulates biopesticides, biofertilizers, and genetically-modified crops that produce pest-resistant compounds like the Bt toxin discussed in Chapter 9.1 Applications of Green Biotechnology. The FDA ensures the safety of food, drugs, and cosmetics. The FDA reviews genetically-modified foods for safety, proper labeling, and nutritional content.

The Future of Green Biotechnology

The future of green biotechnology is exciting and full of possibilities. Some examples include the development of climate-resistant plants that can survive droughts, floods, and extreme temperature by enhancing their **abiotic stress tolerance,** the continued creation of biofortified foods that are rich in specific vitamins, minerals, and antioxidants, and expansion of the use of plants in phytoremediation. However, some new areas are entering the field include the use of CRISPR-based systems to fine-tune desired plant traits (e.g. pest resistance, faster growth) with extreme accuracy. To read more about CRISPR, go to Chapter 8.1 Applications of Animal Biotechnology.

Key Concepts

Green biotechnology offers a more precise approach to developing desirable traits in plants and has numerous benefits.

Some important concepts to remember are:

- green biotechnology techniques can improve crop production, lead to the development of biofortified foods, improve the production of phytochemicals by the plant, and have environmental benefits
- biofortified foods contain increased amounts of a desired compound, like a vitamin or mineral
- plants can be used in the production of edible vaccines
- concerns about plants produced through green biotechnology can be regulated by multiple agencies like the APHIS, the EPA, and the FDA
- regulation of genetically-modified organisms involved the Coordinated Framework for the Regulation of Biotechnology

Glossary

Abiotic - non-living physical or chemical factors in the environment, like temperature, sunlight, or soil quality

Agriculture - the science and practice of growing crops and raising animals for food, fiber, and other products to support human life

Agricultural biotechnology - the use of scientific tools and techniques, like genetic engineering, to improve crops, animals, and farming methods; often called
"green" biotechnology

Antigen - a substance (usually a protein or sugar) that triggers an immune response in the body; often used in vaccines

Anti-inflammatory - a substance that reduces swelling, pain, or irritation in an organism

Antioxidant - a compound that helps protect cells from DNA damage caused by free radicals

Biofortification - the process of increasing the nutritional value of crops through breeding and/or genetic engineering

Biotic - the living components of an ecosystem, such as plants, animals, fungi, and bacteria

Carotenoid - a natural pigment found in plants that gives yellow, orange, or red color; many act as antioxidants; examples include beta-carotene, lutein

Crop - a plant that is cultivated and harvested for food, fiber, or economic purposes

Flavonoid - a type of phytochemical found in fruits, vegetables, and tea; known for antioxidant and anti-inflammatory properties

Genetically-Modified (GM) Crop - a crop whose DNA has been altered using genetic engineering techniques to introduce traits like pest resistance, herbicide tolerance, or improved nutritional content

"Green" biotechnology - a branch of biotechnology focused on developing genetically modified crops, biofertilizers, and biopesticides to promote sustainable farming

Pest - any organism that damages or interferes with crops, livestock, food, or property—commonly includes insects, weeds, fungi, and rodents

Pesticide -a chemical or biological agent used to kill or control pests, including insects (insecticides), weeds (herbicides), fungi (fungicides), and others

Phytochemical - a naturally occurring compound in plants that is not essential for life but may offer health benefits; examples include antioxidants

Vaccine - a biological preparation that stimulates the immune system to protect against a specific disease

Vitamin - an essential nutrient that the body needs in small amounts to function properly; examples include vitamin C, vitamin D

9.4: Benefits of "Green" Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

10: Medical Biotechnology – “Red” Biotechnology

10.1: Principles of Medical Biotechnology

10.2: Diagnostic Biotechnology

10.3: Therapeutic Biotechnology

10.4: Regenerative Medicine

10: Medical Biotechnology – “Red” Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

10.1: Principles of Medical Biotechnology

Biotech Focus

A brief history of medical biotechnology:

- ~6000 BCE – 1000 BCE: early humans use fermentation to make bread, beer, and wine
- ~500 BCE: traditional Chinese and Indian medicine begins using plant-based remedies
- 1796: Edward Jenner develops the first vaccine to protect against smallpox
- 1865: Gregor Mendel discovers the basic laws of heredity
- 1885: Louis Pasteur develops the rabies vaccine
- 1919: Károly Ereky coins the term “biotechnology”
- 1953: Watson and Crick discover the double-helix structure of DNA
- 1973: Cohen and Boyer develop recombinant DNA technology by cloning a gene into a bacterial plasmid
- 1978: first synthetic human insulin (Humulin) produced using genetically-engineered bacteria
- 1982: FDA approves Humulin
- 1986–1990s: development of vaccines (e.g., Hepatitis B) and monoclonal antibodies using biotechnology techniques
- 1990: launch of the Human Genome Project to map all human genes
- 1999–2000: first gene therapy trials for genetic disorders begin
- 2003: completion of the Human Genome Project
- 2012: introduction of CRISPR-Cas9 for gene editing
- 2020: mRNA vaccines approved for COVID-19

Introduction

Medical biotechnology is the branch of biotechnology that focuses on using living cells, cell materials, and biological processes in order to develop products and technologies that help prevent, diagnose, and treat diseases. It's an area where biology, medicine, and technology come together to improve human health.

As scientists began to understand biological systems, they began to use them to develop new diagnostic tools and therapeutic strategies. With time, the field of medical biotechnology began to emerge. Key aspects of medical biotechnology include:

1. **Production of medicines and vaccines:** uses genetically-engineered organisms, such as bacteria or yeast to produce therapeutic compounds like drugs, hormones, antibodies and vaccines
2. **Gene therapy:** treats genetic diseases by repairing, replacing, or modifying faulty genes inside a patient’s cells
3. **Tissue engineering and regenerative medicine:** grows artificial tissues and organs in the lab to replace damaged ones
4. **Diagnostic tools:** develops advanced biotech-based tests to quickly and accurately detect diseases
5. **Personalized medicine:** tailors medical treatments based on an individual’s genetic profile to maximize effectiveness and minimize side effects

In many cases, medical biotechnology can lead to more cost-effective treatments and diagnostics compared to traditional methods. However, medical biotechnology raises numerous ethical concerns. For example, ethical debates surround the long-term effects of gene therapy, as well as the potential for modifying the human germline. Concerns about the use of genetic information and patient privacy also have been raised. There are many people who are worried about ensuring equitable access to new biotechnology-based treatments and diagnostics, particularly in underserved populations. How can someone who is economically disadvantaged afford the high costs of tech treatments created using medical biotechnology? Despite these concerns, it is hard to deny that medical biotechnology has revolutionized the medical field and improved human health.

Concept in Action

Red Biotechnology: The Future of Medical Science

Learning Objectives

Medical biotechnology blends biology, medicine, and technology to create solutions for diagnosing, treating, and preventing diseases. At the end of this page, you should be able to:

- Define medical biotechnology
- List and explain the major principles of medical biotechnology

Principles of Medical Biotechnology

Medical biotechnology relies on the manipulation of biological systems at both the molecular and cellular level so that the resulting treatments are effective, safe, and ethical. To do so, medical biotechnology uses the following (Figure 10.1.1):

1. **Genetic Engineering:** the manipulation of DNA to alter the genetic material of an organism
   - involves the use of recombinant DNA technology
   - often used to insert health genes in order to correct genetic disorders
   - e.g., the production of hormones using genetically modified bacteria
   - to read more about genetic engineering, go to Chapter 4: Genetic Engineering and Recombinant DNA Technology
2. **Cell Culture Technology:** the growth of cells outside of their natural environment in controlled settings, used for research, vaccine production, and regenerative medicine
   - can include the use of stem cells or organ culture
   - to read more about cell culture, go to Chapter 6: Cell Culture Fundamentals
3. **Molecular Diagnostics:** uses molecular biology techniques to detect genetic mutations, pathogens, or diseases at the molecular level
   - includes areas such as next generation sequencing, microarrays, and PCR
   - to learn about PCR, go to Chapter 3.2: Replication of DNA
   - to read more about DNA sequencing, go to Chapter 5.3: Genome Sequencing
4. **Biomanufacturing:** the production of biological products such as vaccines, therapeutic proteins, and antibodies on a large scale using living systems like bacteria, yeast, or mammalian cells
   - to read more about biomanufacturing, go to Chapter 11.2: Bioprocess Engineering
5. **Immunotechnology:** engineers the immune system for the treatment or prevention of diseases
   - e.g., developing monoclonal antibodies for cancer therapy
   - e.g., CAR-T cell therapy
6. **Bioinformatics:** the application of computational tools to understand biological data, such as analyzing gene sequences or modeling biological processes
   - includes areas such as genomics, proteomics

- to read more about genomics, proteomics, and bioinformatics, go to Chapter 5: Genomics and Proteomics

7. **Ethical and Regulatory Compliance:** ensures that biotechnology applications meet ethical standards and are approved by regulatory authorities
   - includes regulatory organizations like the FDA and compliance procedures, such as GMP

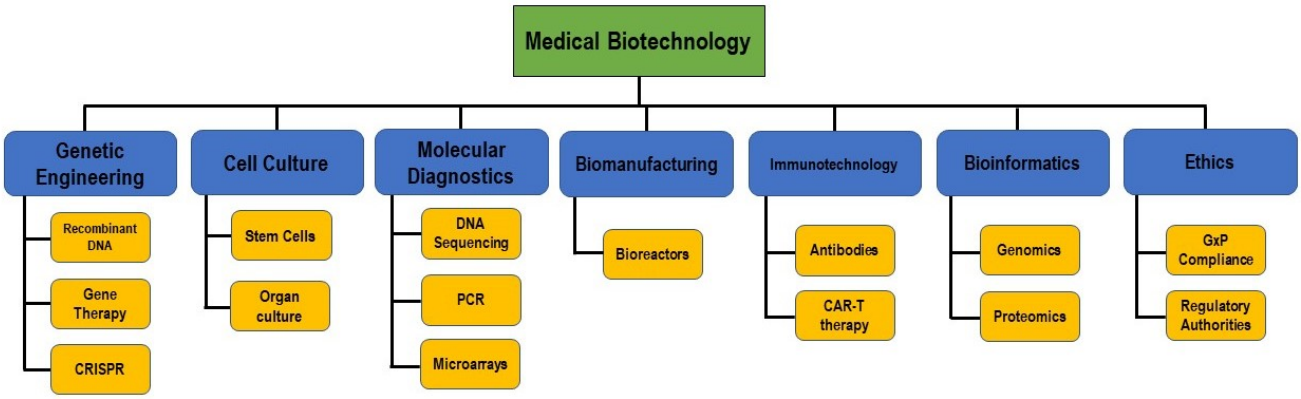


Figure **10.1.1**: Medical Biotechnology Principles. (Medical Biotechnology by Patricia Zuk, CC BY 4.0)

Key Concepts

Medical biotechnology uses biological systems in the production of therapeutic compounds, like drugs and vaccines.

Some important concepts to remember are:

- Medical biotechnology manipulates biological systems at the molecular and cellular level to create treatments are effective, safe, and ethical
- Medical biotechnology involves areas such as genetic engineering, cell culture, bioinformatics, and biomanufacturing

Glossary

Bioinformatics - an interdisciplinary field that combines biology, computer science, mathematics, and statistics to analyze and interpret biological data

Biomanufacturing - the production of biological products, such as vaccines, therapeutic proteins, or enzymes using living cells or organisms in industrial settings

Cell Culture - a laboratory technique used to maintain and/or grow eukaryotic cells in vitro; usually refers to mammalian cells

Genetic engineering - the direct modification of an organism's DNA using biotechnology; often done to introduce new traits, to improve resistance to disease, or to produce beneficial products

Immunotechnology - the use of immune system-based tools and techniques for diagnostics, vaccine development, and treatment of diseases

Molecular diagnostics - techniques used to detect and measure specific genetic material or proteins in a sample in order to diagnose diseases at the molecular level

10.1: Principles of Medical Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

10.2: Diagnostic Biotechnology

Biotech Focus

Conventional laboratory techniques for diagnosing metastatic breast cancers include a combination of imaging, tissue analysis, and biomarker testing. Tumor biopsies are tested for common breast cancer biomarkers like the ER marker (estrogen receptor) and the HER2 marker (human epidermal growth factor receptor 2; also called HER2/neu) using immunological methods, like FISH, and the BRCA-2 marker using molecular techniques such as DNA sequencing. These methods can be time-consuming and expensive to perform.

However, researchers at Hacettepe University and Abant İzzet Baysal University in Turkey have created a potential means of rapidly diagnosing HER2-positive breast cancer using a unique biosensor called a **quartz crystal microbalance (QCM).** A QCM is a **piezoelectric sensor**, means that it uses a thin quartz crystal that oscillates at a specific frequency. When molecules accumulates on the crystal, the change in mass of the crystal decreases its oscillation frequency. This frequency shift is directly proportional to the mass of the material that has bound to the crystal surface, even at the nanogram or even picogram level. To detect breast cancer, researchers can coat the crystal with nanoparticles bound to transferrin, a iron-binding protein. As a result of their enhanced metabolic activity, aggressively-growing breast cancer cells are known to overexpress the receptor to transferrin (i.e., the transferrin receptor). As a result, breast cancer cells will bind to this sensor via their transferrin receptor and change its oscillation frequency.

To test the QCM biosensor, breast cancer cells were added to a transferrin-coated crystal to allow for binding. The oscillation frequency was measured as an indication of the concentration of this cancer biomarker. Researchers found that the QCM biosensor function correlated well with the number of transferrin receptors on the cells, making this a potential diagnostic tool for identifying the early stages of breast cancer, monitoring its progression, or evaluating a patient's response to therapy.

To learn about recent advances in using a QCM in detecting breast cancer, include coating the crystal's nanoparticles with antibodies to the HER2/neu biomarker, check out this article.

Introduction

Diagnostic biotechnology involves the use of biological tools and techniques to detect and/or measure **biomarkers** associated with diseases, genetic conditions, or infections. The exploitation of these tools and techniques has led to the industrial production of biological products for diagnostic purposes. These products are now used in clinical laboratories around the world. A **biomarker, or biological marker**, is a measurable indicator of some biological state or condition. Biomarkers are often used to indicate a normal or abnormal process, identify a disease, or evaluate a response to treatment. In medicine, biomarkers are usually molecules found in blood, other body fluids, or in tissues.

Biomarkers are classified according to their use and include:

- **Diagnostic biomarkers:** used to diagnose a condition like a disease
  - e.g., estrogen and progesterone receptors used in the diagnosis of breast cancer
- **Prognostic biomarkers:** markers that assess the progression of a diagnosed disease, regardless of treatment
  - e.g., Brain Natriuretic Peptide (BNP) expression used to assess heart failure prognosis
- **Predictive biomarkers:** markers that predict a response to a specific treatment
  - e.g., HER2 receptor used to predict response to chemotherapies
- **Monitoring biomarkers:** markers that track disease progression or treatment response over time
  - e.g., troponin synthesis used to monitor heart disease
  - e.g., creatinine levels used to monitor kidney disease
- **Susceptibility biomarkers:** markers that identify an increased risk of developing a disease
  - e.g., BRCA1/BRCA2 mutations used to assess the risk of breast cancer
  - e.g., LDL levels used to assess atherosclerosis

- **Pharmacodynamic/Response biomarkers:** markers that indicate a biological response to a drug in order to assess whether the drug is working
  - e.g., decrease in hemoglobin A1c (HBA1c) levels in response to diabetes treatment
- **Safety biomarkers:** markers that indicate toxicity of adverse effects of a specific treatment
  - e.g., liver enzymes monitored for drug-induced liver injury

Here are some key areas used in diagnostic biotechnology:

1. **Molecular Diagnostics**
2. **Immunodiagnostics**
3. **Point of Care (POC) Diagnostics**

Learning Objectives

Diagnostic biotechnology uses biological tools and techniques to diagnose diseases, disorders, and conditions. At the end of this page, you will be able to:

- List and define the key areas of diagnostic biotechnology
- Explain next-generation sequencing
- Explain the DNA microarray
- Explain Fluorescent In Situ Hybridization (FISH)
- Describe the structure of an antibody
- Explain the different types of ELISAs
- Explain how the "sandwich" ELISA is performed and how this protocol differs from the direct and indirect ELISA
- Define point-of-case diagnostics (POC)
- Explain how a lateral flow assay (LFA) works
- Define biosensor and explain how one works in POC
- Explain how the glucose monitor works as a biosensor
- Explain the lab-on-a-chip

Molecular Diagnostics

Molecular diagnostics is a field of medical testing that uses molecular biology techniques to detect and measure specific DNA, RNA, or protein markers in cells or tissues. These tests are used to diagnose diseases, predict risk, guide treatment, and monitor health conditions at the molecular level.

Key techniques used in molecular diagnostics are:

1. **Polymerase Chain Reaction (PCR):** amplifies specific DNA or RNA sequences
   - used to detect the presence of specific genetic markers associated with diseases and infections
   - to read more about PCR, go to Chapter 3.2: Replication of DNA
2. **Real-Time PCR (qPCR):** quantifies the amount of genetic material in a sample
   - used to quantitate the expression level of genetic markers
   - to read more about PCR, go to Chapter 3.2: Replication of DNA
3. **Next-Generation Sequencing (NGS):** sequences entire genomes or targeted regions for mutations
4. **Microarrays:** analyzes the expression levels of thousands of genes at once
   - two major types: DNA microarrays and protein microarrays
   - to read about protein microarrays, go to Chapter 5.6: Proteomics
5. **In situ hybridization (e.g., FISH):** detects specific DNA or RNA in cells

Next Generation Sequencing

While modern day Sanger sequencing instruments are still used by the majority of research facilities to quickly sequence a small DNA construct, they are limited by the relatively small number of sequencing reactions that can be analyzed at one time (e.g. 8 to

96). **Next-generation sequencing (NGS)** is a powerful platform that enables the sequencing of thousands to millions of DNA molecules simultaneously, thus allowing for the sequencing of an entire genome in one day. In NGS, large-scale DNA libraries are first created by shearing the genome into smaller fragments, denaturing them into single strands and attaching specialized oligonucleotide sequences, called "adaptors" to each end of the DNA fragment (Figure **10.2.1**). These adaptors allow for hybridization of the DNA library fragments to complementary sequences called DNA "linkers". These linkers are found coupled to a specialized glass surface, called a **flow cell**. The adaptors also contain small stretches of sequence that can serve as an "index" to identify the bound fragment once sequencing is complete.

Once bound to their linker, each DNA library fragment is amplified through a multi-step process called **bridge amplification**. This unique amplification method produces thousands of DNA copies identical to the original bound DNA fragment. Because these copies are located at a specific area of the flow cell, they are called "clusters". The DNA in each cluster becomes the starting point for a subsequent sequencing reaction called **Sequencing by Synthesis or (SBS)**. SBS uses using fluorescently-labeled "terminator" nucleotides that are similar to those used in Sanger sequencing. However, these nucleotides are said to be "reversible". During SBS, a DNA polymerase synthesizes a DNA chain that is complementary to the bound library fragment using these fluorescent nucleotides. The addition of each nucleotide is known as a "round" or "cycle". A camera takes an image after each round in order to detect the fluorescent signal. Following incorporation of the nucleotide, the fluorescent tag and terminator are removed, thus permitting a new round. The process of nucleotide incorporation and terminator/fluorescent tag removal is repeated for 50 to 300 cycles, depending on the fragment size.

At the end of these SBS phase, the fluorescent images from each cluster are converted into a sequence of bases called a "read". The reads from each cluster are analyzed using bioinformatic programs that align the DNA sequences using a reference genome or assemble them de novo into a genomic sequence.

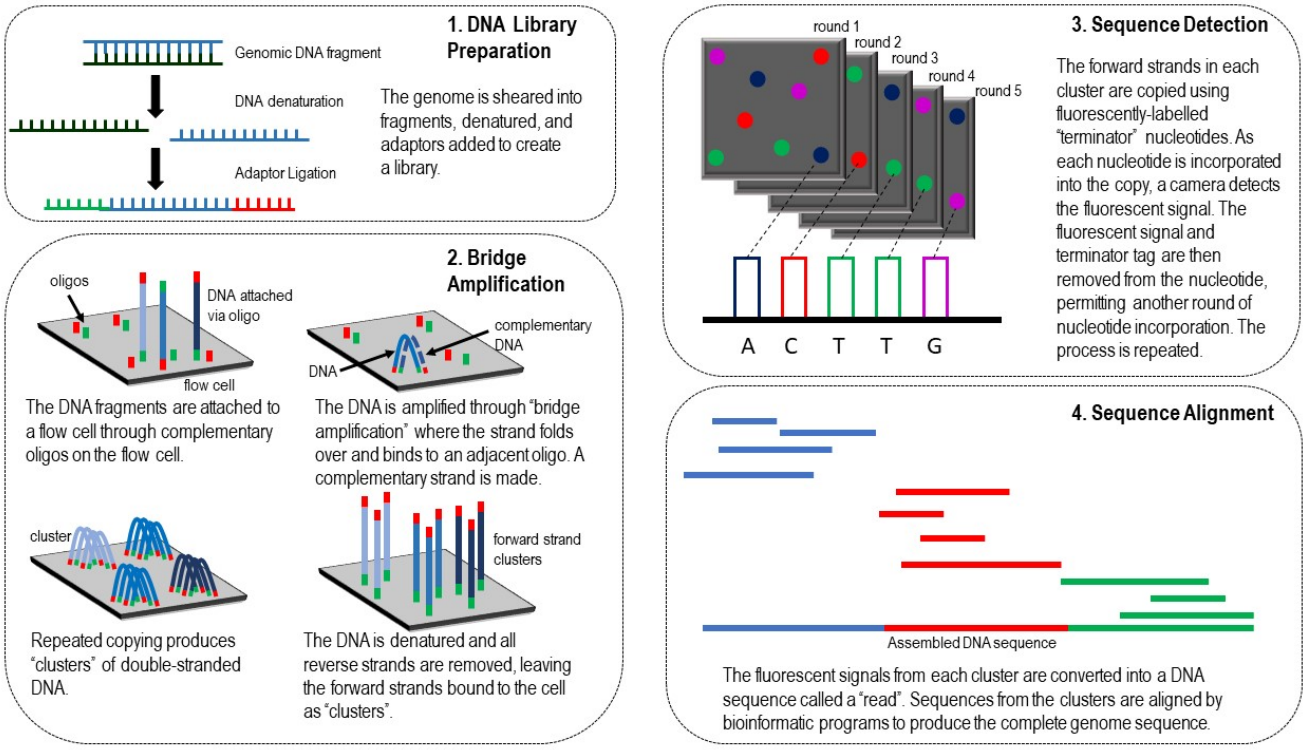
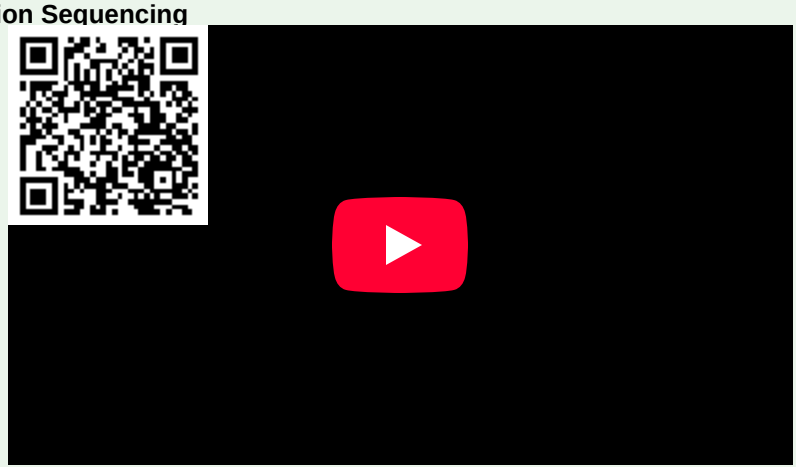


Figure **10.2.1**: The Next Generation Sequencing (NGS) workflow. NGS can sequence multiple genomes in a day. (1) DNA Library Production: The genome is sheared into smaller fragments. Each fragment is flanked by two types of adaptors to create a DNA library for sequencing. (2) Bridge Amplification: Through their adaptors, DNA library fragments are bound to complementary DNA oligos located at specific locations on a "flow cell". The bound genomic fragments are then amplified through "bridge amplification" where the DNA strand folds over and binds to an adjacent oligo. A complementary strand is made. Repeated copying produces "clusters" of double-stranded DNA. The DNA is denatured and all reverse strands are removed, leaving the forward strands bound to the cell as "forward strand clusters". (3) Sequence Detection: The forward strands in each cluster are copied using fluorescently-labeled "terminator" nucleotides. As each nucleotide is incorporated into the copy, a camera detects the fluorescent signal. The fluorescent signal and terminator tag are then removed from the nucleotide, permitting another round of nucleotide incorporation. The process is repeated. (4) Sequence Alignment: The fluorescent signals from each cluster are converted into a DNA sequence called a "read". Sequences from the clusters are aligned by bioinformatic programs to produce the complete genome sequence. (Next Generation Sequencing (NGS) Workflow by Patricia Zuk, CC BY 4.0)

NGS offers researchers a high-throughput option by giving labs the capability to sequence the genomes from multiple individuals at the same time, allowing a genomic comparison between normal individuals and those with a disease or disorder. Through this work, diseases and disorders can be linked to specific DNA mutations.

Concept in Action:

Video: Next Generation Sequencing



Microarrays

DNA Microarrays can be used to measure the expression of thousands of genes simultaneously and are often used to compare gene expression between different cell types or conditions. The microarray is a solid surface onto which short DNA sequences, called **DNA probes (or probes)**, are affixed in a grid pattern. Each DNA probe corresponds to a specific gene in an organism's genome. To perform a microarray, **total RNA** is extracted from a sample being studied and converted into **complementary DNA (cDNA)**. A fluorescent label is then added to the cDNA. The labeled cDNA sample is added to the microarray, where the cDNA molecules will bind to their complementary probe sequence. The unbound cDNA is washed away, leaving only the labeled cDNA molecules bound to ther DNA probes. The microarray is then scanned with a laser, exciting the fluorescent labels and capturing the fluorescent signal from each spot. Binding of a cDNA molecule to its probe indicates expression of a specific gene. The intensity of fluorescence at each probe spot indicates the expression level of that gene.

Multiple samples can be analyzed using microarrays, ranging from hundreds of samples analyzed by "high-throughput" platforms (e.g., Affymetrix or Illumina) to just two samples (i.e., a dual-color microarray). In a dual-color microarray, the cDNA from each sample is labeled with a different fluorescent label, such as green for the control sample and red for the experimental sample. The samples are applied to the microarray as described above. The fluorescent color and intensity is measured at each DNA probe spot and then used to compare gene expression levels between the two samples (Figure **10.2.2**).

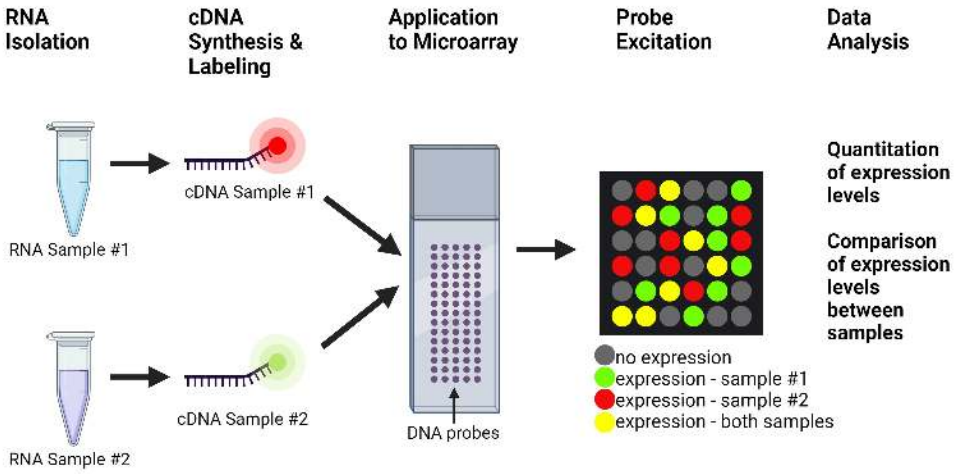


Figure **10.2.2**: The steps of dual-color microarray analysis. (1) RNA Isolation: RNA is isolated from two different samples (RNA Sample #1 and RNA Sample #2). (2) cDNA Synthesis & Labeling: cDNA samples are synthesized from these RNA samples and labeled with different fluorescent molecules (cDNA sample #1 with red probe, cDNA sample #2 with green probe). (3) Application to Microarray: the labeled cDNA samples are added to a microarray slide made of thousands of short DNA sequences (DNA probes). Binding of a labeled cDNA to the complementary DNA probe represents expression of a specific gene. (4) Probe Excitation: Excitation of the fluorescent probes produces fluorescent signals that are detected by a fluorescent reader. Spots with red fluorescence represent gene expression in sample #1. Spots with green fluorescence represent expression in sample #2. Spots with yellow fluorescence (i.e. green plus red) represent gene expression by both samples. Empty spots (gray color) indicate no cDNA binding to a probe and no expression of that gene. (5) Data analysis: quantitation of gene expression levels can be determined by measuring fluorescent levels on the slide. Comparison of expression levels between cDNA samples can also be performed. (Microarray Analysis by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. 2025)

Microarrays allow researchers to assess gene expression profiles across thousands of genes simultaneously. Like NGS, data obtained from DNA microarray analysis can be used to compare the genetic "signatures" of a disease or infection to its healthy counterpart. However, proper data analysis is important since the amount of data generated by a microarray can be significant.

Concept in Action

DNA Microarray Methodology

Fluorescent *In Situ* Hybridization (FISH)

Fluorescent *In Situ* Hybridization (FISH) is a molecular technique that uses a **fluorescently-labeled probe** to detect specific DNA or RNA sequences in a cell or tissue. Because this hybridization occurs within a cell or tissue, it is considered to be *"in situ"* (i.e., in place). Using a fluorescent microscope, FISH allows scientists to confirm the presence and location of a genetic sequence within the sample. The probes used for FISH can be either DNA or RNA. These DNA or RNA probes are chemically synthesized and are typically designed to be small in length (~20 to 50 nucleotides). The DNA probe is designed to be complementary to a

specific DNA sequence in the sample, while an RNA probe is complementary to a specific mRNA or miRNA sequence. Once synthesized, the DNA or RNA probe is then labeled with a fluorescent dye. However, DNA probes can also be labeled with other compounds like fluorescently-bound antibodies. The actual process of FISH will depend on whether the probe is RNA or DNA and how the probes are labeled. To perform FISH, the sample for analysis is prepared by "fixing" the cell or tissue onto a microscope slide (Figure **10.2.3**). Chemicals such as paraformaldehyde or formaldehyde are used. Following fixation, the sample is permeabilized with a detergent to allow for penetration of the probe into the sample and the DNA on the slide is denatured. The probe is allowed to bind, or hybridize, to its target sequence in the sample. Excess probes are washed away and the sample is viewed under a fluorescent microscope.

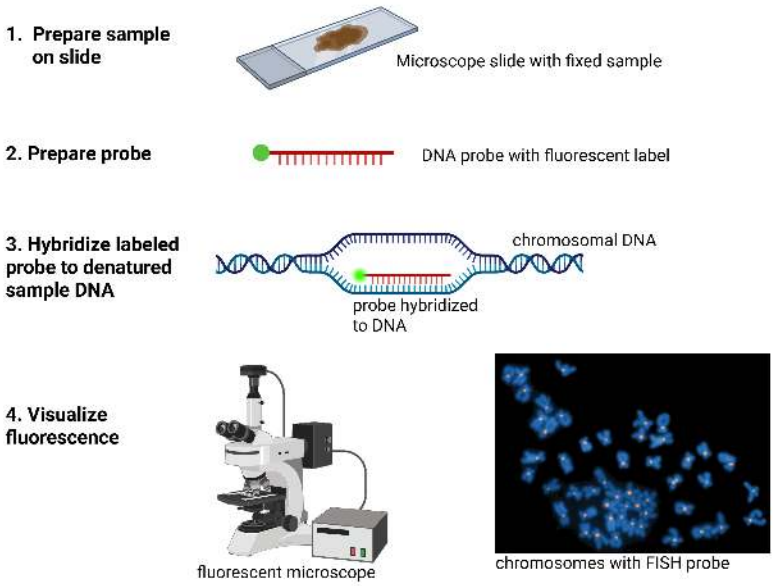


Figure **10.2.3**: Fluorescent in situ hybridization (FISH). (1) Prepare sample on slide: samples a fixed to a microscope slide, permeabilized with detergent and the DNA in the sample denatured. (2) Prepare probe: DNA (or RNA) probes are synthesized to be complementary to a target DNA sequence in the sample and then labeled with a fluorescent probe (green circle). (3) Hybridize labeled probe to denature sample DNA: the probe is hybridized to the sample to allow for the binding of the probe sequence to its complementary target on the sample's chromosome. (4) Visualize fluorescence: fluorescence can be viewed using a fluorescent microscope. The physical location of probe binding to chromosomes (shown in blue) is seen as orange fluorescent dots. (FISH by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025); image credit: Karyotype of Zebrafish by Freeman et al., CC BY-2.0)

FISH is a fast and specific molecular technique that has numerous medical applications, including the detection of chromosomal abnormalities during pre-natal testing (e.g., Down's syndrome), the diagnosis of cancer (e.g. HER2 in breast cancer), the identification of microbes within a sample, the expression of a gene within a tissue, or the mapping of genes to a specific chromosome. Examples of diseases and syndromes that can be detected using FISH include, acute lymphoblastic leukemia, chronic myelogenous leukemia, Down's syndrome, Cri-du-Chat, and Angelman syndrome. In medicine, FISH is also used to evaluate disease prognosis and remission of a disease like cancer. Treatments can then be tailored to fit the patient.

Immunodiagnostics (Immunoassays)

Immunodiagnostics is a group of diagnostic techniques that use the specificity of **antibodies** to detect and measure substances in biological samples. An **antibody,** also known as an **immunoglobulin**, is a protein produced by the B-cells of the immune system in response to the presence of a foreign substance known as an **antigen**. Antigens are typically sugars or proteins found on the surface of cells (i.e., bacterial, fungal, mammalian) and viruses. The antibody is made of four polypeptide chains, two **heavy chains** and two **light chains**, that link together to form a Y-shape (Figure **10.2.4**). The tips of these four chains is known as the **variable region (or antigen-binding site)**. This region changes its shape from antibody to antibody to "match" a specific antigen, giving the antibody the critical property of specificity. The remaining regions of the antibody, called the constant region, can bind to other immune cells or immune components to heighten the immune response. Once bound to the antigen, the antibody can neutralize the

antigen, identify the invading cells for destruction by the immune system, and activate the complement system, enhancing the immune response.

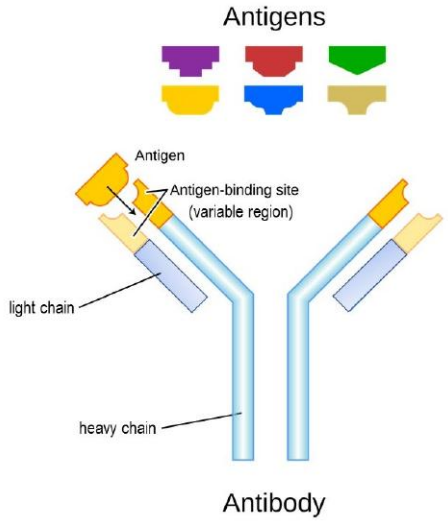


Figure **10.2.4**: The structure of an antibody. The antibody is comprised of four polypeptides known as two heavy chains and two light chains. The ends of the light and heavy chains are known as the antigen-binding site or the variable region (shown in yellow). This variable region changes its shape to reflect the specific antigen that binds to this region. (Antibody Structure by Patricia Zuk, CC BY 4.0; modified from Antibody by Fvasconcellos, public domain)

Immunodiagnostic tests are used to identify infectious diseases (e.g., HIV, hepatitis, COVID-19), autoimmune diseases (e.g., rheumatoid arthritis, lupus), and cancer markers (e.g., PSA, CA-125). They are also used in allergy testing and can quantitate substances like hormones (e.g., TSH, insulin) and growth factors (e.g., EGF, VEGF). They are highly specific and sensitive due to the precise interaction between antibodies and antigens. As such, immunodiagnostic tests can detect minute amounts of substances like hormones, proteins, microbes, or antibodies.

Common immunodiagnostic techniques include:

1. **ELISA (Enzyme-Linked Immunosorbent Assay)** – for detecting antigens or antibodies.
2. **Western Blotting** – for detecting specific proteins in a sample.
3. **Immunofluorescence** – uses fluorescent-labeled antibodies to detect targets in cells or tissues.
4. **Lateral Flow Assays** – like pregnancy tests or rapid COVID-19 tests.
5. **Radioimmunoassay (RIA)** – uses radioactive isotopes to detect small amounts of substances.

The Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA stands for **Enzyme-Linked Immunosorbent Assay**. The ELISA is a common laboratory technique that is used to detect and measure an antigen, such as a protein, found in a sample. Samples that are assayed using an ELISA can range from blood and urine (assayed in clinical labs) or cell lysates (analyzed in clinical and research labs). At the end of the ELISA, a "signal" is produced that can be detected and measured. Antigens that can be detected through ELISAs include antibodies, hormones, growth factors, and toxins. ELISAs are used to diagnose diseases (e.g. HIV, hepatitis), detect allergens in food, monitor hormone levels in blood and urine, test for toxins or pathogens in water or food, and measure antibody levels (i.e., titers) in blood following vaccination.

Concept in Action

The ELISA

There are several types of ELISAs (Figure 10.2.5), including the **direct ELISA** and **indirect ELISA**. In both of these ELISAs, the sample is added to a solid surface in order to bind the antigen to it. The direct ELISA uses an enzyme-bound primary antibody to directly bind the antigen. The direct ELISA is quick and easy to perform but produces a low signal intensity as there is only a single type of antibody used. The antigen in the indirect ELISA is bound by a primary antibody, followed by an enzyme-coupled secondary antibody. Because multiple secondary antibodies can bind a single primary antibody, the indirect ELISA produces an amplified signal. It is also a more flexible ELISA because different types of secondary antibodies can be used. The direct and indirect ELISA use an antigen-coated solid support. In contrast, the **"sandwich" ELISA** starts with a solid support coated with an antibody. In the "sandwich" ELISA, the sample protein to be detected is captured or "sandwiched" in between the antibody-coated surface and the detection antibodies used for quantitation. The sandwich ELISA has high sensitivity and amplification, but non-specific antibody binding can interfere with the results.

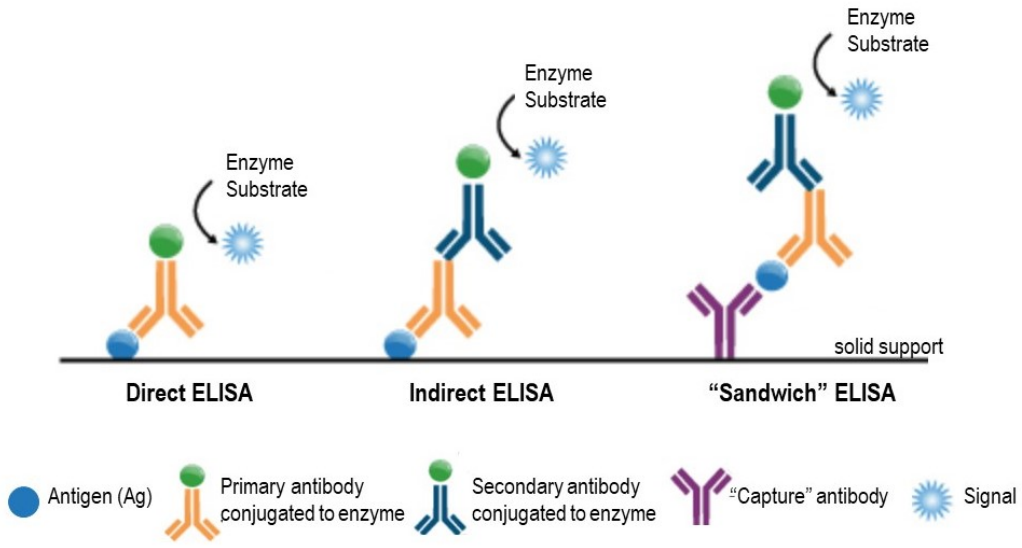


Figure 10.2.5: ELISA types. Some of the major types of ELISAs are direct, indirect, and "sandwich". In the direct ELISA, a primary antibody, coupled to an enzyme binds the antigen bound to the solid support. In the indirect ELISA, a primary antibody binds the antigen, which is then bound by the enzyme-coupled secondary antibody. In the "sandwich" ELISA, the solid surface is coated with a "capture" antibody. This capture antibody binds the antigen. The bound antigen is then detected with a primary antibody and enzyme-coupled secondary antibody. For each ELISA type, the antibody-bound enzyme processes a substrate into a signal that can be detected and quantified. (ELISA Types by Patricia Zuk, CC BY 4.0; modified from ELISA Types by Allan.richard5093, CC BY-SA 4.0)

To perform an "sandwich" ELISA, the following steps are taken:

1. **Coating:** A solid surface, usually a 96-well plate, is coated with an antibody

2. **Blocking:** Non-specific binding sites on the plate are blocked to prevent background signal
3. **Sample addition:** The sample is added and binding of the target to the antibody occurs
4. **Probing:** A target-specific primary antibody is added to the plate to bind and identify the bound target
5. **Washing:** Excess primary antibody is removed
6. **Detection:** A secondary antibody that is linked to an enzyme is added to bind the primary antibody
7. **Washing:** Excess secondary antibody is removed
8. **Substrate Addition:** A substrate specific for the enzyme is added; the enzyme reacts with the substrate to produce a color change or a fluorescent or luminescent signal
9. **Measurement:** The intensity of the signal is measured using a spectrophotometer or luminometer; this measurement is proportional to the amount of the target molecule

The procotols for direct and indirect ELISAs are very similar to the "sandwich" ELISA. Each protocol is summarized below in Table 10.2.1.

Table 10.2.1: ELISA Protocols

ELISA Step	Direct ELISA	Indirect ELISA	"Sandwich" ELISA
1. Coating	Coat with sample	Coat with sample	Coat with "capture" antibody
2. Blocking	Yes	Yes	Yes
3. Sample addition	No	No	Yes
4. Probing	Yes, primary antibody coupled to enzyme	Yes; uncoupled primary antibody	Yes; uncoupled primary antibody
5. Washing	Yes	Yes	Yes
6. Detection	No	Yes, secondary antibody coupled to enzyme	Yes, secondary antibody coupled to enzyme
7. Washing	No	Yes	Yes
8. Substrate Addition	Yes	Yes	Yes
9. Measurement	Yes	Yes	Yes

Lateral flow assays

A **lateral flow assay (LFA)** is a simple, fast, and portable test used to detect the presence of a substance in a liquid sample like water, blood, or saliva. These substances can include proteins, pathogens, or chemicals. The best example of an LFA is a pregnancy test. However, other LFAs include those that will detect viral infections (e.g., the COVID-19 test), bacterial contamination, and the presence of heavy metals in water.

The LFA (Figure 10.2.6) is made of the following parts:

- a **sample pad** where the sample is introduced
- a **conjugate pad** that contains detector molecules, such as antibodies or DNA probes, that mix with the sample
  - the detector molecules are tagged with a visible label
- a **nitrocellulose membrane** as the core of the LFA
  - multiple membranes could be present with each strip performing a specific function
  - the membrane has a **test line** that indicates whether the target is present with a physical line and a **control line** that confirms whether the test worked
- an **absorbant pad (wicking pad)** that pulls the liquid through the strip and soaks up excess fluid

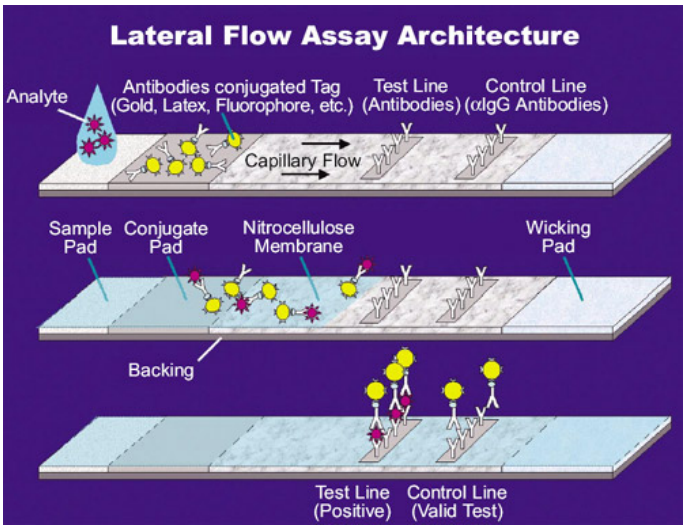


Figure **10.2.6**: The lateral flow assay. The lateral flow assay (LFA) test strip is made of a sample pad, a conjugate pad containing antigen-specific antibodies conjugated to a tag like a gold nanoparticle, a nitrocellulose membrane with an antibody-bound test line, and a wicking pad. The sample (i.e. the analyte) is applied to the sample pad and capillary action draws the sample through the remaining parts of the LFA test strip. The antigens found in the sample bind to the conjugated antibodies in the conjugate pad. If the antigen also binds these antibodies found in the test strip, the conjugated tags will form a physical line and indicate a positive result. (Lateral Flow Assay, no author, public domain)

LFAs are easy to use. The sample is added to the sample pad and the liquid flows along the conjugate pad and into the nitrocellulose pad due to capillary action. If the target substance is present it will bind to the tagged detector molecules. The resulting complexes will stick to the test line found in the nitrocellulose membrane and create a visible line to indicate a positive test result.

Point of Care Diagnostics

The LFA describe above is an example of **Point of Care Diagnostics (PCD)**. PCD are medical tests done at or near the place where the patient is located. PCD allows for rapid diagnostics, rather than waiting for a distant lab to provide results. PCD are relatively easy to perform and can be performed by medical personnel and even the patient themselves. The rapid turn around time gives medical personnel the chance to make quick decisions for the health and well-being of the patient.

Examples of PCD include:

- **Lateral Flow Assays (LFAs)**
- **Glucose Monitors**
- **Lab-on-a-Chip**

Each of these PCD examples provide fast results (often within minutes), are portable and easy to use, require minimal training to operate, and can be used at home, in clinics, ambulances, and even at remote locations where clinics are unavailable.

Lab-on-a-Chip

More advanced PCD are being made possible thanks to **lab-on-a-chip (LOC)** technology. An LOC is a device that integrates one or several laboratory functions on a single integrated circuit, known as the "chip" (Figure **10.2.7**). This chip is only a few millimeters to a few square centimeters and is designed to achieve automation and high-throughput screening (HTS). LOCs can handle extremely small fluid volumes of blood, urine, or saliva, even down to less than a picoliter (i.e., a trillionth of a liter). The LOC is very similar to an LFA, but can provide more complex analysis.

To use an LOC, a tiny drop of fluid is placed into the chip. **Microfluidic channels** move the liquid within the chip using either capillary action, electric fields, pressure, or vacuum. As the liquid moves through the chip, it passes through reaction chambers where it mixes with reagents that allow for the detection of targets like DNA, proteins, or bacteria. The results can appear as a visual color change, a fluorescent signal, or an electrical signal that is sent to an computer or a phone app.

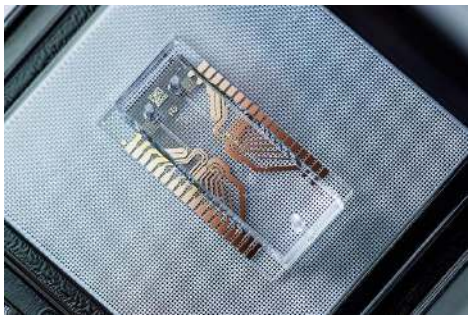


Figure **10.2.7**: a Microfluidic chip. The microfluidic chip is a key element of lab-on-a-chip devices that are outside hospitals. The chip consists of multiple microfluidic channels and a detection system of metal electrodes on a glass substrate. (Microfluidic Chip by FMNLab, CC BY-SA 4.0)

Like immunodiagnostic tests, LOC technology can diagnose diseases, measure hormones and growth factors, and detect toxins. However, their more advanced technology also allows for the analysis of DNA or RNA (i.e. portable DNA sequencers), blood and urine. LOC technology may soon become an important part of efforts to improve global health, as a part of PCD. In countries with few healthcare resources, LOCs may be able to provide rapid and accurate diagnosis of things like infectious diseases.

Biosensors

A **biosensor** is a device that uses a biological component, like an enzyme, antibody, or DNA sequence, to detect a specific substance. The biosensor then converts that detection into a readable signal, such as light, color, or an electric signal. The results are usually displayed as a digital reading, a color change or graph.

The main parts of the biosensor (Figure **10.2.8**) are the:

1. **Bioreceptor** - the biological part that recognizes and binds the target being detected
2. **Transducer** - converts the biological reaction into a signal
3. **Display** - shows the result

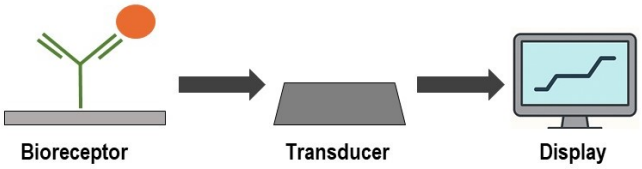


Figure **10.2.8**: The biosensor. The biosensor is made of three components: the bioreceptor for detection of the target, the transducer which converts the detection into a measurable signal, and the display which displays the results (The Biosensor by Patricia Zuk, CC BY 4.0)

Perhaps the most well-known biosensor is the **glucose monitor**. The bioreceptor in this sensor is an enzyme called **glucose oxidase**. Glucose oxidase binds glucose and, through oxidation, converts it to gluconolactone and hydrogen peroxide. This enzyme is physically bound to an electrode. The oxidation reaction releases electrons which flow through the electrode as an electrical current. The amount of current flowing through the electrode is proportional to enzyme activity and glucose concentration in the blood.- The result in then displayed as milligrams per deciliter (mg/dL) or millimoles per liter (mmol/L). In addition to the glucose monitor, biosensors are also used in biotechnology research, drug development, environmental monitoring, and food safety.

Key Concepts

Diagnostic biotechnology involves the use of biological tools and techniques to detect diseases, genetic conditions, or infections. Some important concepts to remember

- molecular diagnostics uses molecular tools like PCR and sequencing to associate DNA sequences with diseases, disorders, and infections
- next-generation sequencing can sequence thousands of DNA fragments at a time and sequence a entire genome in a few hours

- DNA microarrays can analyze sequences from multiple samples at the same time
- NGS and DNA microarrays allow researchers to compare normal genomic sequences to those associated with a specific disease, disorder, or infection
- FISH is used to physically identify the location of a specific DNA or RNA sequence in a genome and can be used to diagnose chromosomal abnormalities, cancer, and microbial contamination
- immunodiagnostics like the ELISA or lateral flow assay (LFA) uses antibodies for diagnosis
- the ELISA is used to measure the amount of a specific antigen or antibody in a sample
- the lateral flow assay (LFA) is a test strip embedded with an antigen-specific antibody
- the LFA is used to quickly identify the presence of an antigen in a sample
- point-of-care diagnostics (PCD) are medical tests performed at local site other than hospital, clinic, or research lab
- PCD include the LFA, biosensors, and the lab-on-a-chip
- a biosensor, like a glucose monitor, uses biological component, like an enzyme, antibody, or DNA sequence, to detect a specific substance in a sample
- advanced biosensors can be combined into a lab-on-a-chip capable of performing multiple chemical steps in a diagnosis

Glossary

Adaptors - short, synthetic DNA sequences attached to a DNA fragment; used in Next Generation Sequencing

Alignment - the process of arranging DNA or amino acid sequences using regions of sequence overlap

Antibody - a Y-shaped protein produced by B cells of the immune system that recognizes and binds to a specific antigen; also called an immunoglobulin

Antigen - a substance (usually a protein or sugar) that triggers an immune response in the body; often used in vaccines

Bioreceptor - a biological molecule or structure that interacts with a target substance

Capillary action - the movement of liquid through a narrow space, like a thin tube or porous material, due to cohesion between water molecules or adhesion between water and the surface

Capillary gel - a thin, glass tube filled with a polymeric gel; used to quickly separate molecules, like DNA fragments, according to their size and electrical charge

Complementary DNA (cDNA) - DNA that is synthesized from an RNA template (usually mRNA) using the enzyme reverse transcriptase

Computational assembly - the process of aligning DNA fragments into a continuous sequence (i.e., a contig) using computer alignment programs

Dideoxynucleotide (ddNTP) - a DNA nucleotide used in Sanger sequencing that lacks the 3'-OH (hydroxyl) group necessary for continued 5'-to-3' DNA synthesis; used to terminate the elongation of the complementary DNA strand

Fluorescently-labeled - a compound that has a fluorescent dye attached to it

High-throughput screening (HTS) - a method for scientific discovery that uses robotics, data processing/control software, liquid handling devices, and sensitive detectors, to allow a researcher to quickly conduct millions of chemical, genetic, or pharmacological tests; used in drug discovery and the fields of biology, materials science and chemistry

Microfluidics - the science and technology of manipulating small volumes of fluids in channels with dimensions of tens to hundreds of micrometers; typically involves volumes of microliters to picoliters

Microfluidic channels - tiny fluid-conducting pathways etched or molded into microfluidic devices; guide fluid flow precisely for chemical reactions, separation, or detection on diagnostics chips or cards

Next Generation Sequencing (NGS) - a high throughput sequencing method that can sequence several genomes in a day; also known as massive parallel sequencing

Oligonucleotide - a short, single-stranded fragment of a nucleic acid, like DNA or RNA, typically containing between 2 and 50 nucleotides; also called an "oligo"

Point of Care Diagnostics (PCD) - medical tests done at or near the place where the patient is located; also called Point of Care Testing (POCT)

Probe - a single strand of DNA or RNA that is complementary to a nucleotide sequence of interest

Reagent - any chemical or biological substance used in a laboratory test or reaction to cause a chemical reaction or detect a target; includes enzymes, antibodies, dyes, and buffers

Sanger sequencing - a DNA sequencing method that uses dideoxynucleotides (ddNTPs) to terminate the synthesis of the complementary DNA chain; also known as chain termination sequencing

Total RNA - the complete set of RNA molecules extracted from a cell or tissue at a given time; includes mRNA, tRNA, and rRNA

Transducer - a cellular component that converts a biological reaction into a signal

---

10.2: Diagnostic Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

10.3: Therapeutic Biotechnology

Biotech Focus

The discovery of insulin in 1922, by Frederick Banting and Charles Best, marked a major breakthrough in medicine and therapy for patients with Type I diabetes. However until the 1970s, Type I diabetics had to rely upon insulin isolated from the pancreas of either cattle or pigs. This necessitated the slaughter of hundreds of thousands of animals each year and could also produce significant immune reactions in certain diabetics. In response, in 1978, David Goeddel and his colleagues at Genentech, successfully inserted the gene for human insulin into *E.coli* cells and confirmed its production by these genetically-modified bacteria. In 1982, recombinant human insulin was approved by the FDA and introduced to the market as **Humulin®** - the first genetically-engineered drug to be approved for human use.

Introduction

Therapeutic biotechnology uses biological systems, organisms, or processes for the development of medical treatments that diagnose, prevent, or cure diseases and disorders. This area of biotechnology relies on the use of tools like recombinant DNA (rDNA) technology, genetic engineering, and gene therapy to create targeted therapies for conditions such as cancer, genetic disorders, autoimmune diseases, and infections. Using these tools, researchers have developed specialized therapies, including monoclonal antibodies, recombinant proteins, and vaccines. Through therapeutic biotechnology, researchers have transformed medicine and significantly improved human health.

Learning Objectives

Through therapeutic biotechnology, researchers can create medical treatments that diagnose, prevent, or cure diseases and disorders. At the end of this page, you will be able to:

- Define therapeutic biotechnology
- Explain gene therapy and some of it approaches
- List some of the pros and cons of viral vectors used in gene therapy
- Explain CAR-T gene therapy and how it can be used to cure cancer
- Explain the potential role of CRISPR in gene therapy
- Define what a therapeutic protein is and explain some major examples
- Explain the difference between a traditional and therapeutic vaccine
- Explain how an mRNA vaccine is made

Gene Therapy

Gene therapy is a therapeutic technique that aims to correct an underlying genetic problem by modifying a person's genes. Through gene therapy a disease can be cured rather than treated. The first step of gene therapy is to identify the faulty gene. Often, the gene at the heart of the issue has been mutated in some way, preventing it from being made or functioning properly. The mutated gene (or genes) can be identified through DNA sequencing and genomic analysis. Once the gene (or genes) has been identified, researchers can begin to design a therapeutic strategy using a gene therapy approach.

There are several approaches used in gene therapy, including:

- Replacing the faulty or missing gene with a normal copy (i.e., "traditional" gene therapy)
- Inactivating or "silencing" the malfunctioning gene using miRNA or siRNA technologies
- Introduction of a new or modified gene to counteract the disease or improve cell function
- Correction of the gene mutation *in situ* through gene editing like CRISPR technology

Traditional gene therapy involves the replacement of a faulty or missing gene through the introduction of "normal" one. This approach has been used in the treatment of diseases, like cystic fibrosis. Cystic fibrosis can be caused by one of several mutations in the **Cystic Fibrosis Transmembrane conductance Regulato**r (CFTR) gene, a gene that codes for a chloride channel found in the plasma membrane of epithelial cells. This chloride channel regulates the movement of chloride and sodium ions across the

plasma membrane and controls the balance of salt and water across the surfaces of epithelial tissues lining the respiratory and digestive systems. When this channel functions properly, it helps keep the mucus coating these epithelial linings thin and slippery. Mutations in the CFTR gene lead to a faulty or absent CFTR protein, resulting in thick and sticky mucus that clogs airways, traps bacteria, and blocks pancreatic enzymes (Figure 10.3.1). Patients suffering from cystic fibrosis have significant breathing problems, lung infections, and digestive issues. Most cystic fibrosis patients will die between the ages of 35 and 50. The introduction of a functional CFTR gene through gene therapy could potentially cure this disease.

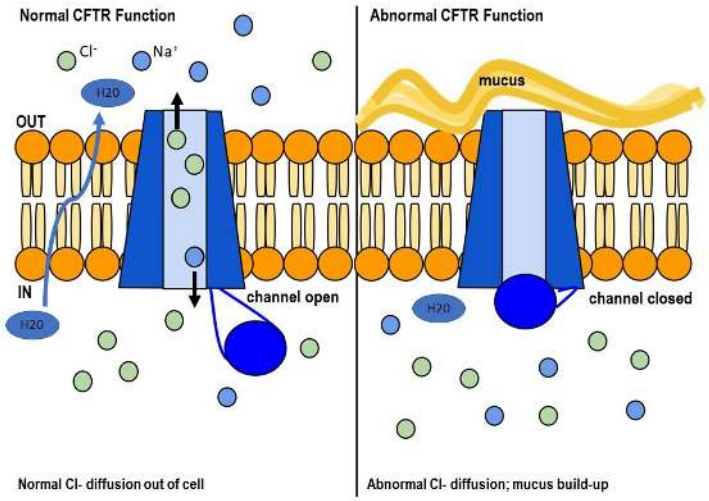


Figure 10.3.1: Cystic fibrosis (CF) and gene therapy. CF is caused by an abnormal CFTR channel protein. The CFTR controls the flow of sodium and chloride ions in and out of the epithelial cells lining the respiratory and digestive systems. In normal CFTR channel function (left panel), the channel is open and chloride ions (Cl-) and sodium ions (Na+) diffuse down their concentration gradients (black arrows). As a result, water (H_20) moves out of the cell through osmosis, minimizing the thickness of the mucus layer covering the cell. In abnormal CFTR function (right panel), the channel is closed to ion diffusion. Abnormal ion diffusion prevents the osmotic movement of water out of the cell and a thick build-up of mucus outside of the cell results. (CFTR Function in CF by Patricia Zuk, CC BY 4.0; adapted from CFTR Protein Panels by Lbudd14, CC BY-SA 3.0)

Traditional gene therapy to treat diseases like cystic fibrosis was first introduced over thirty years ago to limited success. Challenges associated with the limitations in the size of genes that could be corrected, coupled to restrictions in the diseases that could be targeted, prompted researchers to explore other approaches. One growing research is the use of gene therapy to deliver **microRNA (miRNA)** or **small interfering RNA (siRNA)**. As described in Chapter 5.5:Functional Genomics, miRNAs are small non-coding RNA molecules (about 22 nucleotides long) that regulate gene expression by binding to messenger RNAs (mRNAs) and either blocking their translation or causing their degradation. In gene therapy, therapeutic miRNAs could be designed and delivered to down-regulate disease-causing genes, such as oncogenes in cancer. In some diseases, certain miRNAs are underexpressed. By replacing them, normal gene regulation and cell function could be restored. Finally, diseases caused by overactive miRNAs that silence beneficial genes could be inhibited by gene therapy-mediated delivery of anti-miRs or "miRNA sponges", a synthetic or naturally occurring RNA molecule designed to bind (i.e."soak up") microRNAs.

No matter the approach, there are some common steps used in gene therapy. The therapeutic gene is inserted into a delivery system which is usually a viral vector that has been engineered to be safe and non-replicating. The genetic material is then introduced either directly into a patient through intravenous or intramuscular injection (i.e., *in vivo* introduction), or a patient's cells can be removed for genetic manipulation and then reintroduced (i.e., *ex vivo* introduction). Once inside the cells, the new gene will be read by the cell machinery, allowing it to produce a functional protein that restores normal function or fights the disease.

While alternatives to the use of viral vectors are being explored in gene therapy, including the packaging of the corrective genetic material into lipid nanoparticles or direct electroporation into cells, the most common delivery system still remains the viral vector. The choice of viral vector will depend on the size of the gene being cloned, the targeted cell, and the possible immune reaction of the patient.

Common viral vectors include:

1. **Adeno-Associated Virus (AAV)**

- Pros: Low immune response, doesn't integrate into the genome and reduces risk of genetic mutations, long-lasting expression in non-dividing cells
- Cons: Small gene capacity (~4.7 kb), requires repeated dosing in dividing cells
- Currently used for: Eye disorders (e.g., Luxturna for retinal disease), spinal muscular atrophy

2. **Adenovirus**
   - Pros: High gene capacity, efficient delivery to many cell types, fast expression
   - Cons: Strong immune response, usually doesn't integrate into DNA (i.e., temporary expression)
   - Currently used for: Cancer gene therapy, vaccines (e.g., some COVID-19 vaccines)
3. **Lentivirus**
   - Pros: Integrates into the genome for long-term expression, works well in dividing and non-dividing cells
   - Cons: Risk of insertional mutagenesis due to genomic integration, more complex production
   - Currently used for: Blood disorders (e.g., sickle cell disease, β-thalassemia), HIV-related therapies

CAR-T Gene Therapy

***Ex vivo* gene therapy** methods (also called cell-based gene therapy) involve the modification of a patient's cells outside of their body, followed by their re-introduction. The most successful method being used currently is **CAR-T gene therapy** for the treatment of a variety of blood cancers, including leukemia, lymphoma, and multiple myeloma. In CAR-T therapy, a patient's T cells are extracted from their blood and genetically modified in a lab through the introduction of a gene for the **chimeric antigen receptor/CAR.** Expression of this gene results in the expression of the CAR on the surface of the T cell. The modified T cells are expanded in a cell culture facility and infused into the patient's bloodstream (Figure **10.3.2**). Through the CAR, the T cells can bind antigens specific to cancer cells and destroy them. While showing promise, significant side effects of CAR-T therapy have been observed including neurological issues and "cytokine release syndrome" which results in fever, shortness of breath, low blood pressure, and in rare cases, heart or lung issues. The FDA is current investigating instances of secondary T-cell lymphomas in people who have been treated with CAR T-cell therapy.

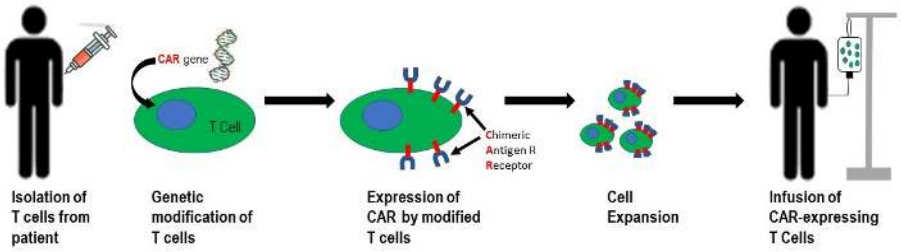


Figure **10.3.2**: CAR-T gene therapy. T cells are isolated from a patient and genetically modified by introducing the gene for the Chimeric Antigen Receptor (CAR gene). Gene expression of this gene leads to the expression of the CAR on the surface of T cells. The modified T cells are expanded in number and then infused back into the patient for the targeted of cancerous cells. (CAR-T Therapy Overview by Patricia Zuk, CC BY 4.0)

Gene Therapy and CRISPR

Early trials in gene therapy approaches showed promise but demonstrated limited effectiveness due to challenges like immune responses and short-lived expression. Newer approaches include CRISPR-based gene editing, which aims to correct the mutation directly in the patient's DNA without the need to introduce additional genetic information. As outlined in Chapter 8.1: Applications of Animal Biotechnology, **CRISPR**, or Clustered Regularly Interspaced Short Palindromic Repeats, is derived from a bacterial defense mechanism that protects bacteria from the integration of viral DNA into the bacterial genome. CRISPR uses a **guide RNA** and the **Cas9 enzyme** to precisely cut DNA at specific locations in the genome. Researchers are exploring its use in correcting, deleting, or replacing genes, making it a powerful tool in combination with gene therapy.

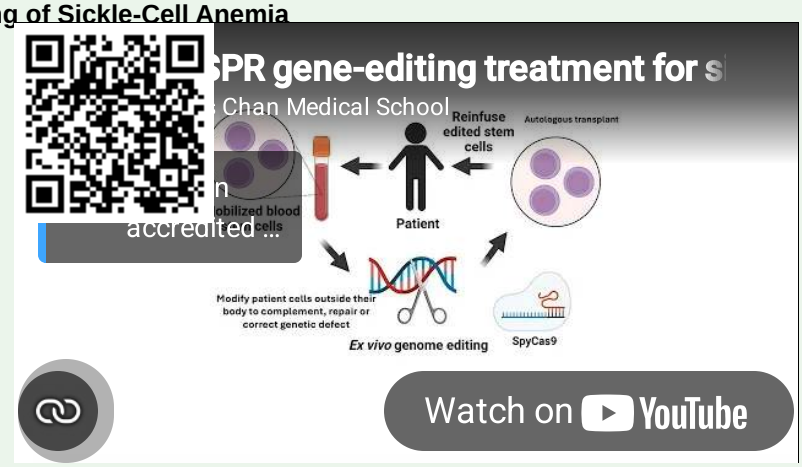
Current gene therapy applications using CRISPR include:

- **Sickle Cell Disease:** used *ex vivo* to edit a patient's blood stem cells in order to "reactivate" fetal hemoglobin and compensate for the defective hemoglobin gene. The edited cells are reinfused into the patient.
- **Leber Congenital Amaurosis (LCA):** uses CRISPR to edit retinal cells *in vivo* to restore vision in patients with this inherited form of blindness
- **Beta-Thalassemia:** similar to sickle cell, CRISPR is being used to edit the genome of bone marrow stem cells to boost healthy hemoglobin production

While the results are exciting, there are limitations to gene therapy and CRISPR. Chief among them is a concern about "off-target" effects. CRISPR may cut unintended parts of the genome and could introduce unintended mutations. Delivery challenges also exist. Getting CRISPR components safely and efficiently into the right cells using viral vectors or nanoparticles is still an obstacle. Finally, an immune response to Cas9 may result in a significant decline in the effectiveness of this technology.

Concept in Action

CRISPR Gene Editing of Sickle-Cell Anemia



Therapeutic Proteins

Therapeutic biotechnology is critical for the development of therapeutic proteins. A **therapeutic protein** is a biologically-engineered protein that is used to treat disease by replacing, enhancing, or modifying a specific physiological function or functions. Therapeutic proteins are typically produced by genetically-modified bacteria, yeast, or mammalian cells that have been modified using rDNA technology. However, therapeutic proteins can also be produced by genetically-modified organisms (i.e., transgenic organisms), such as plants and animals.

There are numerous types of therapeutic proteins with specific functions, including:

- **Enzyme Replacement Therapy (ERT) proteins**: replace missing or defective enzymes in patients with genetic disorders; examples include:
  - lactase: produced to treat lactose intolerance
  - alpha-glucosidase (i.e., Myozyme^{TM}); used to treat for Pompe disease
- **Hormones**: used to regulate biological processes like metabolism, growth, and reproduction; examples include:
  - Insulin: produced by either genetically-modified bacteria or yeast; used to treat Type I diabetes without the need for animal donors
  - Human Growth Hormone (HGH): produced using genetically-engineered bacteria; used to treat growth hormone deficiencies in children and adults.
  - Erythropoietin (EPO): produced by genetically-modified mammalian cells; used to stimulate red blood cell production in patients with anemia; also used/abused by endurance athletes such as marathon runners, triathletes, and long-distance cyclers
- **Clotting Factors:** used in the treatment of blood clotting disorders; examples include:

- Factor VIII and IX: produced using genetically-engineered mammalian cells; used to treat hemophilia and ensure safety and supply without relying on human donors.

- **Monoclonal Antibodies**: target specific antigens for treating cancer, autoimmune diseases, and infections; examples include:
  - Trastuzumab^{TM} (anti-HER2 receptor antibody; Herceptin): produced by genetically-modified mammalian cells; used to treat certain types of breast and gastric cancers by binding the Human Epidermal Growth Factor Receptor Type 2 (HER2), blocking its function and triggering its destruction by the immune system
  - Adalimumab^{TM} (anti-TNF alpha antibody; Humira): produced by genetically-modified mammalian cells; used to treat certain autoimmune disorders by blocking the function of Tumor Necrosis Factor-alpha (TNF-alpha)
- **Immune modulators:** produced to stimulate or regulate the immune system; examples include
  - Interferons: produced by genetically-modified bacteria or yeast; used to treat certain cancers, multiple sclerosis, and viral infections
  - Interleukin-2: produced by genetically-modified bacteria; used to boost the immune response during chemotherapy

Most therapeutic proteins are produced by genetically-modified bacteria, yeast, or mammalian cells. The production of the protein starts with the selection of the desired gene. This gene is then cloned into a suitable plasmid and the resulting recombinant plasmid is then introduced into the genome of the "host cell" (e.g bacteria or mammalian cell). The modified cells are then grown in the laboratory and the protein isolated. Following extensive confirmation that the therapeutic protein is made properly and functions effectively, the protein is then introduced into the commercial market and made through precise manufacturing processes. To learn more about this DNA cloning process, go to Chapter 4.2: Creating Recombinant DNA.

While the approach for genetically-modifying cells for the production of therapeutic proteins is generally the same, the technique used to introduce the genetic information into a cell depends on the cell type (Figure **10.3.3**). For bacteria, this technique is called **transformation**. For mammalian cells, genetic information is introduced through liposomal **transfection**. In transformation, bacteria are treated with ice-cold solutions to weaken their outer wall and plasma membrane, making them "competent" for transformation. They are then mixed with the recombinant plasmid and "heat shocked" to induce internalization of the plasmid. For transfection, the recombinant plasmid is coupled to microscopic **liposomes** that bind to the mammalian cells and are internalized through endocytosis. To learn about transformation, go to Chapter 13.6 Lab Technique: Bacterial Transformation. To learn about transfection, go to Chapter 13.10 Lab Technique: Liposomal Transfection into Mammalian Cells.

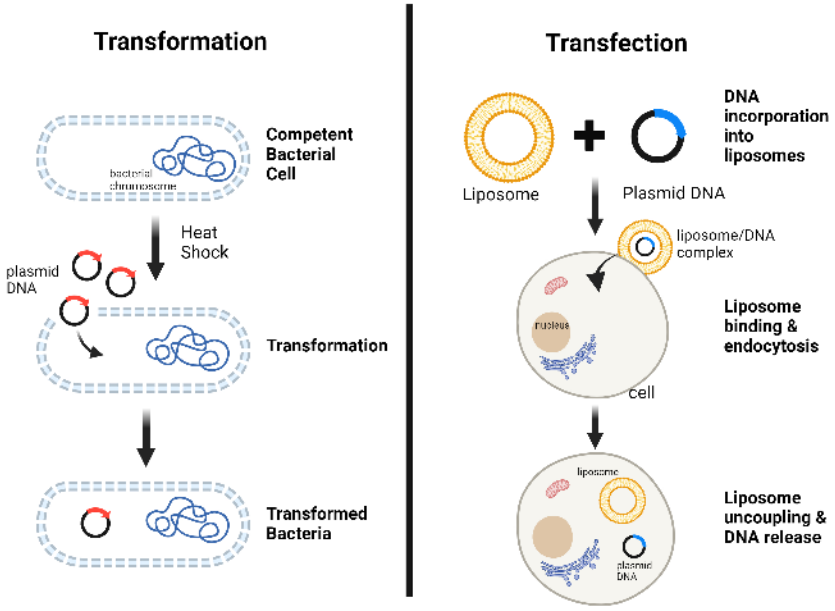


Figure **10.3.3**: Transformation vs. Transfection. In transformation, competent bacterial cells undergo a "heat shock" to internalize DNA such as plasmid DNA. The transformed bacteria with its incorporated plasmid DNA begin expression of the new gene product. In transfection, DNA (e.g. plasmid DNA) is added to liposomes to form a liposome/DNA complex. The complex binds eukaryotic cells and is internalized into the cell through endocytosis. Once inside the cell, the complex undergoes uncoupling and the DNA is released into the cytoplasm. (Transformation vs Transfection by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

Vaccines

There are two basic kinds of vaccines: traditional and therapeutic. The **traditional vaccine** stimulates the immune system in order to prevent an infection, whereas a **therapeutic vaccine** is designed to treat cancer, an existing disease, or chronic infection (like HIV or hepatitis) rather than prevent it. rDNA technology is used in the creation of both types of vaccines. To produce a vaccine using rDNA technology, highly specific viral or bacterial proteins that trigger an immune response without causing disease are produced in the lab. The process for this is very similar to recombinant protein production. A gene crucial for the pathogen's ability to infect a host cell or illicit an immune response is cloned into a plasmid and introduced into a host cell for expression. The expressed protein is purified extensively to ensure that the vaccine contains only the desired protein and no harmful components from the host organism. The purified protein is then used to formulate a vaccine.

While the typical vaccine is made using expressed proteins, the process can be time-consuming. However, the development of **mRNA vaccines** has enabled scientists to dramatically cut down the generation time for a vaccine. Making an mRNA vaccine uses a few steps in common with the traditional vaccine described above (Figure **10.3.4**). A gene for a target protein is identified and cloned into a plasmid. However, rather than expressing this gene as a protein in a cell, the mRNA transcript is produced by a cell-free system and isolated. The mRNA is then coupled to lipid nanoparticles that protect the delicate mRNA. In the final step, the mRNA-nanoparticle mixture is injected into muscle tissue whereupon the mRNA enters into the muscle cells and is expressed as a protein. The expressed protein is displayed on the surface of the muscle cell and triggers a immune reaction.

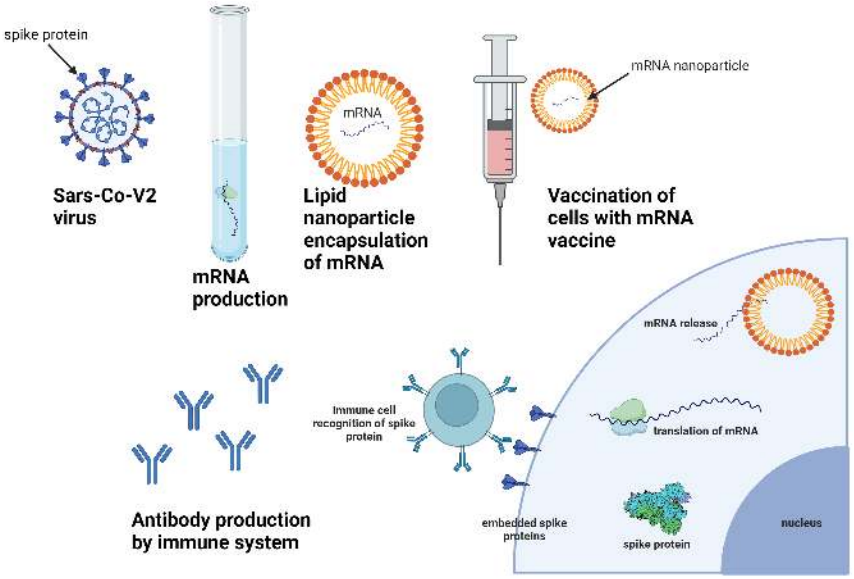


Figure **10.3.4**: mRNA vaccine production. Vaccines against the spike protein of the Sars-CoV-2 virus can be made using mRNA. mRNA that codes for the spike protein is synthesized using a cell-free system (mRNA production). The mRNA particles are encapsulated into lipid nanoparticles (lipid nanoparticle encapsulation of mRNA). The mRNA-lipid nanoparticles are used to vaccinate host cells (vaccination of cells with mRNA vaccine). Once inside the cell, the mRNA is released from the nanoparticle and translated by the host cell's ribosomes. The resulting spike proteins are embedded within the cell membrane and recognized by the host's immune cells. Antibodies to the spike protein are made by the host (antibody production by immune system). (mRNA vaccine production by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

Molecular Protocols

- Lab Technique: Bacterial Transformation
- Lab Technique: Transfection of Mammalian Cells

Key Concepts

Therapeutic biotechnology can be used to treat a wide range of diseases and disorders. Some major concepts to remember are:

- therapeutic biotechnology uses biotechnology tools to diagnose, treat, cure, or prevent diseases or disorders

- gene therapy can potentially treat or cure diseases through the introduction of a gene (or genes) that either replaces a "faulty" one or counteracts its harmful effects
- genes can be introduced for gene therapy using viral vectors like adenovirus, adeno-associated virus, or lentivirus
- new methods for the introduction of genes for gene therapy include the use of nanoparticles
- gene therapy can either be performed by introducing corrective genetic information into an organism directly (*in vivo* approach) or by removing the affected cells, genetically manipulating them, and then re-introducing them back into the patient (*ex vivo* approach)
- CAR-T therapy is a type of gene therapy that genetically-manipulates a patients T cells in order to treat cancer
- CRISPR technologies are currently being explored for use in gene therapy
- most therapeutic proteins are made using bacteria, yeast, or mammalian cells that have been modified with recombinant DNA
- some therapeutic proteins are made through genetically-modifying the entire organism with recombinant DNA
- examples of therapeutic proteins are monoclonal antibodies, hormones, an immune modulators
- therapeutic vaccines use recombinant DNA to produce specific antigens for vaccine production

Glossary

CAR (Chimeric Antigen Receptor) - a synthetic receptor engineered into immune cells (usually T cells) that allows them to recognize and bind to specific proteins on the surface of cancer cells

CAR-T therapy (Chimeric Antigen Receptor T-cell Therapy) - a type of immunotherapy where a patient's T cells are collected, genetically modified in the lab to express CARs, and then re-infused for targeted destruction of a cancer cell

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) - a gene-editing tool that uses a guide RNA and a Cas9 enzyme to find and cut specific DNA sequences

Ex vivo - refers to a process or event that happens outside of the body

Gene therapy - a technique that treats or prevents diseases by modifying the genetic make-up of a person using viral or non-viral vectors

In vivo - refers to a process or event that happens inside of a body

Liposome - a spherical vesicle made of phospholipids that mimics the structure of a cell membrane; used in transfection of mammalian cells

mRNA (messenger RNA) - a sequence of RNA produced through the process of transcription; specifies the sequence of amino acids following translation; comprised of a coding sequence and flanked by a 5' methylated cap and a 3' poly-A tail

mRNA vaccine - a vaccine containing mRNA molecules surrounded by lipid nanoparticles

Nanoparticle - a tiny particle that ranges in size from 1 to 100 nanometers (nm) in at least one dimension; can be made of materials such as biodegradable polymers, metals, and lipids

Recombinant DNA (rDNA) - DNA that is created in a laboratory by combining genetic material from different sources

Therapeutic protein - a protein-based compound used to treat, prevent, or cure diseases and medical conditions; often produced using recombinant DNA technologies

Therapeutic vaccine - a vaccine used to prevent diseases; often produced using recombinant DNA technologies

Transformation - the process of inserting DNA into a bacterial host cell

Transfection - the process of introducing DNA into a mammalian cell using liposomes

Vaccine - a biological preparation that stimulates the immune system to protect against a specific disease

10.3: Therapeutic Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

10.4: Regenerative Medicine

Biotech Focus

In 1956, several workers performing an experiment involving uranium fuel rods at the Vinča Nuclear Institute in Yugoslavia were exposed to high doses of radiation. Soon after, these men began experiencing the symptoms of acute radiation syndrome and were immediately transferred to the Curie Institute in Paris, where they underwent pioneering **bone marrow transplants** by the French oncologist Georges Mathé. All but one of the workers survived. This transplant represented the first use of stem cells as a medical treatment. Five years later, Canadian scientists Dr. James Till and Dr. Ernest McCulloch at the Ontario Cancer Institute in Toronto published their discovery of the **hematopoietic stem cell (HSC)**, the stem cell that gives rise to all blood cells. Till and McCulloch injected bone marrow into irradiated mice and observed the formation of colonies in the spleen. Each colony originated from a single HSC and exhibited both self-renewal and differentiation - the two traits of a stem cell. Thanks to their pioneering work, the stem cell field, and related fields like tissue engineering, were made possible.

Introduction

Regenerative medicine is a branch of medicine that focuses on the repair, replacement, or regeneration of human cells, tissues, or organs in order to restore normal function. It takes advantage of the the body's natural healing processes and often uses advanced technologies to accelerate or enhance those processes.

Key components of regenerative medicine include:

1. **Stem Cell Technology**: uses stem cells to develop into specific cell types and repair damaged tissues
2. **Tissue Engineering:** creates biological tissues in the lab using a combination of scaffolds, cells, and biologically active molecules
3. **Biomaterials and scaffolds:** uses synthetic or natural materials to support cell growth and tissue formation

Regenerative medicine has a wide range of applications, including the treatment of bone defects, damaged heart muscle, and spinal cord injuries, to potentially curing diabetes, Parkinson's, and even organ failure without the need for transplantation. One of the most successful early applications of regenerative medicine was in the treatment of skin defects and burns. Early approaches involved the use of tissue grafts taken from the patient's own skin (i.e., autografts), cadavers (i.e., allografts), or animals like pigs (i.e., xenografts). However, each of these came with their own problems, like the secondary defect associated with an autograft or immune rejection associated with a xenograft. These problems led to the need for alternatives that regenerative medicine could provide. Today, physicians can use synthetic skin grafts made of bioengineered scaffolds, seeded with human epidermal and dermal cells, to treat non-healing wounds and partial-thickness burns (e.g., Apligraft, Stratagraft) or "cultured autografts" made of a patient's own keratinocytes grown in the laboratory (e.g. Epicell). With each advancement, regenerative medicine continues to expand its applications in medicine and offers hope for those living with disease, defect, or injury.

Learning Objectives

Regenerative medicine is a branch of medicine focused on repairing, replacing, or regenerating human cells, tissues, or organs to restore normal function. At the end of this page, you will be able to:

- Define key terms associated with regenerative medicine, like stem cell, differentiation, and potency
- List and explain the three key components of regenerative medicine
- List and explain the three major types of potency associated with a stem cell
- Compare and contrast the three major categories of stem cells
- Describe some commonly used stem cells
- List the components used in tissue engineering
- Explain the different types materials used in making a scaffold
- Explain some methods commonly used to make a scaffold
- Explain what a bioactive molecule is and list some types used in tissue engineering

Stem Cell Technology

A **stem cell** is an undifferentiated (i.e., unspecialized) cell that can differentiate (i.e., specialize) into a specific cell type like a muscle cell or a blood cell (Figure **10.4.1**). Stem cells also exhibit the property of **self-renewal**, in that they divide to produce a daughter cell that continues on as a stem cell and another daughter cell that is destined to differentiate. **Differentiation** is the biological process in which a stem cell develops into a specialized cell type with a distinct structure and function. This differentiation is critical for the determination of a cell's **lineage**, the developmental history of a cell as it divides and differentiates into other cell types. In order for a lineage to be determined, stem cells "choose" a cell fate that directs the type of cell they will become. The tissue continues its development and the lineage is set. For example, a stem cell population whose cell fate is restricted to the osteogenic lineage will differentiate into osteoblasts, followed by direct bone tissue formation.

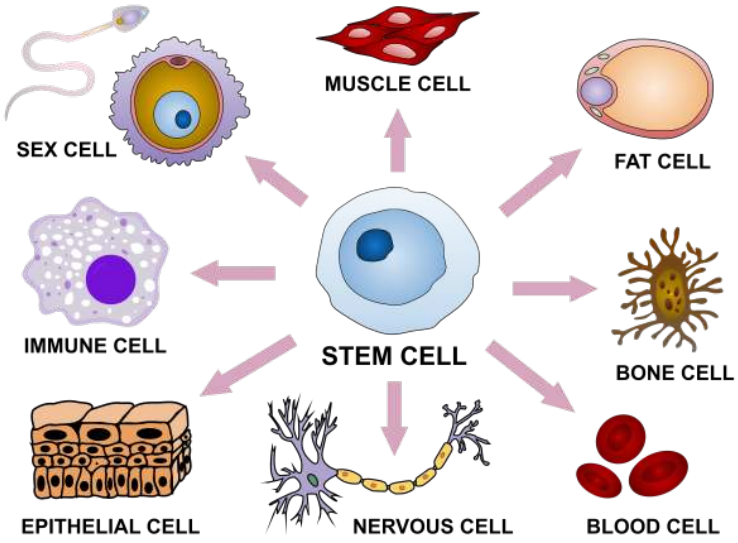


Figure **10.4.1**: Stem cell differentiation. The stem cell differentiates in specific cell types such as epithelial, immune, sex, muscle, fat, bone, blood, and nervous cells (i.e., neurons) (Stem Cell by Haileyfournier, CC BY-SA 4.0)

Differentiation of a stem cell is directed by the expression of specific genes that restrict the stem cell's potency and result in its morphological and metabolic transition to a new cell type. Even today, researchers continue to study the cellular and molecular mechanisms that underlie stem cell differentiation in the hopes of more effectively using stem cells in regenerative medicine. The ability of a stem cell to differentiate is determined by their **potency**. Stem cell potency refers to a stem cell's ability to differentiate into different types of cells. Most stem cells can be described as having one of three types of potency (Figure **10.4.2**):

- **Totipotent**: stem cells can develop into any cell type in the body and the extraembryonic tissues.
  - e.g., early-stage embryonic stem cells (i.e. up to the 16-cell stage)
- **Pluripotent**: stem cells can become any cell type in the body (i.e., all three germ layers: ectoderm, mesoderm, endoderm), but not extraembryonic tissues
  - e.g., embryonic stem cells and induced pluripotent stem cells
  - Widely used in regenerative medicine
- **Multipotent**: stem cells have limited potency and can differentiate into a limited number of cell types within a germ lineage (e.g., mesoderm)
  - e.g., hematopoietic stem cell (HSC), mesenchymal stem cell (MSC), and the adipose-derived stem cell (ASC)
  - Limited use in regenerative medicine that is restricted to the corresponding cell lineage - e.g. HSCs for generation of blood cells; MSCs for the generation of mesenchymal tissues (i.e., fat, bone, cartilage, muscle)

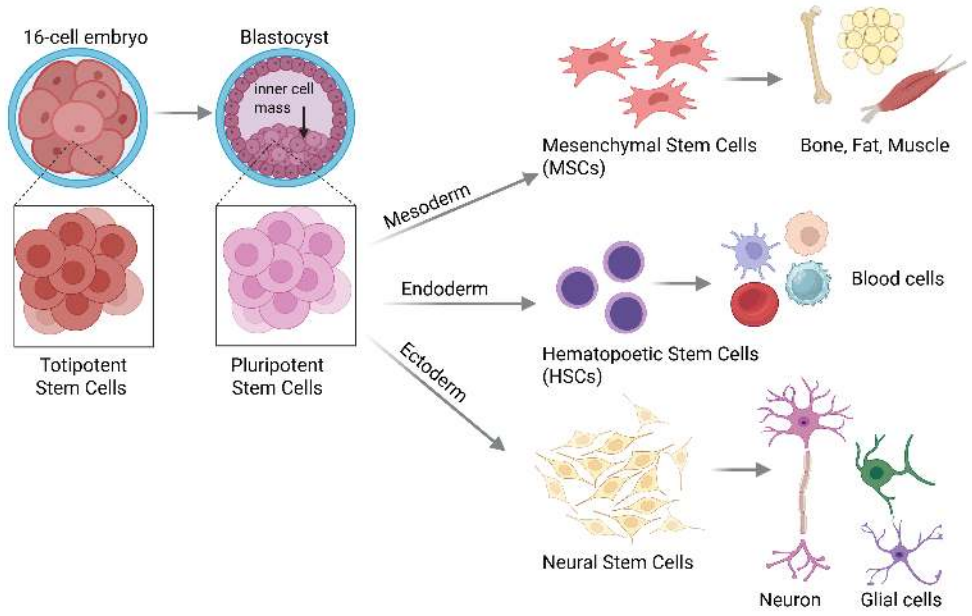


Figure **10.4.2**: Stem cells have specific potency. The 16-cell embryo is made of cells called totipotent stem cells. Over time the embryo develops into a blastocyst. The inner cell mass of the blastocyst is a population of pluripotent stem cells. These stem cells develop into the mesoderm, endoderm, and ectoderm germ lines. Germ line development results in the formation of several kinds of multipotent stem cells like mesenchymal stem cells (MSCs) that can develop into bone, fat, and muscle cells, hematopoietic stem cells (HSCs) that form the blood cells, and neural stem cells that develop into neurons and the glial cells of the nervous system. (Stem Cells by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. 2025)

Embryonic development results in the loss of stem cell potency as the totipotent stem cell population of the 16-cell zygote is replaced with the pluripotent stem cells of the inner cell mass. These pluripotent stem cells will become distributed throughout the embryo and will restrict their potency to become the various populations of multipotent stem cells. The loss potency during embryonic development is directed, in part, by environmental signals found in the developing tissue. In humans, the majority of tissues contain multipotent stem cells that direct tissue repair following damage.

With each passing year, there seems to be a new population of stem cells being discovered and used in regenerative medicine. However, there are only three recognized categories of stem cells: embryonic, fetal, and adult. Each stem cell population is classified into one of these three categories. **Embryonic stem cells (ES) cells, fetal stem cells,** and **adult stem cells** differ in their origin, potency, and applications (Table **10.4.1**).

Table **10.4.1**: Embryonic vs. Adult Stem Cells

Feature	Embryonic Stem Cells (ES cells)	Fetal Stem Cells	Adult Stem Cells
Origin	isolated from the **inner cell mass** of a blastomere (animal) or blastocyst (human)	isolated from fetal tissues after 8 weeks of gestation	isolated from tissues such as bone marrow, adipose tissue, and skin
Potency	totipotent or pluripotent	mostly multipotent	mostly multipotent
Self-renewal ability	high	lower in comparison to ES cells	relatively low; self-renewal depends on the type of adult stem cell
Potential for tumor formation	high - can form teratomas (tumors) due to uncontrolled growth	lower in comparison to ES cells	low
Potential for immune rejection	high - ES cells may be rejected if not genetically matched to the recipient	lower in comparison to ES cells; similar to adult stem cells	low; often isolated from autologous tissues
Applications	• regenerative medicine - organ regeneration and tissue repair (e.g. Parkinson's, diabetes, spinal cord injuries) • disease modeling • drug testing	• regenerative medicine - organ regeneration and tissue repair (e.g. Parkinson's, diabetes, spinal cord injuries) • disease modeling • drug testing	• regenerative medicine - organ regeneration and tissue repair (e.g. Parkinson's, diabetes, spinal cord injuries) • disease modeling • drug testing
Ethical considerations	high - use of human ES cells requires destruction of human blastocysts	high - used of fetal stem cells requires the use of fetal tissues	low - human adult stem cells are isolated from consenting donors

In practice, a stem cell is identified by its ability to repair or regenerate tissue. Since the discovery of hematopoietic stem cells (HSCs) in the 1960s, stem cells have been utilized in a wide variety of regenerative medicine applications, such as:

1. **Hematopoietic Stem Cells (HSCs):** for the treatment of blood cancers (like leukemia, lymphoma), aplastic anemia, and some immune disorders.
    - uses several sources of HSCs, including bone marrow, peripheral blood, or umbilical cord blood
    - the most widely used and established stem cell therapy worldwide
2. **Epidermal stem cells:** for the treatment of large-area burns using autologous skin grafts
    - use of autologous epidermal stem cells donated by the patient
    - products like Epicel (cultured epidermal autografts) are FDA-approved
3. **Mesodermally-derived stem cells:** for the regeneration of a variety of mesodermally-derived tissues
    - use of autologous stem cells from bone marrow (i.e., MSCs) or adipose tissue (e.g., ASCs)
    - regeneration of cartilage in patients with joint injuries or early osteoarthritis (e.g., Carticel)
    - treatment of spinal cord injuries (e.g., Stemirac)
    - treatment of graft vs. host disease in young children(e.g., Ryanocil)
4. **Adipose-derived Stem Cells (ASCs):** for the repair of complex perianal fistulas that do not respond to conventional treatments
    - use of allogeneic ASCs
    - approved in the EU (e.g., Alofisel),with strong efficacy and safety data

The majority of regenerative medicine applications using stem cells utilize adult stem cells due to their ease of isolation and lack of ethical concerns. This is in direct contrast to the use of ES cells. The ethical considerations that continue to surround the isolation and use of human ES cells has led researchers to develop the **induced Pluripotent Stem Cell (iPSC)**. iPSCs have been artificially reprogrammed to behave like ES cells through the introduction of four specific genes (Oct4, Sox2, Klf4, c-myc) into fully

differentiated cells, such as skin or blood cells. This genetic reprogramming appears to return the somatic cell to a more pluripotent state. Because of their high level of potency and their derivation from adult cells, iPSCs have been suggested as a replacement for ES cells. As such, iPSCs are used in disease modeling, drug testing, personalized medicine and regenerative medicine. However, their use in personalized and regenerative medicine has not been as widely explored as ES cells and adult stem cells. Furthermore, the significant degree of genetic changes required to create an iPSC may eliminate them from many applications in regenerative medicine. Despite this, stem cell therapy continues to be promising due to their potential therapeutic application in replacing defective or damaged cells resulting from a variety of disorders and injuries, such as Parkinson disease, heart disease, and diabetes.

Tissue Engineering

Tissue engineering is a multidisciplinary field that combines biology, engineering, and medicine in order to focus on the development of biological substitutes to restore, maintain, or improve tissue function. Tissue engineering combines components:

1. **Cells:** the living units responsible for forming functional tissue
    - can be precursor cells or stem cells
    - can be either **autologous** (harvested from the patient in order to minimize immune rejection) or **allogeneic** (donated cells)
2. **Biological scaffolds:** 3D structures that provide a biocompatible, physical structure for the attachment, proliferation, and differentiation of cells
    - made of natural, synthetic, or composite materials
3. **Bioactive molecules:** growth factors and other signaling cues that guide cell behavior and tissue development within the scaffold

In the typical tissue engineering approach, these three components interact with one another inside a **bioreactor**, a controlled environment designed to mimic physiological conditions and support tissue formation.

By integrating cells, scaffolds, and signaling molecules, tissue engineering aims to repair or regenerate damaged tissues and organs. Examples of tissues generated through tissue engineering include:

- **Skin:** bioengineered skin substitutes consisting of keratinocytes and fibroblasts layered on a scaffold (e.g., Apligraf, Dermagraft) used for chronic wounds and burns
- **Cartilage:** autologous chondrocyte implantation (ACI) or stem-cell seeded scaffolds to repair joint damage
- **Bone:** hydroxyapatite or tricalcium phosphate scaffolds combined with pre-osteoblast precursors of adult stem cells
- **Blood vessels:** vascular grafts engineered from endothelial and smooth muscle cells for the replacement of damaged vessels; e.g. coronary bypass vessels

Tissue engineering is also being used in the generation of whole organs such as liver, kidney, and heart tissues. Miniature versions of organs, called organoids, are being developed in lab settings, although clinical applications remain limited.

The use of tissue engineering principles in tissue repair is promising. However, there are several obstacles to overcome. Vascularization is one of them. Supplying nutrients to thick tissue layers remains a significant challenge. While autologous cells are often used to decrease the chances of an immune response, such as response can occur due to scaffold materials or altered cell behavior. Regulatory hurdles must be cleared in order to ensure the safety and efficacy of engineered tissues. These hurdles can delay the transition of the clinic. Lastly, manufacturing scale-up remains an issue as generating tissues at a large scale both reproducibly and cost-effectively is a significant challenge.

Biomaterials and Bioscaffolds

A **biomaterial** is any natural or synthetic material that is designed to interact with a biological system (e.g. cells, tissues) for a medical purpose, such as the therapeutic delivery of drugs or cells for tissue repair. Biomaterials must be **biocompatible** and not provoke an immune reaction. They should **bioactive** to promote the attachment, proliferation, and/or the differentiation of cells. They should match the mechanical demands of the target tissue (e.g, load bearing vs. non-load bearing). Finally, many biomaterials are designed to be **biodegradable** so that they degrade safely in the body.

A **bioscaffold (i.e., scaffold)** is a three-dimensional structure made of a biomaterial. The composition of a bioscaffold is chosen to be compatible with the cells being used and the tissue being regenerated. The scaffold must promote the viability of the cells seeded onto the scaffold. In addition, it should also integrate into the surrounding tissue so that the tissue being regenerated is compatible. Scaffold compositions used in tissue engineering are **natural polymers**, **synthetic polymers**, or **composite materials** that combine both natural and synthetic biomaterials. Each of these compositions have advantages and disadvantages that must be considered before

being used in a tissue engineering application. For example, synthetic polymers have the advantages of consistent composition, higher mechanical strength, and controlled degradation *in situ*. However, their degradation may create toxic materials. The composition and design of the scaffold ultimately used must consider porosity, mechanical strength, biodegradability, and surface chemistry so as to support cellular function and integration.

Natural polymers include collagen, fibrin, alginate, and chitosan (Table **10.4.2**). These materials have the advantages of being bioactive and biocompatible. However, they can be variable in composition and have lower mechanical strength.

Table **10.4.2**: **Natural Scaffold Materials**

Material	Source	Tissue Engineering Application	Key Features
Collagen	animal connective tissues	skin, bone, cartilage	biodegradable, cell-friendly
Fibrin	blood plasma	wound healing, cardiovascular patches	promotes angiogenesis
Alginate	brown seaweed	soft tissue engineering	mild gelation, controlled stiffness
Chitosan	crustacean shells	cartilage, wound healing	antimicrobial, highly biodegradable
Hyaluronic acid	connective tissue matrix	cartilage repair, cell delivery system	hydrating, similar to cartilage matrix

Common synthetic materials used in biological scaffolds include **polylactic acid (PLA)** and **polyglycolic acid (PGA),** in addition to calcium phosphate-based scaffolds like **hydroxyapatite** (Figure **10.4.3**) and **tricalcium phosphate** (Table **10.4.3**).

Table: **10.4.3**: **Synthetic Scaffold Materials**

Material	Type	Tissue Engineering Applications	Key Features
PLA (Polylactic acid)	thermoplastic polyester	bone, skin, guidance of nerves	biodegradable, FDA-approved for load-bearing tissues like bone
PGA (Polyglycolic acid)	synthetic polymer	cartilage, nerve, skin	fast degradation
PLGA (Poly(lactic-co-glycolic acid))	co-polymer	bioactive factor delivery, tissue patches	controlled degradation
PCL (Polycaprolactone)	synthetic polymer	ligaments and tendons, cartilage, vascular tissue	slow degradation, flexible
PEG (Polyethylene glycol)	hydrophilic polymer	cartilage, nerve, vascular tissue, cornea	mimics soft tissue
Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)	calcium phosphate-based material	bone	slow degradation, good for load-bearing tissues, mimics ECM of bone
Tricalcium phosphate (TCP)	calcium phosphate-based material	bone	fast degradation

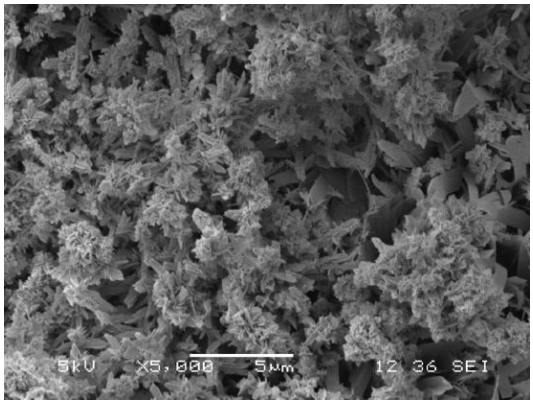


Figure 10.4.3: Hydroxyapatite crystals. This electron micrograph shows an extensive network of hydroxyapatite crystals that closely mimics the matrix of bone tissue. (Crystals of Hydroxylapatite by Nabilah, CC BY-SA 4.0)

Composite scaffolds combine natural and synthetic materials for optimized performance, balancing biocompatibility and mechanical integrity. Examples include collagen-PLGA and hydroxyapatite–PCL for bone and cartilage repair.

Bioscaffolds can be produced using several methods including:

1. **Solvent Casting and Particulate Leaching (SCPL):** used for PLGA, PGA, PCL, hydroxyapatite, tricalcium phosphate scaffolds
   - Production method: a polymer is dissolved in a solvent and mixed with salt or sugar particles (i.e. **porogens**)
     - the mixture is cast into a mold
     - the solvent is evaporated and immersed in water to dissolve the porogen
   - Pros: simple and inexpensive to make; pore sizes controlled by porogen; good for cell infiltration, can be coated with natural materials like collagen
   - Cons: limited control over pore interconnectivity; residual solvent or porogen may be toxic if not fully removed
   - Used for: bone and cartilage
2. **Electrospinning:** used for PLA or PCL synthetic scaffolds and numerous natural materials (e.g., collagen)
   - Production method: a polymer solution is fed through a needle with a high-voltage electric field to create fine fibers that collect on a grounded surface
   - Pros: excellent surface area for cell attachment; mimics native ECM structure well
   - Cons: typically made as a 2D or thin 3D scaffold; poor cell infiltration unless the scaffold is layered
   - Used for: skin, nerve, vascular, and soft tissue
3. **3D printing:** most natural and synthetic scaffold materials
   - Production method: layer-by-layer printing of scaffold material using a 3D printer and its software
   - Pros: Can be customized to print a variety of scaffold shapes
   - Cons: Cost; requires a 3D printer and software
   - Used for: vascular grafts, cartilage, bone scaffolds

Concept in Action

Electrospinning Technology

Bioactive Molecules

A **bioactive molecule** is any compound that affects tissues or cells by producing a specific physiological response. Common bioactive molecules include growth factors, peptides, and hormones. In order to be a bioactive molecule, the compound must bind to receptors on the cell surface and trigger signaling pathways that influence cell fate and behavior (e.g., migration, growth, differentiation). In tissue engineering, bioactive molecules promote the adhesion, proliferation, and differentiation of cells (including stem cells), promote angiogenesis, nerve regeneration and remodeling of tissues, and modulate the immune response to implants. Table: **10.4.4** below outlines some common bioactive molecules used in tissue engineering.

Table: **10.4.4**: **Bioactive Molecules**

Type of Bioactive Molecule	Function	Example
Growth Factors	Stimulate cell growth, migration, or differentiation	BMPs, VEGF, FGF, EGF, TGF-β
Cytokines	Modulate immune response or inflammation	IL-1, IL-6, TNF-α, IFN-γ
Peptides	Mimic protein domains for cell signaling	RGD peptide (cell adhesion), IKVAV
Hormones	Regulate cell metabolism and function	Insulin, estrogen, parathyroid hormone
Extracellular Matrix (ECM) Proteins	Provide structural and signaling support	Collagen, fibronectin, laminin
Small Molecules	Modulate cell signaling	Dexamethasone, retinoic acid, valproate
Antibodies	Target or block specific receptors or proteins on cells	Anti-VEGF, anti-TNF antibodies
Nucleic Acids	Regulate gene expression	siRNA, miRNA

Bioactive molecules used in tissue engineering can be incorporated into scaffolds for the slow release of the molecule, attached to the surface of a biomaterial to affect surrounding cells, and encapsulated in microspheres/nanoparticles. Today, "smart biomaterials" are being designed to release bioactive factors in response to stimuli like pH, temperature, or electric fields.

Emerging Breakthroughs

Today, there are several promising approaches being proposed for tissue engineering. One such approach is called **"bioprinting"**. Bioprinting is based on 3D printing technology but uses **"bioinks"** which are combinations of biomaterials, living cells, and bioactive

materials. Like 3D printing, bioprinted scaffolds are layer by layer in a 3D printer. However, these printers have been modified to use bioinks. Bioprinting is highly precise and can be customized to create complex structures seeded with living cells and embedded with bioactive compounds. However, bioprinting remains expensive, as the bioprinter is more advanced then the standard 3D printer used in labs today. In addition, the bioinks must finely tuned to balance printability with biocompatibility. Bioprinting is currently being used for the creation of soft tissue scaffolds like vascular grafts and tubes. Researchers are using bioprinting to create miniature versions of organs (i.e., **organoids**) in order to advance bioprinting to the point where life-sized organs can be made.

Concept in Action

Bioprinting Human Tissues

Another approach is the use of **decellularized tissues** (i.e. decellularized ECM or dECM). Tissue-specific scaffolds can be created by removing cells from a donor tissue while preserving the overall 3D structure of the tissue and its matrix proteins. Examples include the experimental use of decellularized trachea, tendons, ligaments, and heart matrix, in addition to the commercial use of decellularized skin grafts (e.g., Alloderm and DermACELL) and small intestine submucosa (e.g., SurgiSIS and Oasis). Decellularized tissues have the advantage of being perfectly suited to the tissue they are repairing. However, the possibility of an immune reaction upon implantation cannot be completely discounted.

Concept in Action

The Science of Decellularization

The progress made in tissue engineering over the last two decades has been astounding. Of course, obstacles still remain. Organ regeneration is complicated by the sheer size and thickness of the organ. As such, the formation of a functional vasculature and innervation remains elusive in large tissue and organ constructs. Scaling up manufacturing without compromising the bioactivity of the engineered construct is challenging. Ensuring long-term integration and safety in humans and navigating the ethical and regulatory hurdles, especially in organ engineering, will need be to addressed. Despite these challenges, the future remains bright for tissue engineering.

Key Concepts

Regenerative medicine is a branch of medicine that focuses on the repair, replacement, or regeneration of human cells, tissues, or organs in order to restore normal function.

Some important concepts to remember are:

- regenerative medicine uses tissue engineering techniques to repair or regenerate tissues
- tissue engineering uses cells, biomaterials, and bioactive molecules to regenerate and repair tissues
- the cells used in tissue engineering can be precursor cells or stem cells
- stem cells are unspecialized cells that can divide and differentiate
- stem cells can be totipotent, pluripotent, or multipotent
- each stem cell population has its own unique set of advantages and disadvantages
- biomaterials are any natural or synthetic materials that are designed to interact with a biological system
- biomaterials include scaffolds
- scaffolds can be made of natural or synthetic biomaterials or a combination of both
- scaffolds can be made through techniques like particulate leaching, electrospinning, or 3D printing
- bioactive molecules are compounds that affect tissues or cells by producing a physiological response
- bioactive molecules include growth factors, peptides, hormones, or matrix proteins
- emerging technologies in tissue engineering include the use of decellularized matrices or bioprinting
- bioprinting uses "bioinks" that are a combination of living cells, bioactive molecules, and biomaterials

Glossary

Bioactive molecule - a substance that interacts with living cells or tissues to influence their behavior, such as promoting growth, differentiation, or healing; examples include growth factors, cytokines, or peptides

Biocompatible - a material or substance that can be safely introduced into the body to supports normal cellular function without causing an immune response or toxicity

Biodegradable - a material that can be broken down naturally by biological processes in the body into harmless byproducts over time

Bioink - a printable biomaterial, containing cells and biocompatible hydrogels, that is used in 3D printing to fabricate tissue-like structures layer-by-layer

Biomaterial - any natural or synthetic material used to interact with or replace a biological tissue

Bioprinting - a form of 3D printing that uses bioinks to create complex biological structures, such as tissues or organs

Bioscaffold - a 3D framework used in tissue engineering that is made of biomaterials designed to support cell attachment, proliferation, and differentiation

Decellularized - a term that describes a tissue or organ that has had all cellular material removed, leaving behind the extracellular matrix to be used as a scaffold

Differentiation - a biological process by which a less specialized stem cell becomes a more specialized cell type

Extracellular matrix (ECM) - a complex network of proteins and molecules surrounding cells in tissues; provides structural support and biochemical signals for cell behavior

Lineage - the development "history" of a cell

Multipotent - a term that describes stem cells that can differentiate into multiple, but limited, cell types within a specific cell lineage; e.g., mesenchymal stem cells into bone, fat, or cartilage

Organoid - a miniature, simplified version of an organ grown in vitro from stem cells, that mimics key structural and functional properties of the real organ

Pluripotent - a term that describes stem cells that can give rise to all cell types in the body; found in the inner cell mass of the embryo

Polymer - a large molecule made up of repeating units called monomers

Potency - the measure of a stem cell's ability to differentiate into different cell types; ranges from totipotent to unipotent

Precursor cell - a cell that is restricted in its lineage to only one type of cell; e.g., a pre-osteoblast precursor becomes an osteoblast

Regenerative medicine - a field of medicine that aims to restore or replace damaged tissues and organs using techniques like stem cell therapy, tissue engineering, and gene editing

Scaffold - a natural or synthetic 3D structure that provides physical support for cells used in tissue engineering; also called a bioscaffold

Stem cell - an undifferentiated cell capable of self-renewal and differentiation depending on its potency

Totipotent - a term that describes stem cells that are able to give rise to all cell types of the body and the extra-embryonic tissues; found in early stage embryos (i.e. up to 16-cell)

Unipotent - a term that describes a cell that has the capacity to differentiate into only one specific cell type, but they still retain the ability to self-renew

---

10.4: Regenerative Medicine is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

11: Industrial Biotechnology – “White” Biotechnology

11.1: Applications of Industrial Biotechnology

11.2: Bioprocess Engineering

11.3: Synthetic Biology

11: Industrial Biotechnology – “White” Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

11.1: Applications of Industrial Biotechnology

Biotech Focus

After the Deepwater Horizon oil spill in 2010, scientists began to study how bioremediation techniques could be used to speed up the natural process of breaking down oil in order to minimize the impact of oil spills on the environment. Their work was based on the fact that certain bacteria, like *Alcanivorax borkumensis*, can use the hydrocarbons in oil as a food source, breaking it down into simpler compounds. *A. borkumensis* was discovered near the island of Borkum (hence the epithet borkumensis) by scientists in 2006. It is classified as a **halophile**, an organism that thrives in salty environments, like the ocean. It can be found all across the world both in coastal environments and oceanic environments and has been found to be more common in oceanic areas containing petroleum - although whether this is from spills, natural fields, or other sources of oil, is not known.

The use of microbes in the clean-up of hazardous materials has since gained in popularity since the Deepwater Horizon spill. *Pseudomonas putida*, a versatile bacteria found in soil and water environments, can break down a variety of hydrocarbons, including benzene and other toxic oil-related compounds. Several species of *Mycobacterium* can degrade complex hydrocarbons, which are found in oils, and are often resistant to harsh environments. Other bacterials strains like those of the *Bacillus* and *Thalassolituus* genera have also become part of the ever expanding bioremediation "toolkit" available to industrial manufacturers.

Check out this video explaining how bacteria, like *A. borkumensis*, are used to "eat" oil spills.

Introduction

Industrial biotechnology, also known as "white" biotechnology, is a branch of biotechnology that uses a **biological system** to develop bio-based products, processes, or services used in industry. These biological systems may be naturally occurring, modified slightly through genetic engineering, or can be redesigned from "scratch" to create synthetic systems. To learn about synthetic biology, go to Chapter 11.2 Synthetic Biology & Bioprocess Engineering.

One popular use of industrial biotechnology is the production of a **"biologic"**, a therapeutic product extracted from a biological organism. The biological systems used in industrial biotechnology include the use of microbes (e.g. bacteria, yeast, other fungi), enzymes, and cell cultures. Industrial biotechnology integrates these biological systems with other areas, like engineering, to create sustainable and efficient solutions for industries such as agriculture, energy, pharmaceuticals, chemicals, and textiles.

Some commonly used organisms used in industrial biotechnology are:

- *E.coli* - a bacterial strain used in recombinant DNA technology for protein production
- *Bacillus subtilis* – a bacterial strain that is used to produce industrially important enzymes like amylases and proteases
- *Saccharomyces cerevisiae* – a yeast strain used in fermentation processes for food (.e.g beer, wine), pharmaceuticals, and bioethanol production
- *Aspergillus niger* – a fungus used for citric acid production

Industrial biotechnology is sustainable, using renewable resources to replace fossil-based fuel processes. Its use of microbial systems, like bacteria and yeast, allows industries to manufacture products efficiently with relatively little energy input. The renewable nature of industrial biotechnology has a relatively small carbon footprint in comparison to conventional manufacturing processes, making this area of biotechnology eco-friendly and sustainable.

Learning Objectives

Industrial biotechnology uses biological systems to develop products, processes, or services for industry. At the end of this page, you will be able to:

- Identify the key areas of industrial biotechnology
- Describe the types of bioplastics
- Describe the enzyme types made through industrial biotechnology
- Explain how industrial biotechnology can make enzymes
- Explain how the pharmaceutical industry and industrial biotechnology are related
- List some of the areas of environmental remediation
- List some of the advantages of industrial biotechnology

Key Areas of Industrial Biotechnology

Industrial biotechnology encompasses several key areas, including:

1. **Bio-based chemicals and materials** - the production of biodegradable plastics and bio-based solvents.
2. **Biofuels and Bioenergy** – the use of microbes, like bacteria and yeast, to produce renewable energy sources, such as bioethanol, biodiesel, and hydrogen
3. **Enzyme Technologies** – the production of enzymes used in food processing, detergents, textiles, and pharmaceutical industries
4. **Biopharmaceuticals** – the large-scale production of vaccines, antibodies, and other therapeutic compounds
5. **Agricultural Biotechnology** – the development of genetically-modified crops, biofertilizers, and biopesticides

Applications of Industrial Biotechnology

Bioplastics

Biodegradable plastics, also known as **bioplastics**, are a category of plastics that are derived from renewable biological sources like plant starches, plant oils, organic waste, and microbes. Unlike conventional plastics, which are made from petroleum products, bioplastics are either biodegradable, compostable, or bio-based and non-degradable, making them eco-friendly alternatives.The uses of bioplastics are wide and varied and can include medical implants and devices, food packaging and agricultural films.

Types of bioplastics include:

- **bio-based, non-degradable** - made from renewable resources but do not degrade easily
  - e.g. bio-based polyethylene (PE) - derived from sugar cane; chemically identical to fossil-fuel based PE
- **biodegradable** - break down naturally in the environment through microbial action
  - cellulose-based (e.g., cellulose acetate, cellophane) - produced through the microbial fermentation of cellulose; source material includes wood pulp, cotton, and agricultural waste; used in food packaging, textiles and fibers, coatings and films, filters and electronics
  - starch-based (e.g. thermoplastic starch) - produced through the microbial fermentation of cellulose;
- **compostable** - break down into water, CO2, and other biological compounds under industrial composting conditions

Production of bioplastics typically begins with a **biopolymer**, a natural polymer made by living organisms. Polysaccharides and proteins are well-known examples of biopolymers, along with more complex compounds that come from microbial sources. Many bioplastics are produced through the fermentation of biopolymers like starch and cellulose. Advances in genetic engineering have led to more efficient microbial strains capable of producing high yields of bioplastics.

Examples of biopolymers used in bioplastic production are:

- **polylactic acid (PLA)** - produced through the microbial fermentation of corn starch or sugar cane
- **polyhydroxyalkanoates (PHA)** - produced from bacteria; used in medical implants, food packaging and agricultural films

Biofuel Production

With the rising demand for sustainable energy, biofuels such as ethanol, biodiesel, and biogas have gained significance. Industrial biotechnology facilitates the conversion of biomass (e.g., sugarcane, corn, and algae) into renewable fuels through microbial fermentation. Algae-based biofuels, in particular, hold promise due to their high lipid content and rapid growth. To read more about biofuel production, go to Chapter 7.4 Industrial Applications of Microbial Biotechnology.

Industrial Enzyme Production

Industrial enzyme production is the large-scale process of culturing microbes, like bacteria or yeast to produce enzymes for commercial applications. These microbes may be genetically-modified or used unaltered for this production. The enzymes made can be used in a wide variety of industries, such as the food, textile, pharmaceutical, and energy sectors.

Examples of industrial enzymes produced, include:

- **Amylases:** break down starches into sugars
  - used in the food industry (e.g., baking, brewing) and in the production of biofuels
- **Proteases:** degrade proteins
  - used in the food industry (e.g., dairy production), consumer product industry (e.g., detergent production), and textile industry (e.g., processing of leather)
- **Lipases:** break down fats
  - used in the food industry (e.g., dairy production), consumer product industry (e.g., detergent production) and production of biofuels
- **Cellulases:** degrade cellulases into simple sugars
  - used in the textile industry (e.g., fabric softening) and production of biofuels
- **Lactases:** used to break down lactose
  - used in the food industry (e.g. dairy production)

While done at a large-scale, industrial enzyme production is relatively straightforward. The basic steps are:

1. **Microbe selection**
   - common microbes are bacteria (e.g. Bacillus), yeast or other fungal species (e.g. Aspergillus)
   - microbes can be genetically-modified to increase their yield of a specific enzyme or to produce a non-native enzyme
2. **Fermentation**
   - microbes are fermented in either a liquid medium (i.e., submerged fermentation) or grown on solid materials like agricultural or food waste (i.e., solid-state fermentation)
   - solid-state fermentation mimics natural environments and is used for the production of specific enzymes like amylases and cellulases
3. **Enzyme extraction and purification**
   - enzymes are harvested and purified using techniques like filtration, centrifugation, and chromatography
4. **Formulation and stabilization**
   - enzymes are formed into liquids, powders, or granules
   - compounds like calcium ions or glycerol can be added to stabilize the product for commercial use

Because it reduces chemical waste (and even uses it in some cases) and requires low amounts of energy, industrial enzyme production is eco-friendly. It is also highly specific and efficient as enzymes target specific reactions in industrial processes. Lastly, industrially-produced enzymes can lower production costs in many industries, including food, pharmaceutical, and energy production.

Pharmaceutical Industry

Industrial biotechnology plays a vital role in drug development, including the production of antibiotics, the development of monoclonal antibodies for treating diseases, the production of vaccines through the use of recombinant DNA technology, and the synthesis of therapeutic compounds (e.g. hormones, vitamins, enzymes) using genetically-modified bacteria. For example, human insulin is now produced at scale through the insertion of the human insulin gene into bacteria. Industrial biotechnology has resulted in the development of customized therapies based on an individual's genetic makeup, including CAR-T cell therapy for treating certain cancers. Like other applications, industrial biotechnology practices in the pharmaceutical industry reduces pollution, greenhouse gas emissions, and saves energy and resources. It can also reduce the production of toxic waste products that result from drug manufacturing. Engineered yeast strains can now produce the compound artemisinin, an anti-malarial molecule that is traditionally extracted from plants. As such, the production of this compound using biotechnology approaches is significantly reducing the impact of this drug on the environment.

Waste Treatment and Environmental Bioremediation

Industrial biotechnology offers numerous solutions for waste management and **environmental remediation** - the process of using bacteria, fungi, or plants to naturally breakdown and remove pollutants, contaminants, or toxins from the environment in order to restore ecosystems and protect human health. Microbes are becoming a key part of remediation approaches that degrade pollutants, including oil spills, heavy metals, and plastic waste (Figure 11.1.1). Bacteria and their enzymes are also used in wastewater treatment to break down organic waste and treat industrial effluents. Organic waste products are also the starting point for conversion into methane-rich biogas for energy generation.

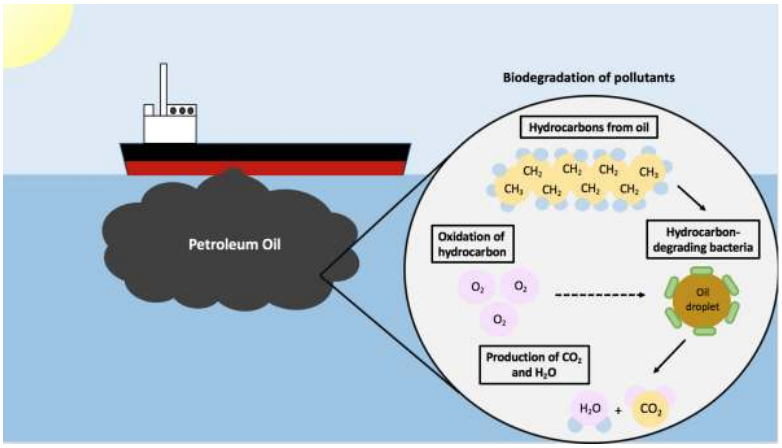


Figure 11.1.1: Bioremediation. Petroleum oil spills can be treated through the biodegradation of pollutants. Hydrocarbons from oil can be converted into H20 and CO2 by hydrocarbon-degrading bacteria that surround the oil droplets. (Biodegradation of Pollutants by Timmer26, CC BY-SA 4.0)

There are numerous areas of environmental bioremediation that utilize industrial biotechnology:

1. **Soil Remediation** – the removal of pollutants like heavy metals, petroleum, or pesticides from soil
2. **Water Remediation** – the treatment of contaminated groundwater, rivers, or oceans by removing toxins, oil spills, or heavy metals
3. **Air Remediation** – the removal of air pollutants such as volatile organic compounds and greenhouse gases

Advantages and Challenges of Industrial Biotechnology

Industrial biotechnology offers manufacturers several advantages, including:

- **Sustainability** – Reduces reliance on fossil fuels and minimizes environmental impact
- **Cost-Effectiveness** – Uses renewable resources and reduces energy consumption
- **Eco-Friendly** – Produces biodegradable products and reduces carbon emissions
- **Innovation** – Facilitates the development of novel materials and drugs

Despite these numerous benefits, industrial biotechnology faces challenges in wide-spread implementation. The high initial cost of setting up a bioprocessing plant requires substantial investment that might discourage manufacturers from considering

biotechnology as an option. Scaling up the required laboratory processes in order to optimize the system(s) can be complex. Strict regulations govern the use of genetically-modified organisms (GMOs) and bio-based products. However, continued improvements in fermentation technology, modifications in synthetic biology systems, the use of CRISPR-based techniques, and artificial intelligence (AI) continue to drive the growth of industrial biotechnology and ensure a more sustainable industrial future.

Key Concepts

Industrial biotechnology is revolutionizing various sectors by offering sustainable, cost-effective, and innovative solutions.

Some important concepts to remember are:

- Industrial biotechnology encompasses areas like bioremediation, biofuels and bioenergy, biopharmaceuticals, enzyme technologies, and agriculture
- Bioplastics are synthesized from biopolymers like starches
- Bioplastics range from biodegradable, compostable, and non-biodegradable but bio-based
- Polylactic acid (PLA) and polyhydroxyalkanoates (PHA) are common biopolymers used in bioplastic synthesis
- Industrial enzyme production is the large-scale process of culturing microbes for the production of enzymes for commercial applications
- Industrial enzyme production reduces chemical waste, requires low amounts of energy, and is eco-friendly and sustainable
- Industrial biotechnology plays a role in the development of antibiotics, monoclonal antibodies, vaccines and therapeutic compounds
- Bacteria, fungi, or plants can be used in bioremediation - a natural process that breaks down and removes pollutants, contaminants, or toxins from the environment
- Bacteria can also be also used in wastewater treatment to break down organic waste and treat industrial effluents

Glossary

Bio-based - materials, products, or processes that are derived from living organisms, especially plants, animals, or microbes, rather than from fossil fuels or synthetic chemicals

Biocatalyst - a natural substance, like an enzyme or a whole microbe, that speeds up (catalyzes) chemical reactions in biological or industrial processes

Biodegradable - a material that can be broken down naturally by biological processes in the body into harmless byproducts over time

Biologic - a product extracted from a living organism

Bioplastic - a type of plastic that is made from a renewable biological source; may or may not be biodegradable

Biopolymer - a natural polymer produced by living organisms, or a synthetic polymer made from biological materials

Biological system - a group of parts in a living organism that work together to perform a specific function and keep the organism alive and healthy

Compostable - refers to a material that can break down naturally into nutrient-rich compost; leaves no harmful residue behind

Industrial biotechnology - the use of living organisms, enzymes, or biological systems to create industrial products and processes; also known as white biotechnology

Industrial enzyme - an enzyme used in large-scale industrial processes to speed up chemical reactions in a cost-effective, efficient, and eco-friendly way

Remediation - the process of fixing, correcting, or cleaning up a problem, especially when it comes to the environment or contaminated areas

11.1: Applications of Industrial Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

11.2: Bioprocess Engineering

Biotech Focus

You can't ship a pharmacy to Mars. Pills expire, conditions change, and space is limited. As a result, bioprocess engineers, working with NASA, are designing mini, portable "biofactories" that can make critical compounds on demand using engineering microbes. One application being studied today is the use of engineering yeast to produce essential nutrients, like **vitamin B12,** which degrades quickly in stored food. Enter the bioprocess engineer.....

Bioprocess engineers are now designing a sealed, automated system comprised of a bioreactor that can provide precise control of culture temperature, oxygen levels, and pH, together with the systems needed to harvest and purify the vitamin. The resulting system will be autonomous, requiring no human operator, would work in microgravity and come equipped with fail-safes and sensors that can adapt the system in real-time to environmental changes.T he whole set-up is being designed to be compact enough to run in a space station—or eventually a base on Mars.

Introduction

Bioprocess engineering uses engineering principles to design industrial biotechnology processes at a scale large enough to make products for commercial sale. In simple terms, bioprocess engineering tries to figure out how to use living cells or biological molecules to make things like medicines, vaccines, food products, and eco-friendly materials like bioplastics. While bioprocess engineering is closely related to industrial biotechnology (Table **11.2.1**), it is not the same thing. Industrial biotechnology *focuses on uses biological systems* like microbes, enzymes, or other biological materials, in order to develop biologic products for commercial use. To learn more about industrial biotechnology, go to Chapter 11.1 Applications of Industrial Biotechnology.

Table **11.2.1**: Industrial Biotech vs. Bioprocess Engineering

Industrial Biotechnology	Bioprocess Engineering
Develops biological tools, like cells and enzymes, for creation of a product	Designs systems to use biological tools at scale
Focuses on innovative biological techniques that are sustainable and eco-friendly	Focuses on implementation and optimization of biological techniques
Often starts in the research & development laboratory	Bridges the gap between the laboratory and industry

In contrast, bioprocess engineering *focuses on engineering principles* in order to make these biologic products at a high-enough quality and quantity that they be sold commercially (Figure **11.2.1**). Bioprocess engineering designs, optimizes, and scales-up the processes used in industrial biotechnology in order to make these products. Like industrial biotechnology, the processes created by bioprocess engineering aim to be be sustainable and eco-friendly.

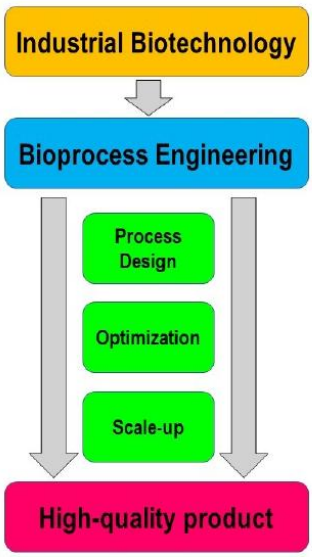


Figure 11.2.1: Industrial biotechnology and bioprocess engineering. Industrial biotechnology uses bioprocess engineering principles for the design of a manufacturing process (process design), its optimization and then application of this process at a large enough scale for commercial use (scale-up). The end result of bioprocess engineering is a high-quality product. (Industrial Biotechnology and Bioprocess Engineering by Patricia Zuk; CC BY-SA 4.0)

Without bioprocess engineering, the principles and techniques that form the foundation of industrial biotechnology could not be applied at a large enough scale. The ultimate goal of bioprocess engineering is to make biology easier to engineer—more predictable, reliable, and scalable.

Learning Objectives

Bioprocess engineering takes the biological techniques used in biotechnology and scales them up for use in a biomanufacturing plant. At the end of this page, you will be able to:

- Distinguish between industrial biotechnology and bioprocess engineering
- Understand the differences between bioprocess engineering and other "bio"-based terms like bioengineering, biochemical engineering, and biomedical engineering
- Explain some of the key areas used in bioprocess engineering
- Explain biomanufacturing
- Define and explain the upstream and downstream processes used in biomanufacturing
- List some areas critical to upstream processing
- List some downstream processing steps

"Bio" Engineering Terminology

Many words have been used to describe engineers that work with biotechnology. For example, the term bioprocess engineering is often used interchangeably with other terms like biomedical engineering, biochemical engineering, bioengineering, and biomolecular engineering. However, each of these terms are different and distinct and are often misunderstood.

The following is a list of some of the commonly used "bio" engineering fields:

- **Bioengineering:** a broad title that includes work in medical and agricultural systems
  - applies engineering principles to biological systems for the development of technologies and products
  - practitioners include many types of engineers, including agricultural, electrical, mechanical, industrial, and chemical
- **Biological engineering:** similar to bioengineering but emphasizes applications to plants and animals
- **Biochemical engineering:** the extension of chemical engineering principles to biological systems in order to bring about a specific chemical transformation
  - subdivided into bioreaction engineering and bioseparation engineering

- **Biomedical engineering**: the application of engineering techniques and design concepts to medicine and biology for healthcare purposes, such as diagnosis, treatment, and rehabilitation
- **Biomolecular engineering**: deals with the interface of biology and chemical engineering with a focus on the molecular level
- **Bioprocess engineering**: applies engineering principles to processes based on living cells or biological components (e.g. organelles, enzymes)
    - includes the work of mechanical, chemical, and industrial engineers

Key Areas in Bioprocess Engineering

Bioprocess engineering is a highly interdisciplinary field of engineering, combining the principles of chemical engineering, biology, and biotechnology. Key areas of bioprocess engineering include:

- **Fermentation Technology:** focuses on the use of microbes to convert raw materials into products
    - optimizes the fermentation process, including the design of bioreactors and the control of fermentation at a large scale
- **Bioreactor Design:** focuses on the design and optimization of bioreactors for efficient cell growth and product formation
    - includes bioreactors for the fermentation of microbes (e.g. bacteria, yeast, fungus) or the growth of plant and animal cells
- **Downstream Processing:** focuses on the development of methods for separating and purifying products produced from a bioprocess
    - includes the field of **bioseparation** - the development of methods for separating and purifying biological molecules
- **Cell Culture Engineering:** focuses on understanding and optimizing cell culture conditions for specific industrial biotechnology applications
- **Bioprocess Modeling:** uses mathematical models to understand and predict bioprocess behavior
- **Biomanufacturing:** focuses on the design and implementation of processes for the large-scale production of bioproducts
    - critical for moving the bioprocess from the lab to industrial production

Biomanufacturing

Bioprocess engineering is a key aspect of **biomanufacturing.** Biomanufacturing facilities use bioprocess engineering principles to commercially produce materials made through synthetic biology (e.g. therapeutics like antibodies, vaccines, biofuels, and food ingredients) at very large scales. The process of biomanufacturing begins with research and development within a laboratory. The lab is responsible for determining the biological system that will be used to make the product and creating the workflow using this system. Once the workflow has been created, bioprocess engineering focuses on how to design and run the process at a large enough scale. A biomanufacturing facility (Figure 11.2.2) then runs these scaled-up processes.

Figure 11.2.2: A biomanufacturing facility. The biomanufacturing facility in this picture contains numerous large bioreactors connected to one another(Copyright; author via source)

Biomanufacturing involves both **upstream and downstream processes** (Table 11.2.2).

"Upstream" is the growth and production phase and refers to the steps involved in growing and preparing the microbes, plant or animal cells that will produce the desired product. Upstream processes happen before the downstream extraction and purification

steps. Because of this, the design of the upstream steps can have a significant effect on what takes place downstream.

The following are critical to upstream processes:

1. **Cell Line Development**
   - the appropriate cells are chosen and/or engineering to make the product
   - cells include prokaryotic (bacteria) or eukaryotic (yeast, plant, animal)
2. **Media Preparation & Optimization**
   - precise mixtures of nutrients (e.g. amino acids, sugars, vitamins, salts) that are critical for cell growth
   - dependent upon the cell chosen for production
   - compositions are optimized for cell viability, growth, and expression of new genes
3. **Bioreactors**
   - where cells grow and make the product
   - provide cells with controlled external conditions - temperature, pH, oxygen (i.e., aeration), agitation/mixing
   - designed for fermentation of microbes or culture of plant of animal cells
4. **Monitoring & Control Systems**
   - sensors and systems track the process in real-time to ensure optimal growth and product formation

"Downstream" in biomanufacturing are the steps that follow production. In general, downstream processes focus on extraction, purification, and packaging of the product. The design of downstream processes are critical to ensuring that the product meets purity, quality, and safety standards.

Depending on the product made and the upstream processes used, downstream steps might include:

1. **Cell Harvesting:** separates cells from the culture medium using techniques like centrifugation or filtration
2. **Cell Disruption/Lysis:** produces a cell lysate containing the product; required if the product is located within the cell
3. **Filtration:** removes large particles or debris from the culture medium or the cell lysate
4. **Chromatography:** purifies the compound of interest from a mixture
5. **Ultrafiltration/Diafiltration:** concentrates and purifies the product; used to improve the yield of the product
6. **Sterilization:** ensures the product is microbe-free
7. **Formulation and Testing:** designs and prepares the product so that the active ingredient(s) can be delivered in a safe, stable, and effective way; mixes the product with stabilizers, preservatives, etc...; tests for stability
8. **Quality Control:** ensures product meets all specifications, including formulation and safety
9. **Filling and Packaging:** transfers the product into the appropriate package for distribution (e.g. vials, syringes, ampules, bottles)

Table 11.2.2 below summarizes the differences between upstream and downstream processing.

Table 11.2.2: **Upstream vs. Downstream Processing**

	Upstream Processing	Downstream Processing
Definition	• First part of the bioprocess. • Results in the crude production of a product	• Second part of the bioprocess • Involves extraction and purification of the product
Major Event(s)	• Creation of the product in bioreactors	• Harvesting, purification, and formulation of the product
Steps Involved	• Selection of microbes or cell line • Development of media conditions • Growth of cells in bioreactors	• Centrifugation, filtration, chromatography, and concentration steps • Quality control • Formulation • Packaging
Challenges	• Sterility • Optimal cell growth • Scalability	• Separation techniques • Purity standards • Quality control

Biomanufacturing facilities must contend with government regulations, production logistics, and quality control. Products that are manufactured for medical or food use must be produced in facilities designed and operated according to **Good Manufacturing Practice (GMP)** regulations. Cleanrooms, that use sterilization and aseptic techniques, are a key component of both upstream and downstream processes. To read more GMP and quality control, go to Chapter 12.1 Quality Assurance and Quality Control in Biotechnology. To learn more about sterilization and aseptic technique, go to Chapter 6.2 Principles of Aseptic Techniques.

Today, thousands of products, including medicines like cytokines, fusion proteins, growth factors, monoclonal antibodies, and vaccines, are made in biomanufacturing facilities across the world. Numerous food and beverages products like amino acids, enzymes, and proteins supplements are also made. Finally, biomanufacturing has played a key role in industrial biotechnology applications such as bioremediation, and the production of detergents and bioplastics.

Key Concepts

Bioprocessing engineering allows for the production of industrial biotechnology products at a large, commercial scale. Some important points to remember are:

- Bioprocessing engineering bridges the gap between a biological system developed in a research and development lab and the biomanufacturing facility that will make this biological system's product
- Key areas of bioprocess engineering include, fermentation system development, bioreactor design and optimization, and cell culture engineering
- Bioprocess engineering is a field that is distinct from many other similar sounding "bio" engineering fields
- The processes designed by bioprocessing engineering are applied at commercial scales in a biomanufacturing facility
- Biomanufacturing is comprised of upstream and downstream processes

Glossary

Biomanufacturing - the production of biological products, such as vaccines, therapeutic proteins, or enzymes using living cells or organisms in industrial settings

Bioprocess - any process that uses living cells to create a product

Bioprocess engineering - an engineering field that designs and develops the processes that use biologic systems to make a product

Bioreactor - a container or vessel used to grow cells, microorganisms, or tissues under controlled conditions

Downstream - refers to the steps in a bioprocess after the cells have made the desired product

Engineering - the science of designing and building complex processes or machines

Fermentation - a biological process where microbes (like bacteria and yeast) convert sugars into other products like alcohol, gases, or acids

Upstream - refers to all the steps involved in preparing and growing the biological system that will produce the desired product

---

11.2: Bioprocess Engineering is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

11.3: Synthetic Biology

Biotech Focus

Can you synthesize an organism's entire genome?

The answer to this question is yes. In 2002, scientists in the US synthesized an "artificial" viral genome for the first time when they recreated the polio virus genome from scratch. Their work brought attention to the risk that synthetic biology could be used to develop biological weapons.The first synthetic bacterial genome was completed 6 years later with the synthesis of the *Mycoplasm genitalium* genome, a bacterium that causes urinary and genital tract infections in humans. In 2017, another group of scientists partially synthesized the genome of *Saccharomyces cerevisiae*, the yeast that is used to make bread and brew wine and beer. Today, researchers are continuing to push the limits of synthetic biology and DNA-synthesis technology in order to help understand how genomes work. Researchers

One group of researchers, called the "Genome Project-Write" (GP-Write)", is seeking to synthesize, or "write" the human genome, in addition to the genomes of other plants and animals important to agriculture and public health. However, like the work on the polio virus, ethical concerns have been raised in association with GP-Write. What is the exact purpose of artificially creating a human genome? Will the work be restricted to cell lines or be used to alter human embryos. How synthetic biology and artificial genomes will continue to develop will definitely be an area to keep an eye on.

To learn more about synthetic biology and what it can do, check out the National Institute of Health (NIH) webpage on synthetic biology.

Introduction

Synthetic biology is a field of science that combines biology and engineering to design and build new biological parts, systems, or organisms. In synthetic biology, organisms are often completely "redesigned" to give them new abilities, like produce a produce a new therapeutic compound, detect a toxin, or make enzymes that turn sugars into biofuels. Alternatively, entirely new organisms can be built from the "ground up". Synthetic biology is analogous to computer programming, but instead of writing code, scientists use DNA, RNA, and proteins to write new genetic "software" that makes "old" cells behave in "new" ways.

Synthetic biology is often confused with genetic engineering. Genetic engineering is the *direct modification of an organism's DNA using biotechnology*. It focuses on using genes that are already found in nature and involves the insertion of new genes into an organism's genome, or the editing of the genome by either deleting a gene or changing its sequence. Based on this, genetic engineering is part of synthetic biology. However, synthetic biology is a more advanced and design-oriented field that builds new biological parts, systems, or even organisms that may not exist in nature at all.

Learning Objectives

Synthetic biology combines the techniques of biology and engineering to create new biological systems. At the end of this page, you will be able to:

- Explain synthetic biology and its importance
- Explain BioBricks and list some of the common parts used

Synthetic Biology Applications

The applications of synthetic biology are wide and varied. Examples include building synthetic circuits in bacteria to make them behave like computers or creating entirely new synthetic genomes like the Synthetic Yeast Project. However, one of the most well-known examples of synthetic biology is the production of lab grown "meat". Since the introduction of the first cultured beef hamburger in 2013, snthetic biology is allowing researchers to rethink meat production by growing meat without animals. To do this, a sample of animal cells (e.g. cow or chicken muscle cells) is collected without harming the donor animal. The cells are placed into a bioreactor containing a nutrient-rich culture medium that mimics what they would be exposed to in the animal's body. The cells are induced to grow rapidly, differentiate into muscle and fat tissue, and then formed into something that resembles meat. Improvements in this technology have allowed for improved taste and texture, enhanced nutritional content, and cell growth using synthetic alternatives

to animal serum. Today the global lab-grown meat "market" has increased, with companies like Upside Foods (USA) and GOOD Meat (Singapore) being among the first companies to sale lab-grown "chicken" to customers.

Figure 11.3.1: The Cultivated Hamburger. Lab-grown meat can be used to make delicious hamburgers. (First cultivated hamburger by Mosa Meat; CC BY 4.0)

Lab-grown meat is a more conventional application of synthetic biology, but there are others that may seem more in the realm of science fiction than science. Today, researchers are exploring the use of synthetic biology in order to produce self-healing materials like concrete, metal, and plastic. For this, scientists engineer microbes or biological components (like proteins or enzymes) and embed them in a material. When damage happens, these biological agents activate and “heal” the material. Engineered bacteria, like *Bacillus subtilis*, have been mixed into concrete to product a "bio-concrete". This bio-concrete, when cracked, allows the entrance of water and activates the bacteria to begin to produce limestone, thus sealing the crack. Synthetic biology and bio-concrete have been proposed as a way of extending the life of roads, bridges, and buildings. Similarly, scientists have embedded engineered yeast or *E. coli* into hydrogels and plastics, enabling them to detect stress or damage and respond by producing repair proteins that mend the material. This approach could be used to make materials more sustainable rather than disposable and could even be used on spacecraft or space stations to repair damage on their own.

BioBricks

BioBricks are standardized DNA sequences (i.e. "parts) that encode biological functions and can be easily assembled together physically to build new biological "circuits". These synthetic biological circuits can then be incorporated into living cells to construct new biological systems.

Examples of BioBrick parts include the:

1. **promoter** which directs gene expression
2. **ribosome binding site (RBS)** that helps start protein production via translation
3. **gene or coding sequence**
4. **terminator** which tells the cell when to stop translating the coding sequence

BioBrick parts are used according to a specific hierarchy (Figure **11.3.2**). Specifically, the BioBrick parts (e.g. promoter, RBS etc...) are combined to form a "device" - a genetic circuit to perform a defined function. A complementary set of devices can then be combined as a biological system to perform a high-level task.

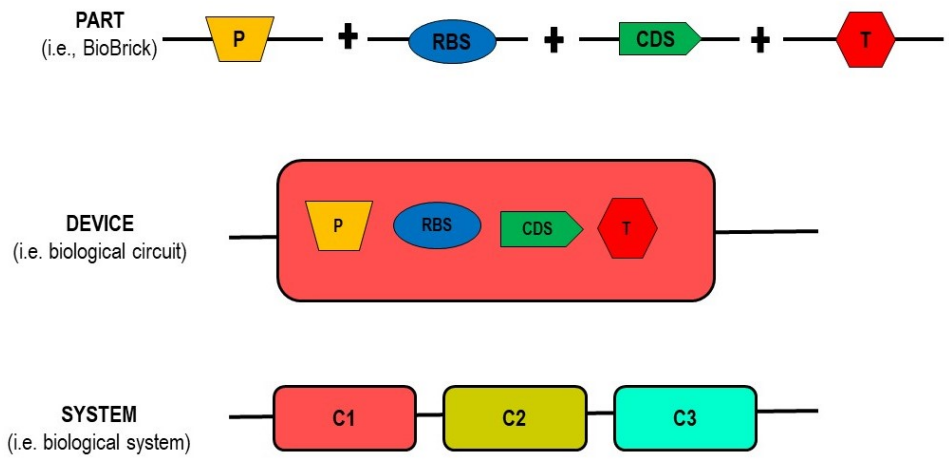
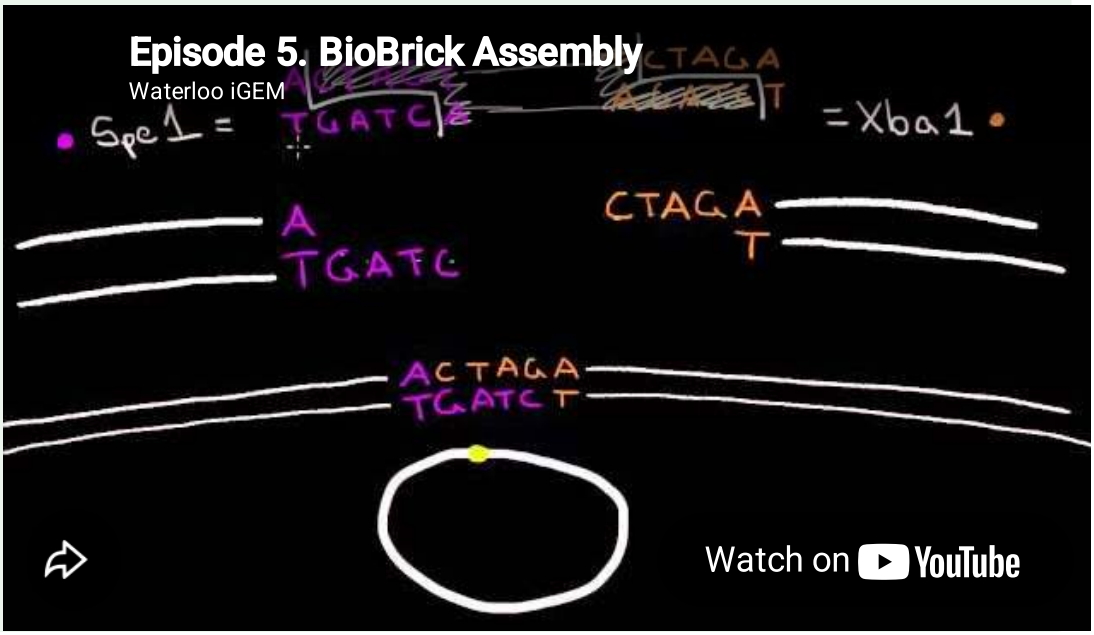


Figure **11.3.2**: Biobricks are used in synthetic biology. Each BioBrick, known as a "part", is a DNA sequence. The BioBrick parts of promoter (P), ribosome binding site (RBS), coding sequence (CDS), and terminator sequence (T) are ligated together in a plasmid to form a "device" or biological circuit. Several circuits are combined to form a "system" or biological system (BioBricks by Patricia Zuk, CC BY 4.0; modified from Abstraction Hierarchy by Shammie23, CC BY-SA 3.0)

To create a synthetic system using BioBricks, each part is cloned into a plasmid. The sequences flanking each part are specific restriction sites that allow the BioBrick parts to be digested out of their plasmid and then ligated into another plasmid in a specific order - not unlike snapping several Lego bricks together to make a bigger toy. These restriction sites are standardized across all BioBricks. The "prefix" restriction site at the start of the part's sequence is either EcoRI, NotI, or XbaI. The "suffix" restriction sites found at the end of the sequence are either SpeI, NotI, or PstI. Ultimately, when these parts are ligated to one another in their desired order, the majority of the restriction sites are eliminated to prevent the accidental removal of a key BioBrick part. However, the flanking sites at the extreme ends are still intact, allowing for the addition of more parts or the transfer of the assembled parts. To learn more about restriction sites and ligation, go to Chapter 4.1 Principles of Genetic Engineering and Chapter 4.2 Creating Recombinant DNA.

Concept in Action

BioBrick Assembly



Testing of BioBrick systems are often done using cell-free systems or a **"chassis"** - a host organism (like *E.coli* or yeast) that is used to "run" synthetic genetic circuits. A chassis in synthetic biology is not unlike the hardware of the computer that runs the newly written software. Choosing the right chassis is important in synthetic biology. This is because different chassis have different metabolisms, growth rates, and tolerances. Others are better for industrial-scale production, drug delivery, or biosensoring.

Common chassis organisms used in synthetic biology include:

- *E. coli* - fast-growing, well-studied, and easy to modify
- *Saccharomyces cerevisiae* - a eukaryotic chassis used for more complex tasks, like producing drugs of biofuels
- Cyanobacteria - used as photosynthetic chassis to produce energy
- Mammalian cells - used for advanced medical or therapeutic applications

Numerous applications of BioBricks are being proposed each and every day. One way could be in the creation of **biosensors** within cells capable of detecting biotoxins like heavy metals in cells (Figure **11.3.3**). Others include detection systems for pests in agriculture, systems for carbon capture, and the creation of chemical "factories" that would produce things like complex flavors, fragrances, and materials.

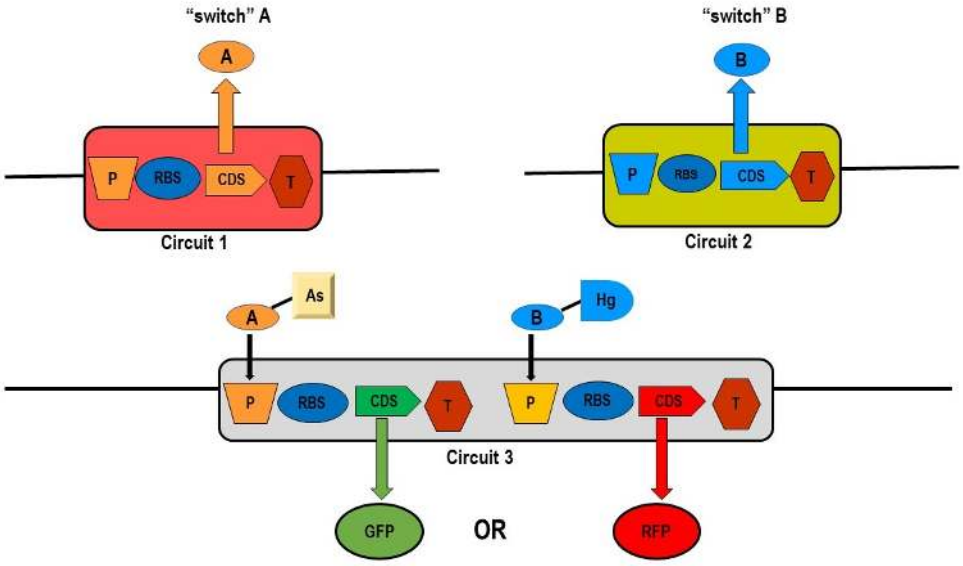


Figure 11.3.3: Heavy metal detection using BioBricks. BioBricks are assembled in Circuit 1 for the expression of a DNA-binding protein that act as a molecular "switch" (Switch A) for the detection of arsenic (As). A similar switch protein (Switch B) that detects mercury (Hg) is expressed using Circuit 2. These switch proteins control gene expression from Circuit 3. Switch protein A binds arsenic (A-As) which then binds to its promoter, driving expression of green fluorescent protein (GFP) from its coding sequence. Switch protein B binds mercury (B-Hg). Binding of this switch to its promoter drives expression of red fluorescent protein (RFP). (BioBrick Detection of Heavy Metals by Patricia Zuk; CC BY 4.0)

Key Concepts

Synthetic biology is a combination of biology and engineering and is used to modify or create genetic "circuits" in cells that changes the function of a cell

Some important concepts to remember are:

- synthetic biology uses engineering principles on biological systems
- synthetic biology treats DNA like software code, editing it or creating it, in order to given an organism a new function
- synthetic biology can be applied to medicine, agriculture, energy, and the environment
- one aspect of synthetic biology is the use of BioBricks
- BioBricks are standardized DNA sequences that can be linked together to form a "circuit" - a system for expression of a gene multiple circuits can be combined to create synthetic biological systems

Glossary

Bioengineering - the application of engineering principles to biological systems for the development of technologies and products

BioBricks - standardized DNA sequences called "parts" with defined functions that can be combined to build synthetic genes and circuits; includes parts like promoters, ribosomal binding sequences, coding sequences, and terminator sequence

Biosensor - a device that uses a biological component, like an enzyme, antibody, cell, or DNA sequence) to detect and measure specific chemicals, substances, or conditions and convert them into a signal (like light, electricity, or color change)

Cell-free system - a synthetic environment outside living cells where biological reactions can occur using extracted cellular components like enzymes and ribosomes

Coding sequence (CDS) - the sequence of DNA that is ultimately translated into a polypeptide by ribosomes

Genetic circuit - a designed combination of genes and regulatory elements that control how a cell behaves, similar to electronic circuits

Genome - the totality of genetic information found within a cell

Promoter sequence (promoter) - a sequence of DNA where the RNA polymerase binds; determines the site of transcription initiation

Restriction enzyme - an endonuclease that cuts the DNA at a specific sequence called a restriction sequence

Restriction sequence - the sequence of DNA cut by a restriction enzyme

Restriction site - the physical location of a restriction sequence in a DNA sequence

Ribosomal Binding Site (RBS) - a short sequence found in messenger RNA (mRNA) that signals the ribosome where to start translating the mRNA into a protein

Synthetic genome - a genome that has been entirely built from synthetic DNA rather than copied from nature

Terminator sequence - a short sequence found in mRNA that signals where the ribosome stops its translation

---

11.3: Synthetic Biology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

12: Quality Assurance and Quality Control in Biotechnology

12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs

12: Quality Assurance and Quality Control in Biotechnology is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

12.1: Principles of Quality Assurance (QA) and Quality Control (QC)

Biotech Focus

In the fall of 2004, the US experienced a severe influenza vaccine shortage due to a production issue at Chiron Corporation's Liverpool, England plant. FDA and British inspectors at the plant revealed violations of "good manufacturing practices" leading to contamination of vials of the vaccine with the bacteria *Serratia marcescens*. Inspectors identified potential errors in the filling of the vials, possibly compromising the sterility of the vaccine. The FDA concluded that none of the Chiron vaccine was safe for distribution in the US and a **recall** was issued of over a million doses of the vaccine shipped to the US. This recall effectively halved the expected US supply of influenza vaccine, necessitating a shift in vaccination recommendations and priority groups.

Read about the Fluvirin recall here.

Introduction

Because biotechnology products impact human health, quality is not optional *it is essential*. From therapeutic proteins to gene therapies, the integrity of biotechnology products (and services) must meet the highest standards. To ensure standards are met, biotechnology facilities rely upon **quality assurance (QA)** and **quality control (QC)** (Figure 12.1.1). Through QA, product quality is built into the process from design to delivery, with an emphasis on identifying defects and errors before they happen. If they do, companies can use QC procedures to correct. As such, both QA and QC are integral components of a biotechnology company's production of safe and effective products.

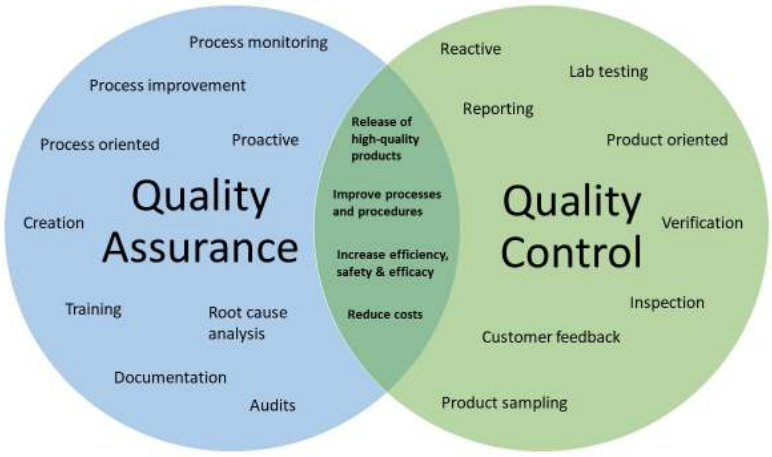


Figure 12.1.1: QA versus QC. Quality assurance (QA) is proactive and process oriented and involves process improvement, monitoring, documentation, audits and training. Quality control (QC) is reactive and product oriented and involves product sampling, lab testing, verification, inspection, and customer feedback. Both emphasize the release of high-quality products, improvement of processes and procedures, increase of efficiency, safety and efficacy, and the reduction of costs. (QA vs. QC by Patricia Zuk, CC BY 4.0)

Learning Objectives

Quality assurance (QA) and quality control (QC) are used by biotechnology companies to identify errors before they happen or to correct them when they do. At the end of this page, you will be able to:

- Define QA and QC and distinguish between them
- Compare and contrast major features of QA and QC
- Explain the major principles of QA and QC
- List the different types of QA and QC
- Explain the role of ISO standards in QA and QC
- Explain the ISO 9000 family of standards

- Explain the purpose of ICH guidelines in pharmaceuticals
- Explain the major categories of ICH guidelines
- Explain the difference between a standard and a specification
- Discuss the roles of QA and QC in biotechnology manufacturing

Quality Assurance (QA) vs. Quality Control (QC)

QA and QC are often confused with one another. While they are related to one another and are both used in product development, they are distinct processes. QA is a systematic approach that ensures all processes involved in the production, testing, and documentation of manufactured products are conducted in accordance with defined standards, specifications, and regulations. QA is process-oriented and pro-active. It focuses on *preventing errors before they occur*. The mantra for QA is "right the first time". QC reviews the quality of all factors involved in production. It is the process of testing and inspection that is used by manufacturers to identify defects or deviations from specifications in raw materials, in-process samples, and final products. QC is product-oriented and reactive. It ensures that outputs meet established quality standards and, when they don't, identifies *where and when errors occurred*. Table 12.1.1: below summarizes the focus, purpose, responsibilities, and activities of QA and QC.

Table 12.1.1: Quality Assurance vs. Quality Control

Feature	Quality Assurance (QA)	Quality Control (QC)
Focus	The process of making a product (from design to delivery)	the product being made
Purpose	Prevention of defects before production (proactive)	Identification and correction of defects during or after production (reactive)
Activities	Documentation, audits, training, SOPs	Testing, sampling, inspection
Responsibility	Quality Assurance department	Quality Control department

Major Principles of Quality Assurance

1. **Compliance with Regulatory Standards:** QA ensures that all products and processes meet regulatory requirements set by regulatory agencies
2. **Document Control:** QA ensures that all procedures are properly documented in **Standard Operating Procedures (SOPs)**, which must be approved, regularly reviewed, and archived
3. **Change Control:** Any proposed changes to materials, methods, or equipment must go through a formal **change control process**, including risk assessment and justification for the change
4. **Training and Qualification:** QA is responsible for ensuring all personnel are properly trained according to internal and external regulations (e.g., Occupational Health and Safety) and their training is documented and regularly updated
5. **Auditing and Self-Inspection:** QA is responsible for conducting internal and external audits that help verify compliance with standards and specifications and uncover opportunities for improvement
6. **Corrective and Preventive Action (CAPA):** QA oversees CAPA systems that investigate the root causes of deviations from standards and specifications and implements actions to prevent recurrence of these deviations

Types of QA

- **Product QA:** focuses on ensuring that the final product meets the specified requirements and standards
- **Process QA:** focuses on ensuring that the processes used to create the product or service are efficient, effective, and consistent
- **Functional QA:** focuses on ensuring that the product functions as intended and meets all functional requirements
- **Supplier QA:** focuses on ensuring that the suppliers of materials or components meet the required quality standard

Major Principles of Quality Control

1. **Raw Material Testing**: All raw materials must be verified for identity, purity, and potency before use in production
2. **In-Process Control (IPC):** QC requires the monitoring of processes while they are being performed (i.e., real-time monitoring) in order to ensure that critical parameters remain within acceptable ranges

- prevents defects and errors by maintaining process conditions within predefined limits
- e.g. bioreactor conditions during fermentation processes

3. **Acceptance Sampling:** Inspection of a random sample taken from a production batch to decide whether the whole batch meets quality standards
   - identifies defects in products as they are being made
   - samples (and the entire batch) are either accepted or rejected
   - e.g. extraction of a sample from a bioreactor
4. **Final Product Testing:** QC performs tests for material identity, potency, purity, sterility, and stability
   - goal is to detect and remove defective items before they reach the customer
   - e.g. laboratory analysis of a drug
5. **Analytical Method Validation:** All analytical methods used in QC must be validated to demonstrate accuracy, precision, sensitivity, and reproducibility
   - method validation is mandatory to meet standards set by regulatory authorities like the FDA
   - e.g. validation of quantitative QC methods used to assay drug purity and safety
6. **Stability Testing:** QC must ensure products remain effective and safe throughout their shelf life by conducting short-term and long-term stability studies
7. **Batch Release and Certificate of Analysis (CoA):** QC provides the final evaluation of product quality before a "batch" of product is released
   - includes a signed CoA with key test results

Quality Management Systems

In biotechnology, QA and QC ensure that each product is made in accordance with regulations set by agencies like the FDA, USDA, or the European Medicines Agency (EMA). To ensure this, a biotechnology company employs some type of **quality management system (QMS).** A QMS is a highly structured framework that helps an organization deliver a high-quality product (or service) in a consistent manner. The QMS defines and documents all processes, procedures, and responsibilities involved in manufacturing a product (or providing a service).

Biotechnology facilities will use one (or both) of the following QMS:

- **GMP (Good Manufacturing Practice)**
  - a QMS that applies to the manufacturing, processing, and packaging of regulated products, such as pharmaceuticals, medical devices, and foods
  - ensures that products are produced to consistently meet all quality standards and are safe for use
  - emphasizes comprehensive documentation of all manufacturing processes, batch records, and product testing in order to ensure traceability and regulatory compliance
- **GLP (Good Laboratory Practice)**
  - a QMS used in the research and development labs of biotechnology companies
  - ensures the credibility and reliability of scientific data
  - establishes standards for the conducting of a study and the collection, analysis and reporting of its data
  - emphasizes adherence to protocols, documentation, and quality control measures to ensure that research findings are accurate and reproducible

ISO Standards in QA and QC

Every company promises an unwavering commitment to quality. However, how does a company provide proof of this commitment? Companies can assure their customers of the quality of their practices and processes using standards established by the **International Organization for Standardization (ISO).** Founded in 1947, the ISO has over 170 member countries and publishes new standards and revisions to existing standards on a regular basis. As of 2024, there are over 24,000 ISO standards that cover virtually every industry and sector. Some of the major categories of ISO standards cover quality management systems, environmental management systems, information security, occupational health and safety, food safety, medical devices, and

laboratory competence. In QA, ISO's promote principles such as continual improvement and a commitment to safety and quality. They encourage documentation, monitoring, and evaluation of processes to prevent defects and errors. In QC, ISO standards are used to define calibration requirements for equipment, specific testing methods, and the criteria used to accept or reject a product. They help organizations develop a structured approach to inspection and testing, so that non-conforming products can be detected and dealt with.

The following ISO standards are used frequently in biotechnology QA and QC:

- **ISO 9001** – Used to create and implement a QMS
- **ISO 22000** - Used for Food Safety Management Systems
- **ISO 2859** - Used for QC sampling procedures
- **ISO 17025** – Used for testing and calibration of laboratories
- **ISO 13485** – Used for a QMS for medical devices
- **ISO 19011** – Guidelines for auditing management systems

ISO 9001 is part of the ISO 9000 "family" of standards. The ISO 9000 family includes three main standards: ISO 9000, ISO 9001, and ISO 9004. **ISO 9001** is the standard that helps organizations develop and implement a robust QMS like GMP or GLP. The most recent ISO 9001 version is called ISO 9001:2015. Companies achieving this certification are permitted to display a logo on company materials like letterhead, labels, and their website (Figure **12.1.1**). This specific ISO is the only one in the ISO 9000 family that is "certified" through an external **ISO audit** - a systematic, independent, and documented process for evaluating whether an organization's management system, such as their QMS, complies with the requirements of a specific ISO standard. Companies will seek "**ISO 9001 certification"** in order to improve their product and service quality, enhance operational efficiency, and support continuous improvement of their processes. This certification can also boost customer satisfaction and confidence and increase market share because it assures customers that the product they are purchasing has a defined level of quality. In certain instances, companies cannot realistically enter a market without ISO 9001 certification. In contrast to ISO 9001, the ISO 9000 and ISO 9004 standards are not certified through an audit. Rather, companies will use ISO 9000 to learn the basic concepts, principles, and terminology important to ISO 9001 certification. If desired, companies can also follow the standards outlined in ISO 9004 in order to go beyond ISO 9001 compliance to achieve long-term success. Because of this, ISO 9000 and ISO 9004 are applicable to any organization regardless of size, industry, or location, whereas ISO 9001 is used by companies that have established a QMS.

Figure **12.1.2**: The ISO 9001:2015 logo. (ISO 9001-2015 by Юкатан, CC BY-SA 4.0)

ICH Guidelines

For pharmaceutical companies, products must be deemed as safe, effective, and of high quality. Moreover, the production, testing, and regulatory processes used in QA and QC must be consistent across major global markets. In short, these processes must be "harmonized". As a result, pharmaceutical companies also follow **ICH guidelines**. These guidelines are internationally harmonized standards developed by the **International Council for Harmonisation (ICH)** of Technical Requirements for Pharmaceuticals for Human Use. The ICH was founded in 1990 and its members include regulatory authorities (e.g., FDA, EMA, PMDA) and industry groups. The goal of ICH guidelines is to "harmonize" pharmaceutical regulations across the U.S., EU, Japan, and more recently, Canada, China, and Brazil. ICH guidelines focus on reducing duplication in drug development, testing, and approval processes. They are recognizes by regulatory agencies worldwide and are often required for drugs that are entering the global market.

ICH guidelines are divided into five categories:

1. **Q – Quality Guidelines:** focuses on chemistry, manufacturing, and controls
    - examples: Q1 (stability testing), Q8 (pharmaceutical quality system)
2. **S – Safety Guidelines:** focuses on preclinical safety and toxicology studies
    - examples: S1 (carcinogenicity), S2 (genotoxicity), S6 (biotechnology products - i.e., preclinical safety)
3. **E – Efficacy Guidelines:** focuses on clinical studies and pharmacology
    - E6 (Good Clinical Practice), E8 (general considerations for clinical trials), E9 (statistical principles)
4. **M – Multidisciplinary Guidelines:** focuses on topics that cross multiple categories
    - M1 (medical terminology), M4 (common technical document), and M9 (biopharmaceutical classification system)
5. **R – Recently Revised/Implemented Guidelines:** introduces updated versions of older guidelines

Quality Standards and Specifications

The terms standard and specification are used frequently in QA and QC. A standard might require or refer to a specification, while a specification might comply with or be derived from a standard. So, while standard and specification are related, they are not the same thing. A **standard** is a broad, consensus-based document that provides the guidelines or rules for a process and product (or service). A standard covers terminology, general principles, and "best practices" and is often applied across an organization or industry. Because of this, the scope of a standard is broad and general. For example, a standard might stipulate that the base of a light bulb be "made of conductive metal". Another example of a standard is the ISO 9001 standard used in a QMS. This standard defines what a QMS should include, but not how every company must implement it.

A **specification** is a more detailed, *technical* document that defines the exact requirements for a product, material, or process. It is often used in manufacturing and engineering. The specification tells a user exactly what the product must be and must do. The scope of a specification is narrow, detailed, and technical. For example, with regards to the light bulb, the specification might stipulate that the conductive metal contain 30 to 40% copper. Lastly, specifications might differ from company to company, but a standard does not change.

Integration of QA and QC in Biotechnology Manufacturing

In biotech manufacturing, QA and QC are deeply integrated into every stage of the process to ensure that biopharmaceutical products are safe, effective, and comply with regulatory standards (e.g., FDA, EMA, ICH). They are embedded in all stages of product development. For example, during upstream processing, QA oversees process validation, while QC tests cell lines and growth media. During downstream processing, QC ensures the purity and integrity of a product. Regulatory agencies like the FDA (and the European Medicines Agency/EMA) require comprehensive QA and QC documentation as part of new drug applications and the licensing of new biologics. Failures in QA or QC can result in clinical trial delays, product recalls, or regulatory action that can result in large fines. Table **12.1.2** below gives several examples of how QA and QC work together in biotechnology manufacturing.

Table **12.1.2**: **Roles of QA and QC In Biotech**

Phase	QA Role	QC Role
Design and Development	• Implement quality manufacturing systems (QMS) like Good Manufacturing Practices (GMP) or Good Laboratory Practices (GLP) early in development • Develops standard operating procedures (SOPs) • Oversees design controls	• Validates analytical test methods • Performs initial raw material testing • Supports method development with data
Raw Material Control	• Approves qualified suppliers • Reviews material handling procedures	• Tests incoming raw materials (e.g., media, buffers) • Ensures material identity, purity, and sterility
Upstream and Downstream Processing	• Audits the compliance of processes to SOPs and batch records • Approves change controls and Corrective and Preventive Actions (CAPAs)	• Performs in-process testing (e.g., cell viability, metabolite levels) • Monitors critical quality attributes (CQAs) • Conducts environmental monitoring in clean rooms
Product Testing and Release	• Reviews and approves batch records and QC data for release • Ensures data integrity and traceability • Manages product disposition decisions	• Conducts release testing of products (e.g., potency, purity, sterility) • Confirms compliance with product specifications • Performs stability testing (short-term)
Validation and Continuous Monitoring	• Oversees the development of strategies for process validation • Implements quality risk management (QRM) principles • Ensures systems remain qualified and calibrated	• Collects and analyzes validation data • Performs trend analysis (e.g., control charts) • Performs and supports long-term stability studies

Key Concepts

Both QA and QC are integral components of a biotechnology company's production of safe and effective products. Some important concepts to remember are:

- QA is proactive; QC is reactive - QA focuses on preventing defects, QC focuses on detecting them
- QA is process-based and focuses on the processes and systems that create the product (or service)
- QC is product-based and tests the final product before it is released
- Biotechnology manufacturers use a quality management system (QMS), like GMP or GLP as part of their QA and QC practices
- QA and QC are conducted using a set of standards established by the International Organization for Standardization (ISO).
- ISO 9001 is the current ISO standard used for the QMS
- Pharmaceutical companies will also use International Council for Harmonisation (ICH) guidelines to ensure that the production, testing, and regulatory processes used in QA and QC are consistent for pharmaceuticals entering the global market

Audit - a systematic, independent, and documented process for evaluating whether an organization's management system complies with the requirements of a specific ISO standard

Batch - a specific quantity of material or product produced during a single manufacturing cycle under uniform conditions; identified by a batch number and must meet defined quality criteria

Documentation - the systematic process of creating, organizing, and maintaining written records that describe processes, procedures, decisions, designs, and results

Good Manufacturing Practice (GMP) - a system of regulations and guidelines used in pharmaceuticals, biologics, medical devices, and food, that ensure products are consistently produced and controlled according to quality standards; enforced by regulatory bodies

Good Laboratory Practice (GLP) - a quality system concerned with ensuring the integrity, reliability, and reproducibility of laboratory data

International Organization for Standardization (ISO) - an independent, non-governmental international organization that develops and publishes voluntary global standards

ISO standards - internationally recognized guidelines published by the International Organization for Standardization (ISO) in order to provide best practices, technical specifications, and requirements for quality, safety, and efficiency across industries

ISO 9001 - the standard that helps organizations develop and implement a QMS like GMP or GLP

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) - an international organization that develops guidelines to ensure safe, effective, and high-quality medicines are developed and registered efficiently and properly

Quality assurance (QA) - a set of planned and systematic activities implemented in a quality system to ensure that quality requirements for a product or service are fulfilled

Quality control (QC) - the operational techniques and activities used to fulfill requirements for quality, typically through sampling, measurement, and testing

Quality Management System (QMS) - a highly structured framework that helps organizations deliver a high-quality product (or service) in a consistent manner

Product testing - the process of evaluating a product's performance, quality, safety, and compliance with relevant standards, regulations, or customer requirements before it is released to the market or approved for use

Standard Operating Procedure (SOP) - a detailed, written instruction describing how to perform a routine or repetitive activity in a consistent and controlled way; ensures compliance, training, quality, and safety

Standard - a documented general agreement containing technical specifications, guidelines, or criteria that can be used consistently by an industry to ensure that materials, products, processes, or services are fit for purpose

Specification - a detailed and exact statement of technical requirements, including measurements, tolerances, materials, and methods for a product, service, or process; defines what is acceptable or expected and is used for quality control, purchasing, and regulatory compliance

---

12.1: Principles of Quality Assurance (QA) and Quality Control (QC) is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

12.2: QA/QC Documentation- SOPs, Batch Records, and COAs

Biotech Focus

While the exact date of creation for the **Standard Operating Procedure (SOP)** is not known, there are some important historical milestones in its evolution. In the 1940s and 1950s, SOP-like procedures were used in military and manufacturing industries to ensure consistency and reliability in products and services used during WWII and the Korean war. In the 1960s and and 1970s, in response to regulatory oversight, SOPs became more common and more formalized in the pharmaceutical and medical industries. SOPs become embedded in most U.S.-regulated industries in 1978 with the publishing of Good Manufacturing Practices (GMP) regulations by the Food and Drug Administration (FDA). In the 1980s, SOPs became standard documentation used in ISO 9001 quality management systems. Since then, SOPs have been increasingly adopted across a wide range of industries, including biotechnology.

Introduction

In Quality Assurance (QA) and Quality Control (QC), **documentation** is essential to ensure processes are followed correctly and products meet required standards. Companies that have a quality management system (QMS) will incorporate specific documents into their system in order to ensure quality in all processes. In addition to these QMS documents, companies will create and use documents that are specific to their QA and QC processes.

Learning Objectives

QA and QC rely on appropriate documentation in order to assure themselves and their customers of their product and process quality. At the end of this page, you will be able to:

- List some of the major documents used in quality management systems (QMS)
- List some of the major documents used in quality assurance (QA)
- List some of the major documents used in quality control (QC)
- Explain the difference between a standard operating procedure (SOP) and work instructions
- Explain the purpose of a batch record and list some of its components

QMS Documentation

ISO 9001 certification requires extensive documentation. These documents can ranges from ones that explain the company's commitment to quality to ones that track non-conformance issues and reviews management protocols. Some QMS documents required for ISO 9001 certification are:

1. **Scope of the QMS:** Defines the scope of the QMS by detailing the products, services, and operations it covers
    - should specify which departments, processes, and locations fall under its scope
2. **Quality Objectives and Plans:** Sets specific, measurable goals that support quality improvement in the company
    - outlines action plans, assigns responsibilities, and allocates resources so the objectives can be achieved
3. **Maintenance and Calibration Records:** Tracks maintenance and calibration of equipment used in monitoring and measuring processes
    - should confirm compliance with industry standards
    - should include details of all approved calibration labs used - both external and internal labs
4. **Competence (Training) Records:** Maintains training and competency records of employees to ensure they have the necessary skills for their roles
    - should document any additional training required for quality management
5. **Design and Development Records:** Documents design inputs, outputs, controls, and modifications
    - should ensure all changes are evaluated and recorded to verify compliance
6. **Internal Audits:** Documents the regular occurrence of internal audits to evaluate QMS effectiveness

- should record findings, issues, and opportunities for improvement

Quality Assurance (QA) Documentation

Through QA documentation, the QA department can link processes to mistakes and defects in the hopes of improving performance and prevent future mistakes. These QA documents include:

1. **Quality Manual**: a high-level document that outlines the company's quality policies, objectives, and overall structure of the QA system
2. **Quality Plans:** project- or product-specific plans that describe the quality standards, practices, and resources to be applied
3. **Standard Operating Procedures (SOPs):** detailed, written instructions designed to ensure specific functions are performed consistently each and every time
   - can contains or refer to detailed work instructions
4. **Work Instructions:** step-by-step guidance for performing a particular task or function
   - more detailed than SOPs
5. **Training Records:** documents that outline staff training, qualifications, and competency evaluations
6. **Audit Plans and Reports:** internal and external audit schedules, checklists, findings, and corrective actions
7. **Supplier Evaluation and Approval Records:** criteria and documentation used to assess and approve suppliers providing materials and equipment
8. **Process Validation Reports:** evidence that the processes used consistently produce the desired results

Standard Operating Procedures and Work Instructions

An **Standard Operating Procedure (SOP)** is a documented procedure that describes how a worker is to carry out a specific process or activity so that what is done is consistent across departments and shifts and is compliant with regulatory requirements and/or an organization's standards. An SOP focuses on a process and describes who does what, when, and why. An SOP is often part of regulatory or quality compliance by a company (e.g. ISO certification, FDA compliance). For example, a worker receiving incoming raw material into their company will use an SOP to ensure that the material meets quality standards before it is used in a process. This SOP might specify the following steps:

1. receive materials from the shipping company
2. inspect the material using established criteria
3. document the receipt and inspection results
4. approve or reject the raw material

The SOP will often contain **work instructions** that provides detailed, step-by-step instructions on how to perform a specific task or activity. They are more granular than SOPs. Work instructions are used by frontline operators or technicians and focuses on a specific task. They often includes images, diagrams, tools needed. The work instruction describes exactly how to do something. For example, the worker receiving the raw material would follow a specific set of work instructions that details how move the material into the facility and where and how to store it. The work instructions would detail the exact steps the worker would use to inspect the material based on the SOP criteria. The work instructions would also outline how each document is to be completed and what the worker would do during accepting or rejecting the material.

Table 12.2.1 below shows how SOPs and work instructions are related.

Table 12.2.1: **SOPs and Work Instructions**

SOP	Work Instruction

Receipt of raw material	• Warehouse instructions:
	  ◦ Receive material from shipper
	  ◦ Verify the materials on the shipping receipt (i.e., packing slip) match what is being received
	  ▪ e.g. quantity,
	  ◦ Sign shipping receipt and make three copies of signed receipt
	  ◦ Record the type of packaging (e.g. styrofoam container, gallon drum) in the appropriate logbook
	  ▪ Place one copy of receipt in the warehouse shipment logbook; forward one copy to the purchasing department; forward one copy to the quality office
	  ▪ Alternative instructions (if applicable): digitally input receipt into the appropriate warehouse database and forward digital copies to the purchasing department and quality office
	  ◦ Assign a unique identification number for each material received and enter information into the appropriate logbook
	  ◦ Create an attach a receiving label to each material that contains the material name, the customer name, the date received, the internal ID number, the status of the material, the storage location of the material
	  ◦ Transfer the received materials to the inspection technician

Inspection of material	• Quality control instructions:
	  ◦ Inspect the received packages for unacceptable conditions like physical damage, moisture, mold, discoloration
	  ▪ For each material received, record each unacceptable condition in the appropriate log book
	  ▪ Document each unacceptable condition with photos, record in the appropriate logbook, and forward to the purchasing office
	  ◦ Verify that the receiving label generated by the warehouse technician includes at least the material name, lot/batch number, quantity, and customer name
	  ◦ Remove the Certificate of Analysis from each material and enter information into the appropriate logbook
	  ▪ Alternative: scan each Certificate of Analysis and enter into the appropriate database
	  ▪ Forward certificate data to the quality lab
	  ◦ Remove a sample from material and perform the appropriate QC tests
	  ◦ Record the QC results using the appropriate form (i.e. paper or digital)
	  ◦ Compare QC results with the Certificate of Analysis
	  ◦ Deem the material as acceptable or unacceptable
	  ◦ Store the material in the warehouse according to the material's requirements (e.g. store the material in the warehouse refrigerated room)
	  ▪ Segregate accepted and rejected materials into the correct areas of the warehouse

SOP	Work Instruction

Documentation	• Warehouse logs (or software database):
	  ◦ raw material receiving logbook
	  ◦ identification number logbook
	• Inspection logs and reports (or software databases):
	  ◦ Condition logbook
	  ◦ Certification of Analysis logbook
	  ◦ QC testing report
	  ◦ Non-conformance logbook

Accept/Reject	• QC instructions:
	  ◦ If material passes QC tests, deem the material as acceptable, sign off and updates status of material to "Approved"
	  ◦ If material fails QC tests, deem the material as unacceptable, sign off and updates status of material to "Rejected"

In summary, the SOP provides the framework and rules for how a process should be managed. Work Instructions provides the instruction for executing specific tasks in that process. Both are essential for a complete and effective quality system.

Quality Control (QC) Documentation

Using QC documents, the QC department identifies defects in products before they are shipped to customers. QC documents record these defects and are often forwarded to the QA department to determine if process improvements are needed. QA documents include:

1. **Inspection and Test Plans (ITPs):** documentation of all inspections and tests required for each process or product
2. **Certificates of Analysis (COA):** documentation provided by a company that verifies the quality and composition of a material or product
   - provides actual test results for specific attributes of a material, such as purity, strength, composition, and other relevant parameters
3. **Test Results:** a record of test results confirming compliance (or non-compliance) with internal specifications outlined by the company
   - test results are compared to results provided in the COA
4. **Non-Conformance Reports (NCRs):** documentation that reports any deviation from specifications or procedures
5. **Corrective and Preventive Action (CAPA) Reports:** documentation of any action taken to fix quality issues and prevent recurrence
6. **Sampling Plans and Records:** records that outline guidelines and evidence for product sampling and testing
7. **Calibration Records:** records that provide verification that measurement equipment is accurate and traceable
   - verification can be done internally or externally
8. **Batch Records/Lot History Records:** traces production and QC activities for each batch/lot of material used
9. **Control Charts and Statistical Process Control (SPC) Data:** used to monitor and control processes over time

Batch Records

A **batch record** is a comprehensive written (or digital) document that is used in a manufacturing processes to record the production details of a specific batch of a product. Batch records are commonly used in industries like pharmaceuticals, biotechnology, food production, and cosmetics. The batch record is not only a quality document that ensures traceability and consistency, but is a legal, compliance document for FDA regulatory standards or ISO requirements.

The batch record is used to standardize batch production, ensuring that each batch is made consistently, following approved procedures. It is used to trace or track the history of every batch for recalls, investigations, or audits. The batch record is part of

quality assurance is it confirms that the product meets quality and safety standards. It is required by regulatory bodies during inspections. Finally, it is used to train new employees on proper procedures and standards.

A batch record contains the following:

1. **Batch identification**
   - Batch/Lot number
   - Product name and code
2. **Manufacturing instructions**
   - Raw materials and quantities in the product
   - Equipment and settings used in the creation of the product
   - Processing steps and timelines required for manufacturing of the product
3. **Personnel and responsibilities**
   - Operator and supervisor signatures
   - Dates and times of each manufacturing step
4. **Quality control checks**
   - In-process test results for specific manufacturing steps
   - Final product specifications
   - Deviations and corrective actions (if applicable)
5. **Environmental conditions**
   - Temperature, humidity, or other required conditions for storing and using the product
6. **Packaging and labeling details**
   - Type and quantity of packaging materials
   - Label batch codes

Companies will create and use a **Master Batch Record (MBR)** which is a standardized template or approved instruction set for manufacturing and testing a product. The MBR is used to ensure consistent production across all batches. The completed version of a batch record that captures the actual data and observations for a specific batch is called an **Executed Batch Record (EBR)**. The EBR reflects what actually occurred during manufacturing. Today, many companies are now moving from paper-based to **Electronic Batch Records (eBRs)** for better efficiency, error reduction, and real-time tracking.

Certificate of Analysis (COA)

A **certificate of analysis (COA)** is a document that is validated and issued by a QC laboratory or QA department. It is required under Good Manufacturing Practices (GMP) and ISO standards and is subject to inspection by regulatory bodies like the FDA, EMA, or Health Canada. The COA confirms the identity, strength, purity, and quality of a product or raw material. The COA will include key test results for these parameters obtained through analytical methods. The COA is a key part of a **batch release decision** in which the product is approved to be used in a company's production process. A QA department uses the COA to determine if a batch is suitable for release into a company's production line or should be referred to the QC department for additional testing or investigation. Issues with the information provided in the COA can result in rejection of the material by the QA department before it is used. The COA is also a key part in **supply chain communication**. By creating a COA, a company can share important information with customers, regulators, or partners about their product in order to provide confidence in their product's quality. The COA provides **traceability**. It helps a company maintain a record of product performance and compliance for audits or recalls. Finally, the COA is used by QC department in raw material testing. Suppliers provide COAs to verify raw materials meet a company's specifications before it is used in manufacturing. The QC department can test raw materials and compare their results to those listed in the COA. Discrepancies between the COA and the QC test results can result in rejection of the material.

The components of a COA can be determined by the industry or by regulatory bodies. However, a typical COA includes:

1. **Product and batch information**
   - Product name and code

- Batch or lot number
- Manufacturing and expiry dates

2. **Specifications and test parameters**
   - Physical and chemical tests (e.g., appearance, pH, assay)
   - Microbiological testing (if applicable)
   - Specification limits or acceptance criteria
3. **Actual test results**
   - Quantitative or qualitative results for each parameter
   - Units of measurement (e.g., %, ppm, CFU/g)
4. **Compliance statement**
   - A declaration that the product "meets" or "does not meet" specification requirements
5. **Signature by authorized QC or QA representative and date**
6. **Laboratory or company information**
   - Name, address, and contact details of the external testing lab or the manufacturer used for testing

QA and QC Documents

Some documents are used in both QA and QC. These documents are:

1. **Change Control Records:** a document that outlines any planned or actual changes to processes, equipment, materials, documents, or systems that could affect quality or compliance
2. **Risk Assessment Analysis and Plans:** a document that identifies and evaluates potential risks to product quality, safety, or compliance
   - also outlines measures to mitigate or eliminate these risks
3. **Customer Complaints and Feedback:** documented reports from customers regarding product issues, dissatisfaction, or improvement suggestions
   - used to identify areas of non-compliance
4. **Regulatory Compliance Records:** records that show how an organization complies with applicable laws, regulations, and industry standards
   - e.g., compliance to FDA, ISO, GMP, OSHA standards and regulations

Key Concepts

Documentation is a key part of quality assurance and quality control. Some important concepts to remember are:

- Quality Management System (QMS) documents include the scope of the QMS, design and development records, and validation reports
- QA documents provide customers with an assurance of a product's quality
- QA documents can link processes to mistakes and defects in the hopes of improving performance and prevent future mistakes
- QA documents include the quality manual, standard operating procedures (SOPs) and work instructions
- QC documents include batch records and certificates of analysis
- Batch records and certificates of analysis have required components product name, product ID code, and batch code
- a batch record documents the production process and quality checks for a specific batch of product
- a certificate of analysis (COA) is issued by a company's QA or QC department to confirm that a product meets all predefined specifications

Glossary

Audit report - an electronic or manual log that tracks all changes, deletions, or additions to a document or system, ensuring transparency and traceability

Batch Record (BR) - a detailed document that records the production process and quality checks for a specific batch (i.e., lot) of product.; includes the raw materials, equipment used, personnel involved, and results of in-process tests

Batch release - the formal process of approving and releasing a batch of product for distribution or sale, after it has successfully passed all required quality, safety, and compliance checks

Certificate of Analysis (CoA) - a document issued by QA or QC that certifies a batch meets all predefined specifications

Change Control - a formal process used to request, assess, approve, and document changes to procedures, equipment, or systems; may impact product quality or compliance

Corrective and Preventive Action (CAPA) - a documented process used to investigate, correct, and prevent the recurrence of deviations, non-conformities, or failures

Deviation - a documented instance where a process or result does not follow the approved procedure or expected outcome

Executed Batch Record (EBR) - the completed version of a batch record that reflects what actually occurred during manufacturing and captures the actual data and observations for a specific batch

Logbook - a chronological record used to document equipment usage, maintenance, calibration, or other quality-relevant activities

Master Batch Record (MBR) - a standardized template used in manufacturing and testing a product to ensure consistent production across all batches

Non-Conformance Report (NCR) - a formal document used to report instances where a product or process does not meet specifications or regulatory requirements

Quality Manual - a top-level document that outlines an organization's quality policies, objectives, and the structure of its quality management system (QMS)

Standard Operating Procedure (SOP) - a detailed, written instruction describing how to perform a routine or repetitive activity in a consistent and controlled way; ensures compliance, training, quality, and safety

Supply chain - the network of people, organizations, activities, information, and resources involved in producing and delivering a product or service from raw materials to the end customer

Traceability - the ability to track and trace the history, application, location, or use of a product, material, or component from its origin to its end use

Validation - the process of proving that a system, process, equipment, or method consistently produces results that meet predefined quality standards

---

12.2: QA/QC Documentation- SOPs, Batch Records, and COAs is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

13: Biotechnology Lab Protocols

13.1: Lab Technique - Pipetting
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
13.4: Lab Technique - Restriction Digest of DNA
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
13.6: Lab Technique - Bacterial Transformation
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
13.8: Lab Technique - Quantitation of DNA
13.9: Lab Technique - DNA Sequencing
13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
13.11: Lab Technique - SDS-PAGE
13.12: Lab Technique - Western Blotting
13.13: Lab Technique - Cell Culture
13.14: Lab Technique - Light Microscopy
13.15: Bacterial Identification Methods
13.16: Basics of Bacterial Culture

13: Biotechnology Lab Protocols is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.1: Lab Technique - Pipetting

Using a Volumetric Serological Pipette

Volumes may be measured and dispensed using volumetric serological pipettes in combination with a pipet-aid (also called a pipette pump). To read more about serological pipettes and pipette pumps, go to Chapter 1.3: Common Biotechnology Equipment.

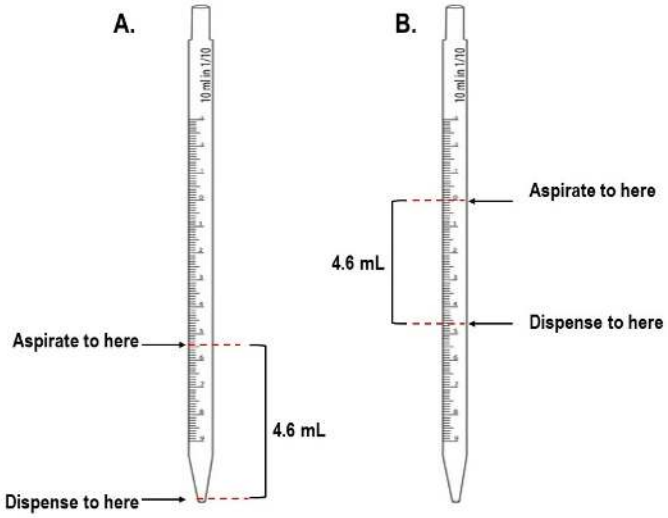


Figure **13.1.1**: Using a serological pipette to dispense 4.6 mL of liquid. (A) The direct aspiration of 4.6 mL of liquid. "TC-In" pipettes would require the complete dispensing of all liquid from the pipette tip for accuracy. (B) Dispensing of 4.6 mL of liquid using the "point to point" technique. Both techniques are equally accurate. (Point to Point Pipetting by Patricia Zuk, CC BY 4.0)

Lab Protocol:

1. Prior to use, verify the type of serological pipette being used by looking at the top of the pipette. Also, note the maximum volume and gradations of the pipette.
2. Insert the top end of the pipette into the pipet-aid (i.e., pipette pump) being careful not to touch the tip of the pipette to anything. This will prevent contaminating the pipette tip and is good pipetting technique. If the tip becomes contaminated, discard the pipette.
3. Carefully insert the pipette into the vessel containing the liquid.
    - Do not put the entire pipette in the liquid – place the tip just below the surface of the liquid (e.g. up to the first major gradation). This will ensure that excess liquid from the vessel does not adhere to the outside of the pipette and inadvertently get transferred.
4. Using the pipet-aid, aspirate the liquid into the pipette a bit above the desired capacity line and then slowly dispense the liquid until the bottom of the meniscus reaches the desired volume.
5. Carefully remove the pipette from the vessel. The tip of the pipette may be gently touched to the inside of the vessel to remove any adherent liquid at the tip.
6. Aseptically move the pipette to the receiving vessel and deliver the content being careful not to touch the tip of the pipette to anything. If the tip becomes contaminated, discard the pipette and the liquid.
7. When done, remove the pipette from the pipet-aid and discard it into an appropriate waste container.

Using a Micropipette

The micropipette (or micropipettor) is used to transfer small amounts of liquid (i.e. less than 1 mL). To do so, the micropipette uses a plunger, that when depressed to a set point and then released, generates a small vacuum that aspirates liquid into a small micropipette tip placed on the end of the shaft. When the plunger is depressed once again, the aspirated liquid is dispensed from the tip. A volume adjustment knob is used to set the volume for aspiration and dispensing. This volume can be observed through a display window found in the body of the micropipette. This display can be either digital or analog. The volume that is set by the adjustment knob will determine how far the plunger can be depressed and how much vacuum can be created. The plunger of a

micropipette stops at two different positions when it is depressed (Figure **13.1.2**). The first of these stops is called the **soft stop or 1st stop** and is the point of initial resistance. *This level of depression will result in the correct volume of liquid being accurately aspirated and transferred.* The plunger has another stopping point called the **hard stop or 2nd stop**. This stop can be found when the plunger is depressed beyond the soft stop until the plunger reaches the body of the micropipette. Increased resistance will be felt if the plunger is depressed past the soft stop and towards the hard stop. The hard stop is only used to ensure all the volume in the pipette tip is dispensed. *It should never be used when aspirating liquid up into the pipette tip.* To read more about micropipettes, go to Chapter 1.3: Common Biotechnology Equipment.

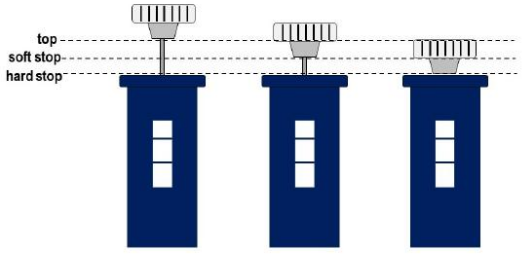


Figure **13.1.2**: The soft and hard stop positions of a micropipette. (Copyright; author via source)

Accurately reading the display requires knowing the volume of the micropipette (Figure **13.1.3**).

- For the **P20,** the numbers in the display window read as "XX.X uL". In many P20 micropipettes used in research labs, the small "tick" marks in the readout are a volume gradation of 2/100th of a microliter (e.g. 1.50 uL and 1.52 uL).
- For the **P200,** the numbers in the display window read as "XXX uL". In many P200 micropipettes used in research labs, the small "tick" marks in the readout are a volume gradation of 2/10th of a microliter (e.g. 152.0 uL and 152.2 uL).
- For the **P1000,** the numbers in the display window read as "XXXX uL". In many P200 micropipettes used in research labs, the small "tick" marks in the readout are volume gradations of 2 uL (e.g. 150 uL and 152 uL).

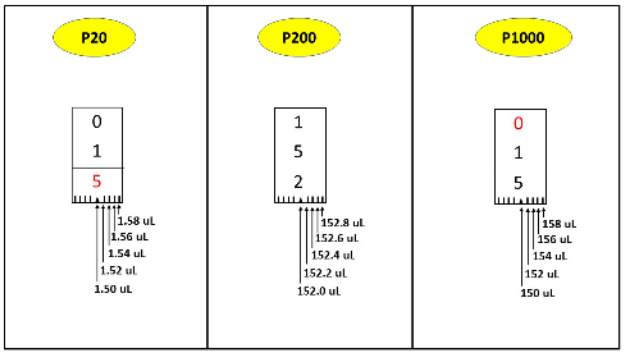


Figure **13.1.3**: The micropipette volume display. The micropipette volume depends on the type of micropipette. The volume of the P20 has gradations of 1/10th and 2/100th of a microliter (e.g. 1.50 ul and 1.58 uL). The volume of the P200 has gradations of 2/10th of a microliter (e.g. 152.0 ul and 152.8 uL). The volume of the P1000 has gradations of 2 uL (e.g. 150 uL and 158 uL). The volume of the P1000 is obtained by multiplying the readout by 10 (e.g. 15 X 10). In many P20 micropipettes, the red digit represents the gradation of 1/10th of a microliter. In the P1000, the red number represents a volume in the 1000s. (Copyright; author via source)

Lab Protocol:

1. Prior to use, ensure that the appropriate micropipette is being used.
2. Set the volume by turning the volume adjustment knob to the desired volume. For some micropipettes, the plunger can also be used to adjust volume.
3. Insert a clean, plastic pipette tip onto the barrel.
4. Depress the plunger to the first/soft stop. Do not go beyond this first stop or the volume aspirated will be inaccurate.
5. Insert the pipette tip into the liquid so that the tip is just below the surface.
6. Slowly, raise the plunger so that the liquid is drawn up into the tip slowly. To prevent bubble formation in the aspirated liquid, do not remove the pipette tip from the liquid until all the liquid is aspirated into the tip.

7. Lift the micropipette from the liquid and move the micropipette to the new container.
8. Slowly depress the plunger to the first/soft stop.
9. Look at the pipette tip. If there is remaining liquid, continue to depress the plunger to the second/hard stop. If all the liquid has been aspirated, this will not be necessary.
10. Without lifting the plunger, lift the micropipette from the liquid. Release the plunger.
11. Dispose of the tip using the tip ejector button or by pulling the tip off the shaft.

13.1: Lab Technique - Pipetting is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.2: Lab Technique - Polymerase Chain Reaction (PCR)

Amplifying DNA with Semi-Quantitative PCR

The protocols given below are standard **semi-quantitative PCR protocols** that can amplify a wide range of target DNA sizes. The first protocol outlines how to set-up a PCR reaction using a PCR "Master Mix". The second protocol sets up the PCR reaction using the individual components found in the master mix. Because a successful PCR relies upon numerous components, a few are discussed here.

Primer Design

Below is a list of characteristics that should be considered when primers are designed. Modern primer software will take these into consideration when designing primers complementary to a DNA target region. Two popular primer design sites are the NCBI Primer design tool and Primer3. Both are free to use.

1. Primer length should be 15-30 nucleotides.
2. Optimal G-C content of the primer should range between 40-60%.
3. The 3' end of primers should contain a G or C in order to "clamp" the end of the primer to the DNA template. A G-C base pairing between a primer and its template sequence increases overall primer hybridization and PCR efficiency.
4. The 3' ends of the forward and reverse primer should not be complementary to each other in order to prevent the formation of primer dimers.
5. The 3' end of a single primer should not be complementary to other sequences in that prime in order to prevent the formation of hairpin loop structures.
6. Optimal melting temperatures (Tm) for primers range between 52-58°C, although the range can be expanded to 45-65°C. The final Tm for both primers should differ by no more than 5°C.
7. Di-nucleotide repeats (e.g., GCGCGCGCGC or ATATATATAT) or single base runs (e.g., AAAAA or CCCCC) should be avoided as they can cause polymerase "slipping" along the primed segment of DNA and or hairpin loop structures to form. If unavoidable due to nature of the DNA template, then only include repeats or single base runs with a maximum of 4 bases.
8. Primer stocks will be provided as a dried pellet. The amount of DNA in each primer sample will be provided. For ease of use, add enough nuclease-free water to each dried primer pellet stock to create a stock concentration of 100 µM. Many primer companies will provide this volume.
9. Dilute the primer stocks to a working stock of 10 µM. This will be diluted to 1 µM in the PCR reaction.

Materials

- Wear gloves to avoid contaminating the reaction mixtures or reagents.
- Barrier pipette tips may be used to decrease contamination of reaction mixtures and reagents.
- Include a negative control reaction in which no DNA is added, and if possible, a positive reaction.
- Set up the PCR reactions in 0.2 mL PCR tubes only. PCR tubes are thin-walled and allow for the efficient temperature changes within the reaction.

PCR Reaction Components

- Arrange all reagents needed for the PCR in a freshly filled ice bucket, and let them thaw completely on ice before setting up a reaction. Keep the reagents on ice throughout the set-up.
- Several companies sell a concentrated PCR "Master Mix" that is comprised of the Taq polymerase, a proprietary buffer at neutral pH, magnesium chloride, and DNA nucleotides (i.e., dNTPs). Common concentrations for these mixes are two-times (2X). They are diluted to 1X after setting up the PCR reaction, providing the optimal concentrations of salts, nucleotides, and Taq polymerase.
- The final concentration of $MgCl_2$ in the reaction should be 1 mM to 1.5 mM.
- The final concentration of forward and reverse primers in the reaction should be between 0.2 and 0.4 µM (20 to 50 pmol)
- The final concentration of dNTPs in the reaction should be approximately 0.2 mM (50 µM of each dNTP). Most companies will sell a dNTP mixture containing dATP, dCTP, dGTP, and dTTP. The concentration of each dNTP in this mixture is the concentration of the mix divided by 4.

- The PCR reaction should contain 0.5 to 25 units of Taq polymerase per 50 µL reaction. Each manufacturer will sell this polymerase at a specific concentration.
- The final concentration of DNA in the reaction should range between 1.0 pg/µL to 200 ng/µL.

The PCR Workflow

The PCR workflow is outlined in Figure **13.2.1**. This workflow is applicable whether the PCR being performed is semi-quantitative or quantitative. However, the thermocycler will be specific to semi-quantitative or quantitative protocols.

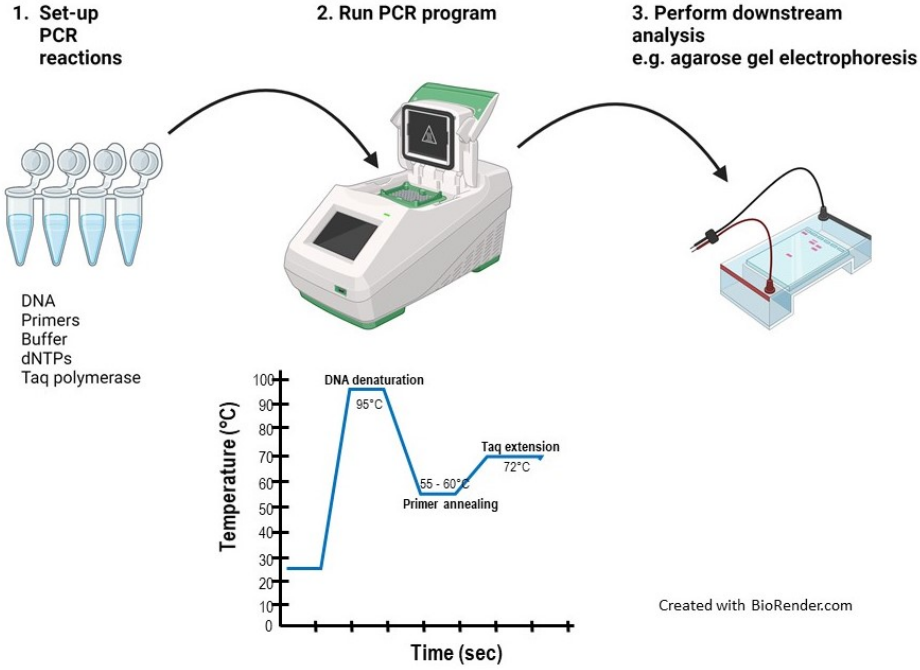


Figure **13.2.1**: The PCR workflow. (1) Set-up PCR reactions: PCR reactions containing DNA, primers, buffer, nucleotides (dNTPs), and a Taq polymerase are set up in PCR tubes. (2) Run PCR program: the tubes are placed into a thermocycler for amplification through a PCR program comprised of repeating cycles of denaturation, primer annealing, and Taq extension steps. (3) Perform downstream analysis: the completed PCR reactions can be analyzed through downstream applications like electrophoresis (PCR Protocol by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2024))

Semi-quantitative PCR Protocol

The protocols for semi-quantitative PCR given below outline the use of a PCR "Master Mix" containing Taq polymerase, buffer, magnesium chloride, and DNA nucleotides and a standard protocol using individual reagents. The Master Mix protocol uses the 2X Master Mix from Promega. Many companies sell a similar type of PCR Master Mix. Table **13.2.1** below provides the final concentration of each component in a 50 µL reaction volume.

Table **13.2.1**: PCR components using a Master Mix

Reaction Component	Concentration	Final Concentration in 50 µL reaction volume
DNA	0.1 ng/µL to 10 ng/µL	2.0 pg/µL to 0.2 ng/µL
Forward Primer	10 µM	0.2 µM
Reverse Primer	10 µM	0.2 µM
2X Master Mix	Taq polymerase: 0.05 Units/µL dNTP mix: 1.6 mM (400 µM of each dNTP) $MgCl_2$: 3 mM	Taq polymerase: 0.025 Units/µL dNTP mix: 800 µM (200 µM of each dNTP) $MgCl_2$: 1.5 mM

The "standard" PCR protocol outlines the PCR reaction set-up using individual components. Table **13.2.2** below provides the final concentration of each component in a 50 µL reaction volume.

Table **13.2.2**: Components of a standard PCR reaction

Reaction Component	Concentration	Final Concentration in 50 µL reaction volume
DNA	0.1 ng/µL to 10 ng/µL	2.0 pg/µL to 0.2 ng/µL
Forward Primer	10 µM	0.2 µM
Reverse Primer	10 µM	0.2 µM
PCR buffer (without $MgCl_2$)	10 X	1 X
$MgCl_2$	50 mM	1.5 mM
dNTP mixture	10 mM (2.5 mM each of dATP, dCTP, dGTP, dTTP)	200 µM (50 µM each of dATP, dCTP, dGTP, dTTP)
Taq Polymerase	manufacturer specific	0.5 to 2.5 units

Lab Protocol: Master Mix

1. Place a PCR tube holder on ice and add the appropriate number of labeled 0.2 mL PCR tubes to the holder.
2. For a 50 µL PCR reaction, add 1.0 µL of DNA sample (concentration between 0.1 ng/µL to 10 ng/µL) to each PCR reaction, except the negative control tube.
3. Add 1.0 µL of 10 µM forward primer to each PCR reaction.
4. Add 1.0 µL of 10 µM reverse primer to each PCR reaction.
5. Add the appropriate volume of PCR master mix to dilute it to a 1X working concentration. For example, for a 2X Master Mix, add 25 µL to the reaction.
6. Add enough nuclease-free water to bring each PCR reaction to 50 µL.
7. Carefully mix each PCR reaction by pipetting up and down. Ensure all components are at the bottom of the tube. If necessary, flick spin to collect the contents at the bottom of the tube.
8. Place the PCR tubes into the thermocycler and initiate the PCR program.
9. Remove the PCR reactions when the program is complete and store them at -20°C.

Note: Design the PCR program to have a 4°C "soak" at the end of the run. The PCR reactions can be kept for several hours at this temperature.

Lab Protocol: Standard Protocol

1. Place a PCR tube holder on ice and add the appropriate number of labeled 0.2 mL PCR tubes to the holder.
2. For a 50 µL PCR reaction, add 1.0 µL of DNA sample (concentration between 0.1 ng/µL to 10 ng/µL) to each PCR reaction, except the negative control tube.
3. Add 1.0 µL of 10 µM forward primer to each PCR reaction.
4. Add 1.0 µL of 10 µM reverse primer to each PCR reaction.
5. Add 5.0 µL of 10X PCR buffer to each PCR reaction. If the PCR buffer does not contain $MgCl_2$, add 1.5 µL of 50 mM $MgCl_2$ to each PCR reaction.
6. Add 1.0 µL of a 40 mM dNTP mixture (10 mM of each nucleotide) to each PCR reaction.
7. Add 1.0 µL of Taq polymerase to each PCR reaction.
8. Add enough nuclease-free water to bring each PCR reaction to 50 µL.
9. Carefully mix each PCR reaction by pipetting up and down. Ensure all components are at the bottom of the tube. If necessary, flick spin to collect the contents at the bottom of the tube.
10. Place the PCR tubes into the thermocycler and initiate the PCR program.
11. Remove the PCR reactions when the program is complete and store them at -20°C.

Note: Design the PCR program to have a 4°C "soak" at the end of the run. The PCR reactions can be kept for several hours at this temperature.

Quantitative PCR (Real-Time PCR)

The protocol given below is based on the Taqman protocol from Thermo Fisher. This protocol uses a 20X Gene Expression Mix, containing custom forward and reverse primers, in addition to a fluorescently-labelled probe that binds between these two primers. Customized Expression Mixes can be made by submitting gene sequences to the manufacturer for primer design. Alternatively, pre-designed Taqman Gene Expression mixes can be purchased from the company's extensive library of primer sequences. The Gene Expression mix is combined with a 2X Master Mix (containing buffer, nucleotides and a DNA polymerase), the cDNA for amplification and nuclease-free water for a final volume of 10 µL. The PCR reactions are set-up in optically clear 96-well plates and run in compatible real-time thermocyclers. Because 96-well plates are used, the expression of several genes can be quantified using the same plate.

Quantitative PCR Protocol

1. Place a 96-well optical PCR plate on ice.
2. Add 2.0 µL of cDNA sample (concentration between 1.0 pg/µL to 50 ng/µL) to each well with the exception of the negative control well.
3. Add 1.0 µL of the 20X Gene Expression Mix to the appropriate wells.
4. Add 10.0 µL of 2X Master Mix to each well.
5. Add enough nuclease-free water to bring each PCR reaction to 20 µL.
6. Cover the optical plate with the provided sealing film. Ensure all components are at the bottom of the tube. If necessary, flick spin to collect the contents at the bottom of the tube. Protect the reactions from light.
7. Place the PCR tubes into the quantitative thermocycler and run the PCR program.
    1. 95°C for 20 seconds - 1 cycle
    2. 95°C for 3 seconds
    3. 60°C for 30 seconds
    4. Repeat steps 2 and 3 for 40 cycles
8. Analyze the resulting quantitative data using either absolute (i.e., standard curve application) or relative (i.e. delta-delta Ct) quantitation methods.

In **relative quantitation**, otherwise known as the "delta-delta Ct" comparative approach, the change in gene expression in an experimental sample versus a control sample is calculated. This method requires that the Ct values for the "target" gene being studied in the experimental and control samples are first normalized, usually to a reference gene whose expression is unaffected by the experimental conditions. Examples of such reference genes are the "housekeeping genes" GAPDH, beta-actin, or 18S. To normalize, the Ct values of the reference gene are subtracted from the Ct values of the target gene (Equation #1). The normalized values between the samples are then directly compared by subtracting the normalized experimental Ct values from that of the normalized control Ct values (Equation #2). This is the change in expression or the "delta-delta" Ct. The relative fold difference in gene expression as a result of the experiment is then calculated as 2 to the power of negative delta-delta Ct (Equation #3).

1. The ΔCt value for each target gene being studied is normalized to a reference gene:

$$\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$$

2. The ΔΔCt value is calculated by comparing the normalized ΔCt value of an experimental sample to the normalized ΔCt value of a control:

$$\Delta\Delta Ct = \Delta Ct_{experimental} - \Delta Ct_{control}$$

3. The relative fold change in expression is given by:

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

In **absolute quantitation**, otherwise known as the "standard curve" approach, known amounts of template DNA are amplified and a standard curve is created. This standard curve is used to calculate the y-intercept and slope. These two values are then used to convert the Ct values of the gene being studied into absolute quantities.

13.2: Lab Technique - Polymerase Chain Reaction (PCR) is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.3: Lab Technique - Agarose Gel Electrophoresis of DNA

Separating DNA by Electrophoresis

Electrophoresis (meaning to “carry with electricity”) separates molecules based on their size using an electric field. In **DNA agarose gel electrophoresis**, fragments of DNA move through a polymer gel made of **agarose**. Agarose is a polysaccharide (primarily galactose) of an approximate molecular weight of 120,000 Daltons or 120 kDa (kiloDaltons). When solidified, it forms a three-dimensional "molecular sieve" made of pores with diameters ranging from 50 nm to >200 nm depending on the concentration of agarose used. Smaller fragments of DNA will pass more easily (and quicker) through the pores of the agarose gel than the larger fragments. Therefore, *the rate at which a DNA fragment passes through the gel is inversely proportional to its size*.

Preparation of the Agarose Gel

Agarose gels are prepared and run in a buffer that provides optimal salt concentration and pH for DNA separation. Most agarose gels are prepared in a **TAE buffer** (Tris-Cl, acetic acid, EDTA; pH of 8.0) because it offers the best separation of a large range of DNA fragment sizes (1 to 10 kb). It is also better if the DNA fragments need to be isolated from the agarose. However, the TAE buffer is prone to overheating so gels prepared in this buffer are usually run for a short duration (approximately 30 to 45 minutes). Agarose gels for the separation of smaller DNA fragments (less than 1 kb) can be prepared and run in a **TBE Buffer** (Tris-Cl, boric acid, EDTA). This buffer has a better buffering capacity and is more resistant to overheating. Therefore TBE-agarose gels are better for longer runs.

The DNA gel is made by pouring the prepared agarose solution into a mold, called a **gel tray**, containing a **“comb”** (Figure 13.3.1). As the agarose cools, it polymerizes inside the tray and around the comb. When the comb is pulled from the polymerized gel, multiple wells are created for the addition of the DNA samples that will run through the gel in parallel “lanes”. A wide variety of gel tray sizes can be used in agarose electrophoresis, ranging from small trays at 5 x 6 cm to larger trays at 12 x 14 cm. The combs can also vary in size and number, providing the user with flexibility in terms of the number of DNA samples that can be run on each gel. The size of the gel trays and the combs that fit within them will depend on the manufacturer.

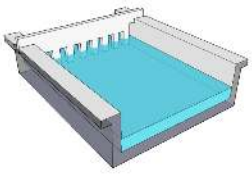


Figure 13.3.1: The DNA gel. The prepared agarose solution is poured into a tray and a comb inserted to create wells in the gel. (The DNA Gel by Patricia Zuk, CC BY 4.0)

An agarose gel of concentration between 1 to 2% can separate a wide range of DNA fragment sizes (50 bp to 10,000 base pairs). Most gels are prepared at a concentration of 1%. The higher the concentration of agarose, the smaller the pores and the better separation of smaller fragments will occur. However, concentrations greater than 2% may be more turbid, making it more difficult to visualize the DNA bands. Concentrations lower than 1% may be fragile and difficult to physically handle. Most manufacturers will have a table outlining the concentration of agarose in a gel and the range of DNA fragments it can separate. Gels may also be poured using Low Melting Point (LMP) agarose. This type of agarose will dissolve within the buffer at approximately 65°C. LMP agarose is more suitable for the analysis of DNA fragments greater that 10,000 bp (i.e., 10 kb).

Visualizing DNA within the agarose gel can be done by either adding a DNA dye to the agarose solution, or by adding the dye to the DNA loading buffer (see the next section). The most common method is the addition of **ethidium bromide (EtBr)** to the dissolved agarose solution. EtBr works by intercalating between the bases of the DNA. When exposed to UV light, EtBr glows orange, allowing the visualization of the DNA fragments. EtBr is carcinogenic because it can structurally distort the DNA helix and stop replication and transcription. As such, gels prepared with EtBR must be disposed of using correct biohazard procedures.

Preparation of DNA Samples

DNA samples are prepared for electrophoresis by mixing with a **DNA loading buffer**. A typical DNA loading buffer contains:

- salts to optimize ionic strength and pH for the DNA sample
- SDS or EDTA to inactive any enzymatic reactions associated with the DNA sample and to inactivate contaminating nucleases

- glycerol to make the DNA “heavy” so that it will sink to the bottom of the well
- one or more **indicator dyes**

The indicator dyes are necessary to track the progress of electrophoretic separation because DNA is colorless and cannot be seen in the gel. The indicator dyes will move through the agarose gel at different rates because, like the DNA fragments, they have different sizes. A typical DNA loading buffer will have at least two indicator dyes. The most commonly used are **xylene cyanol** (green color) and **bromophenol blue** (blue color), although alternate indicator dyes can be found in commercially-sold loading buffers. In an agarose gel of 1% prepared with TAE buffer, bromophenol blue will mimic a DNA fragment of about 350 bp while xylene cyanol runs as if it is approximately 4,000 bp. The distance that these two dyes will run is dependent on the agarose concentration and the buffer being used (Table **13.3.1**). The further the distance between these two dye "fronts" in the gel, the longer the gel has been running and the better resolution you will have between DNA fragments. Good resolution in a DNA gel is important so that individual DNA fragments of similar sizes can be distinguished from one another.

Table **13.3.1**: Indicator Dye Sizes versus Agarose Concentration

Agarose Concentration (%)	Bromophenol Blue size in TAE Buffer (base pairs)	Bromophenol Blue size in TBE Buffer (base pairs)	Xylene cyanol size in TAE Buffer (base pairs)	Xylene Cyanol size in TBE Buffer (base pairs)
0.5	1,150	750	16,700	13,000
1.0	370	220	4,160	3,030
1.5	190	110	1,840	1,300
2.0	120	65	1,040	710

Most DNA loading buffers are purchased or made at either a 6X or 10X concentration. This will necessitate dilution of the loading buffer in the DNA sample so that it is at a 1X working concentration.

Determining the size of the separated DNA fragments requires the running of a **DNA ladder** alongside the DNA samples. A DNA ladder contains multiple DNA fragments where the size is known. A wide variety of DNA ladders can be purchased, ranging from a small-range "100 bp ladder" where the DNA fragments increase in size from 100 bp to 1000 bp in 100 bp increments, to larger scale ladders where the fragments can range from 100 bp to 15,000 bp. DNA ladders are typically sold already mixed with a loading buffer and are "ready to use".

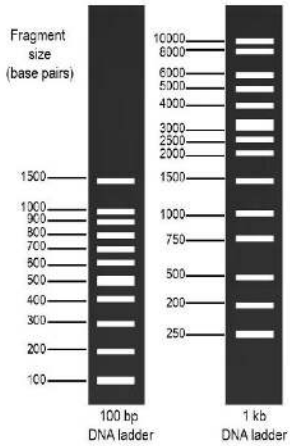


Figure **13.3.2**: DNA ladders. DNA samples are run alongside DNA ladders made up of DNA fragments of known sizes. DNA ladders can vary from small range (100 bp DNA ladder) to larger scale ladders made up of bigger DNA fragments (1kb DNA ladder). (DNA ladders by Patricia Zuk, CC BY 4.0)

Electrophoresis of DNA

To run the agarose gel, the gel is placed into an electrophoresis "tank" and the tank is filled with the same buffer used to make the gel, so as to create the electric field needed for electrophoretic separation. The tank will have an **anode** at one end (black electrode)

and a **cathode** at the other end (red electrode). The electric field will flow through the TAE buffer in the tank in a circular pattern from anode to cathode and then back (Figure **13.3.3**). Because DNA is negatively charged, the DNA fragments will be pushed away from the negative anode (due to repulsion) and attracted toward the positive cathode. The voltage at which the gel is run is important. Too low and insufficient resolution between DNA fragments will result. Too high and the DNA fragments will run in a distorted pattern. The size of the gel, the voltage of the gel, and the anticipated sizes of the DNA fragments will all determine how long the gel should be run (Table **13.3.2**). Higher concentration gels will need less time. Running a gel at a higher voltage should translate into a short duration. In contrast, better separation between gel fragments will require longer run times.

Table **13.3.2**: Summary of Gel Size and Voltage

DNA size range	Voltage	Optimal Running Buffer
less than 1 kb	5 to 10 V per cm gel length	TBE
1 to 5 kb	4 to 10 V per cm gel length	TAE or TBE
greater than 5 kb	1 to 3 V per cm gel length	TAE

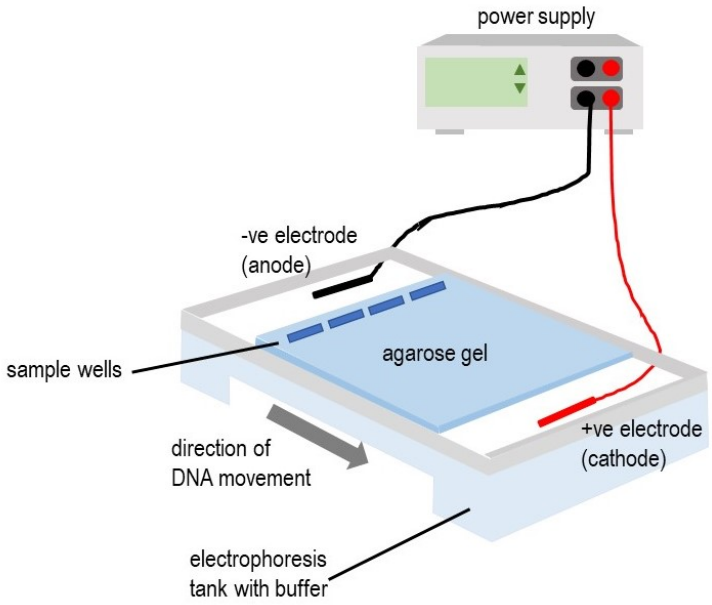


Figure **13.3.3**: DNA electrophoresis. The polymerized agarose gel is placed into an electrophoresis "tank" so that the sample wells are closest to the anode (the "-ve" electrode). A buffer is added to the tank and the electrodes connected to a power supply set to a specific voltage. The resulting electric field will result in the movement of the DNA towards the cathode (the "+ve" electrode). (DNA Electrophoresis by Patricia Zuk, CC BY 4.0)

Visualizing DNA Bands

DNA fragments within an agarose gel can be visualized using nucleic acid stains, fluorescent dyes, and even radioactive markers. The type of dye will determine the protocol used. For example, nucleic acid stains such as methylene blue are used after the gel has been run, while fluorescent dyes and radioactive markers are used "in-gel". In-gel staining, in which the dye is incorporated either into the agarose gel or added to the DNA sample via the loading buffer, is more convenient and requires significantly less dye. However, these dyes can only be used once and could alter the mobility of the DNA in the gel due to the binding of the dye to the DNA. Post-gel staining does not affect sample mobility. However, this method adds time to the workflow as individual staining and destaining steps will need to be performed. Moreover, some stains may not be as sensitive as the in-gel dyes.

As described above, the most common method of visualizing DNA fragments is the addition of the fluorescent dye **ethidium bromide (EtBr)** to the agarose solution. Today, the non-toxic fluorescent dye **SYBR** is a popular alternative to EtBr. Both dyes are fluorescent upon UV excitation. The intensity of fluorescence is direct proportional to the amount of DNA bound by the dye. EtBr is more sensitive than SYBR and can provide better visualization of smaller DNA bands. Visualizing both EtBR- and SYBR-stained DNA fragments will require the use of a **UV transilluminator**. The UV bulbs of a transilluminator are positioned below the

gel and can provide short, medium and long-range UV wavelengths for visualization. UV light is extremely dangerous and proper safety protocols must be used to prevent damage to lab personnel. Many UV transilluminators come as part of a **DNA visualization/documentation system** in which the DNA gel is placed into a closed chamber containing the transilluminator (Figure **13.3.4**). In this way, the user is protected from UV exposure. A detector within the chamber provides an image of the gel on a screen. Exposure times can be adjusted to optimize the image of the gel and the image can be saved and printed out.

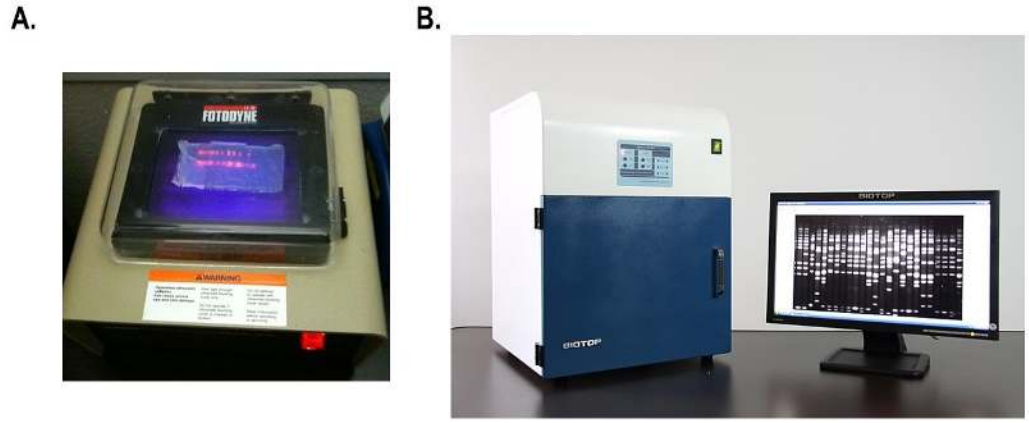


Figure **13.3.4**: DNA documentation can be done by placing the gel on a UV transilluminator (A) or using a DNA documentation system, comprised of a UV transilluminator in a closed chamber connected to a monitor (B). (Agarose gel with ladders by Joseph Elsbernd, CC BY-SA 2.0; Gel Doc with PC by Bonnvenn, CC BY-SA 3.0)

Agarose Gel Electrophoresis Protocol

Lab Protocol: Preparing the Gel

1. For a 1% agarose gel, weigh out 1.0 g of electrophoretic grade agarose and add to a clean 125 mL glass flask.
2. Add 100 mL of 1X TAE buffer (or 1X TBE) to the flask and gently swirl to mix.
3. Heat the mixture to near boiling (90 to 95°C) to dissolve the agarose within the buffer. Ensure all agarose has been dissolved within the buffer. Allow to cool slightly.
4. Prepare the DNA tray according to the manufacturer's instructions.
5. Insert the comb at the top of the DNA tray.
6. Pour enough dissolve agarose solution for gel of approximately 5 mm thickness into a plastic centrifuge tube and allow to cool slightly. A volume between 40 and 50 mL is typically sufficient. Gels that are too thin will break upon handling. Gels that are too thick will produce "fuzzy" DNA bands. If using ethidium bromide to visualize the DNA bands, add enough ethidium bromide to this dissolved agarose sample for a final concentration of 0.5 ug/mL. Use correct biohazard procedures when handling the EtBr stock solution and agarose solution containing EtBR.
7. Carefully pour the slightly cooled agarose solution into the DNA tray.
8. Let stand at room temperature until the gel has completely polymerized. Making sure that the tray is level and will not be disturbed as it polymerizes.

NOTE: Hot agarose is dangerous. **Vigorously mixing hot agarose will cause it to boil over and may cause significant burns. As such, place the hot agarose mixture on the bench top and mix using a glass stir rod or long weighing scoop.

Lab Protocol: Sample Preparation

1. Depending on the size of the comb used, pipette the appropriate amount of DNA sample into a clean 1.5 mL microfuge tube. Most wells will accommodate volumes between 10 uL and 40 uL.
2. Add the correct volume of 10X DNA loading buffer to dilute its concentration to 1X. For example, if the DNA sample is 20 uL, add 2.0 uL of loading buffer.
3. Vortex to mix and flick spin to collect the contents at the bottom of the tube.

Lab Protocol: Running the Gel

1. Once the gel has fully polymerized, carefully pull out the comb to create the wells. Prepare the gel tray to remove the ends of the tray and to allow for buffer movement.
2. Place the tray in the electrophoresis tank so that the wells are closest to the black electrode.
3. Slowly fill the tank with 1X TAE or 1X TBE buffer. Fill the tank so that the entire surface of the gel is covered with the buffer. A “layer” of buffer on top of the gel with a depth of 3 to 5 mm is sufficient. Less than 3 mm could result in evaporative loss of buffer and drying of the gel. More than 5 mm of buffer will impede the electric field and the mobility of the DNA fragments in the gel.
4. Slowly pipette the DNA samples in the appropriate wells, being careful to introduce air bubbles into the well. In order to assess the size of the DNA fragments, it is important to pipette a DNA ladder into one of the wells.
5. Once the gel has been loaded, place the lid on the tank and hook it up to the power supply. Ensure that the black electrode (i.e., the anode) is closest to the DNA wells.
6. Set the voltage on the power supply to the desired voltage (see Table **13.3.2**).
7. Allow the gel to run so that the fastest running indicator due (e.g bromophenol blue) has traveled approximately 60 to 70% of the length of the gel.
8. Stop the gel when desired, remove the gel, and visualize the DNA fragments.

Lab Protocol: Visualizing DNA Bands "In-gel"

1. Prepare the agarose solution as detailed above.
    1. For EtBr staining, add enough to the dissolved agarose to a final concentration of 0.5 ug/mL.
    2. For SYBR staining, add the SYBR dye concentrate to the DNA loading buffer to a final concentration of 1X.
2. Load the DNA samples and DNA ladder and run the gel.
3. Carefully remove the gel and visualize the bands using a DNA documentation system or a UV transilluminator. If using a transilluminator, use proper UV safety protocols, including using a UV-blocking face shield and wearing a fully buttoned lab coat, long pants, and closed toe shoes. Make sure that all skin is protected from exposure to UV light, including face, neck, hands and arms.

Lab Protocol: Visualizing DNA Bands "Post-gel"

1. Prepare the agarose gel without any stains or dyes.
2. Run the DNA as detailed above.
3. Carefully remove the gel and place it into a staining container.
4. Add the desired stain and stain according to the manufacturer's specifications.
5. Remove the stain.
6. De-stain (if required) according to the manufacturer.
7. Visualize the bands using a DNA documentation system or a UV transilluminator.

Safety Considerations

- Hot agarose can cause burns. Do not vigorously mix hot agarose. Stir gently to dissolve agarose powder.
- Ethidium bromide (EtBr) is a carcinogen. Discard all materials containing EtBr using proper institutional guidelines.
- UV light can damage DNA. For UV transilluminators, use a UV-blocking face shield, a fully buttoned lab coat, long pants, and closed toe shoes. Make sure that all skin is protected from exposure to UV light, including face, neck, hands and arms.

Agarose Reagents

10X TAE (Tris, Acetate, EDTA)

- dissolve the following in 800 mL distilled water:
    - 48.4 g Tris base
    - 11.4 mL Glacial acetic acid
    - 7.4 g of disodium-EDTA
- adjust water to 1 L with distilled water (pH of the buffer will be approximately 8.0)

- store at room temperature
- final concentration:
  - 400 mM Tris
  - 200 mM acetate
  - 20 mM EDTA

1X TAE

- add 100 mL of 10X TAE to 900 mL of distilled water
- store at room temperature
- final concentration:
  - 40 mM Tris
  - 20 mM acetate
  - 2 mM EDTA

10X TBE (Tris, Borate, EDTA)

- dissolve the following in 800 mL distilled water:
  - 108 g Tris base
  - 55 g Boric acid
  - 9.3 g of disodium-EDTA
- adjust water to 1 L with distilled water (pH of the buffer will be approximately 8.3)
- store at room temperature
- final concentration:
  - 890 mM Tris
  - 890 mM boric acid
  - 20 mM EDTA

1X TBE

- add 100 mL of 10X TBE to 900 mL of distilled water
- store at room temperature
- final concentration:
  - 89 mM Tris
  - 89 mM boric acid
  - 2 mM EDTA

Ethidium Bromide (10 mg/ml)

- dissolve 200 mg ethidium bromide powder in 20 mL distilled water
- do not expose to light
- wear gloves
- discard gloves and weigh boat in a biohazard container
- store at room temperature

0.5M EDTA

- dissolve 186.1 g of disodium EDTA (Na_2-EDTA) in 400 mL distilled water
- pH to 8.0 with 10 N NaOH
- adjust to 1 L with distilled water
- store at room temperature

10X DNA Loading Buffer

- combine the following:
  - 2.5 mL 80% glycerol (20% final)
  - 0.5 mL of 10% SDS (1% final)

- 0.2 mL of 0.5M EDTA (10 mM final)
- 500 uL of 1% bromophenol blue (0.05% final)
- 500 uL of 1% xylene cyanol (0.05% final)

- add nuclease-free water to 10 mL
- store at room temperature
- final concentration:
  - 20% final glycerol
  - 1% SDS
  - 10 mM EDTA
  - 0.05% bromophenol blue
  - 0.05% xylene cyanol

13.3: Lab Technique - Agarose Gel Electrophoresis of DNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.4: Lab Technique - Restriction Digest of DNA

Digesting DNA with Restriction Enzymes

The restriction digest uses a specialized endonuclease, called a **restriction enzyme** to "cut" or digest double-stranded DNA. The restriction enzyme recognizes a specific sequence of DNA, known as a **restriction sequence**. The enzyme breaks phosphodiester bonds between specific nucleotides within that sequence. One break will occur in one DNA strand and a second cut will occur in the second DNA strand. The cuts produced by most restriction enzymes are not aligned with each other and produce DNA "overhangs" called **sticky ends**. A smaller number of enzymes will produce aligned cuts known as **blunt ends**. Restriction digests can be performed using a single restriction enzyme or can be conducted using two different enzymes. Using two different enzymes is known as a **double digest**. For more information about restriction enzymes, go to Chapter 4.1: Principles of Genetic Engineering. To read more about restriction digests, refer to Chapter 4.2: Creating Recombinant DNA.

Key Components of a Restriction Digest

The key components of a restriction digest are as follows:

- **DNA sample:** typically a piece of linear DNA or a circular plasmid
  - Amount used in the reaction depends on the application of the DNA (e.g. cloning, sequencing, mapping)
  - Recommended amount for molecular cloning is 1 µg
- **Restriction Enzyme:** supplied in glycerol so that it will not freeze
  - Sold at a specific concentration that is expressed as "units per microliter" (U/µL)
  - The volume of restriction enzyme used depends on the amount of DNA being digested. ***One unit of enzyme will cut 1 µg of DNA in a 50 µL reaction in 1 hour***
  - Stored at -20ºC
  - Should be kept on ice until needed
- **Restriction Buffer:** a concentrated mixture of salts provided with the restriction enzyme
  - Usually provided as a ten-times (10X) concentrated buffer but other concentrations (2X, 5X) are also possible
  - The volume used will depend on the final volume of the restriction digest
  - When diluted to a 1X concentration in the digest, provides an optimal salt concentration and pH for the restriction enzyme
  - Restriction enzymes require the use of restriction buffers with specific compositions. One specific restriction buffer may be suitable for use with several restriction enzymes. However, some restriction enzymes have unique buffers (e.g. EcoRI).
  - When performing a double digest with two enzymes, the buffer with the lower salt concentration is typically used in the reaction to ensure maximal activity of both enzymes. Restriction enzyme activity in several buffers can be verified using the company's webpage (e.g., Double Digest Finder; New England Biolabs)
  - Stored at -20ºC and should be thawed completely prior to use
- **100X BSA (optional):** a concentrated mixture of bovine serum albumin
  - May not be required for every restriction enzyme
  - The volume used will depend on the final volume of the restriction digest
  - When diluted to 1X concentration in the digest, provides stability to the restriction enzyme and increases the efficiency of the digest
  - Stored at -20ºC and should be thawed completely prior to use
- **Distilled water:** used to provide the final volume of the restriction digest
  - The final volume of a restriction digest can vary. Typical final volumes include 20 µL to 50 µL

The Double Digest

A double digest uses two restriction enzymes. Double digests are commonly used to insert a piece of DNA into a plasmid in a specific orientation (i.e. "directional cloning"). To read more about directional cloning, go to to Chapter 4.2: Creating Recombinant DNA. Table **13.4.1** lists the components found in a double digest.

Table **13.4.1**: Components of a Double Restriction Digest

Component	Amount	Volume for 50 uL Reaction
plasmid or insert DNA	1.0 µg	dependent upon DNA concentration (A)
10X Restriction Buffer	1X final concentration	5.0 µL (B)
100X BSA (optional)	1X final concentration	0.5 µL (C)
Restriction Enzyme #1	1 unit per microgram DNA	dependent upon DNA amount (D1)
Restriction Enzyme #2	1 unit per microgram DNA	dependent upon DNA amount (D2)
Nuclease-free water		water to 50 µL (i.e., 50 µL-[A+B+C+D1+D2])

Lab Protocol: Double Restriction Digest

1. Thaw all frozen components and place on ice along with the desired restriction enzyme(s).
2. Label the desired number of 1.5 mL microfuge tubes appropriately (e.g. "plasmid", "insert").
3. Add the desired amount distilled water to each tube. The amount of distilled water will depend on the final volume of the digest.
4. Add 1.0 µg of the appropriate DNA to each tube (e.g. plasmid DNA, insert DNA).
5. Add the correct volume of restriction buffer to each tube. The exact restriction buffer to be used will be dependent on the restriction enzyme being used. The correct volume will based on the concentration of the buffer and the final volume of the reaction.
6. Optional: Add the correct volume of 100X BSA to each tube based on the final volume of the reaction.
7. Add the correct volume of each restriction enzyme to each tube.
8. Vortex each tube and "flick spin" in a centrifuge to collect the contents at the bottom of each tube.
9. Incubate at 37ºC for 1 hour.
10. Heat inactivate the enzymes by incubating for 10 minutes at 75ºC
11. Store the reaction(s) at -20ºC until needed.

13.4: Lab Technique - Restriction Digest of DNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.5: Lab Technique - Ligation of Insert DNA into a Plasmid

Inserting DNA into a Plasmid

Once the DNA insert and plasmid have been prepared through restriction digestion, they can be ligated to one another to form the recombinant plasmid. However, several steps must be performed prior to the ligation reaction (Figure **13.5.1**).

1. In the first step, the restriction enzymes must be inactivated and removed from the DNA samples to prevent interference with the ligation step.
2. In the second step, the plasmid DNA must be incubated with a **phosphatase** to remove the exposed 5' phosphate groups from the DNA to prevent reannealing of the plasmid.
3. In the third step, the plasmid DNA is then purified.
4. In the final step, the plasmid and insert DNA are combined and phosphodiester bonds between them are formed using a **DNA ligase**, producing a recombinant plasmid.

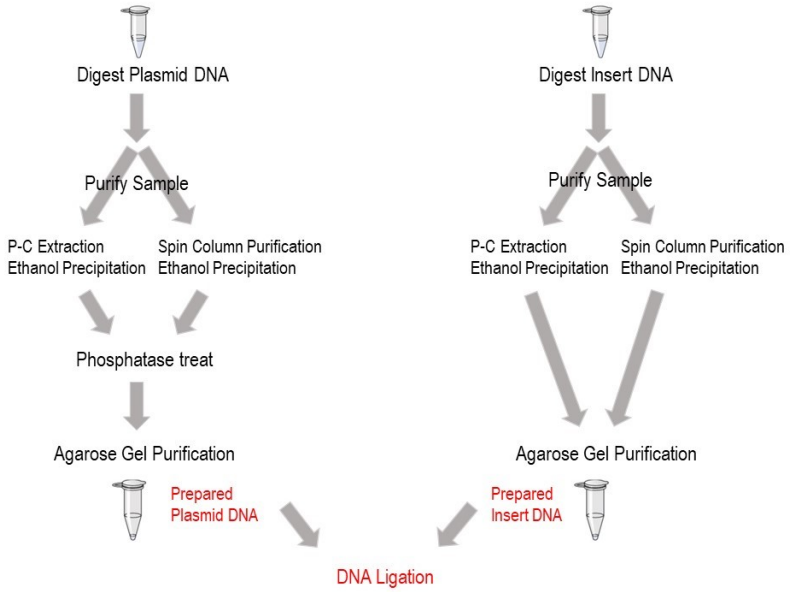


Figure **13.5.1**: The ligation workflow. To ligate a DNA insert into a plasmid, both are digested with the same restriction enzymes. Both DNA samples are then purified either through phenol-chloroform extraction (P-C) or using spin columns. Following purification, the DNA samples are precipitated with ethanol. The plasmid DNA is treated with a phosphatase to remove 5' phosphate groups. Both plasmid and insert DNA are then run on agarose gels and the DNA bands excised and purified from the gel. Both the plasmid and insert DNA are now prepared for DNA ligation. (The Ligation Workflow by Patricia Zuk, CC BY 4.0)

Step #1: Purification of Restriction Digests Prior to Ligation

The removal of contaminants from a DNA sample, such as restriction enzymes, can be done several ways, including:

- extraction using **phenol:chloroform**
- purification of the DNA using **spin columns**
- isolation of the DNA from agarose gels

Phenol:Chloroform Extraction & Ethanol Precipitation of Insert and Plasmid DNA

Phenol-chloroform extraction is a technique used to extract DNA (and RNA) from contaminating proteins and lipids. In this technique a phenol-chloroform (P-C) mixture of equal volume is added to the material and then centrifuged to create two distinct liquid layers:

- **Organic phase:** The lower layer, containing contaminants such as proteins
- **Aqueous phase:** The upper layer, containing the DNA

The aqueous phase containing the purified DNA is then removed. When followed by an ethanol precipitation step, the purified DNA can then be concentrated.

Lab Protocol: Phenol/Chloroform Extraction

1. Digest both plasmid and desired insert using the appropriate restriction enzymes and buffers in a 50 µL volume.
2. Extract both digests using an equal volume of phenol:chloroform (P-C; 25 uL of each for 50 µL total). The phenol should be saturated to ensure neutral pH and prevent damage to the DNA sample.
3. Gently invert to mix and centrifuge in a benchtop micro-centrifuge for 5 minutes at 14,000 rpm at room temperature.
4. Remove the top aqueous layer and place into a new 1.5 mL microfuge tube. The top layer should be approximately 50 µL. Be careful not to remove any of the P-C layer.
5. Add two times (2X) the volume of ice-cold 95% ethanol to the aqueous layer (i.e., 100 uL). Alternatively, add 2.5 times the volume of ice-cold 95% ethanol (i.e., 125 uL) and 0.5 times the volume of 7.5M ammonium acetate (NH_4OAc).
6. Vortex and place on ice for 30 minutes to precipitate the DNA. Precipitation can be done at -20ºC overnight but may increase salt contamination of the DNA sample.
7. Centrifuge for 30 minutes at 14,000 rpm.
8. Pour off (i.e., decant) the supernatant.
9. Wash the pellet with 125 µL of ice-cold 70% ethanol.
10. Centrifuge for 5 minutes at 14000 rpm and decant the supernatant.
11. Air dry the pellet.
12. Re-suspend the dried insert DNA in 20 µL of TE buffer (or nuclease-free water) and place at -20ºC until needed. This sample will be run on an agarose gel and purified prior to ligation.
13. Re-suspend the dried plasmid DNA sample in the CIP Reaction Cocktail for phosphate removal (see below).

DNA Extraction Reagents

TE Buffer (Tris-EDTA Buffer)

- combine the following in 50 mL distilled water:
  - 1.21 g Tris base
  - 0.037 g EDTA
- add water to 800 mL
- pH to 8.0 with 10 N HCl
- add distilled water to 1 L
- store at room temperature
- final concentration:
  - 10 mM Tris-Cl
  - 1 mM EDTA

1M Tris-Cl

- dissolve 12.14 g of Tris base in 800 mL distilled water
- adjust to pH 8.0 with 10N HCl
- add distilled water to 1 L
- store at room temperature

TE-saturated phenol

- mix room temperature phenol with an equal volume of distilled water and allow to separate into two layers overnight
- discard the top layer of water in a biohazard liquid container
- mix the phenol with an equal volume of 1M Tris-Cl, pH 8.0 and allow to separate into two layers overnight
- discard the top Tris-Cl layer in a biohazard liquid container
- mix the phenol with an equal volume of 1M TE buffer, pH 8.0 and allow to separate into two layers overnight
- discard the top TE layer in a biohazard liquid container
- store at 4ºC

70% Ethanol

- combine the following:
  - 368.4 mL of 95% molecular grade ethanol
  - 131.6 mL distilled water
- store at -20ºC

Spin Column Purification & Ethanol Precipitation of Insert and Plasmid DNA

Several companies sell "spin columns" that can be used for DNA (and RNA) purification. These columns use a solid phase component within the column (usually a silica gel) to which the DNA will bind. Centrifugation is used to flow the DNA sample through this solid phase. While the details of the protocol will differ according to the company selling the spin column, the major steps of column purification are the same.

- **Lysis** of target cells to release the DNA or RNA into the lysate (this step may not be required if the DNA is already in the sample)
- **Binding** of the DNA or RNA to the silica membrane within the column. To prepare the DNA for binding to the membrane, a buffer is often added to the DNA sample. This buffer is included in the purification kit.
- **Washing** away of unbound particulates and inhibitors from the silica membrane. The wash buffer will be provided in the purification kit.
- **Elution** of the DNA or RNA from the membrane into a collection vessel. Most purification kits will provide an elution buffer design to optimize the removal of the DNA from the colum. However, elution can be done using a TE buffer (pH 8.0) or nuclease-free water. The EDTA in the TE buffer can interfere with subsequent enzymatic reactions like ligation. Moreover, elution efficiency is dependent on pH with the maximum elution efficiency achieved between pH 7.0 and 8.5. Therefore, when using nuclease-free water, the pH value should be within this range.

Lab Protocol: Spin Column Extraction

The protocol given below is typical of many spin column protocols. This protocol summarizes the steps of the Qiaquick® Purification kit from Qiagen.

1. For each DNA sample, add 10 volumes of Buffer PN to 1 volume of DNA sample and mix. For example, add 500 µL Buffer PN to a 50 µL restriction digest.
2. Place a QIAquick spin column in the provided 2 mL collection tube.
3. To bind the DNA to the column, pipette the DNA sample to the center of the spin column and centrifuge in a benchtop micro-centrifuge for 1 min at 6000 rpm at room temperature.
4. Discard the flow-through and place the QIAquick column back into the same collection tube.
5. To wash the QIAquick column, add 750 µL of Buffer PE to the column and centrifuge for 1 min at 6000 rpm.
6. Discard this flow-through and place the QIAquick column back in the same tube.
7. To remove any residual PE buffer, centrifuge for an additional 1 min at 14,000 rpm. The removal of this residual buffer requires that the collection tube be emptied in the step before.
8. Place the QIAquick column in a clean 1.5 mL micro-centrifuge tube.
9. To elute the DNA from the column, add 50 µL of Buffer EB (10 mM Tris-Cl, pH 8.5) or nuclease-free water (pH 7.0–8.5) to the center of the column and let stand for 1 min. Centrifuge the column for 1 min at 14,000 rpm.
10. Store the insert DNA sample at -20ºC until needed.
11. Precipitate the plasmid DNA using 2 volumes of ice-cold 95% ethanol. Vortex and place on ice for 30 minutes. Decant the supernatant. Wash the precipitated DNA with 125 uL of ice-cold 70% ethanol. Centrifuge for 5 minutes at 14000 xg and decant the supernatant. Air dry the pellet.

Step #2: Phosphatase-treatment of Digested Plasmid DNA

Restriction digests of plasmids will produce exposed ends of DNA that will bear 5' phosphate groups or 3' hydroxyl groups. The 5' phosphates can spontaneously reform phosphodiester bonds with the 3' hydroxyls and result in the plasmid reforming into a circular piece of DNA before the insert can be added to the plasmid. To prevent this, plasmids used for ligation are treated with an enzyme called a **phosphatase** that removes these phosphate groups. The most common source of this phosphatase is **calf intestinal**

phosphatase (CIP). In the protocol below, the precipitated and dried plasmid DNA sample is simply re-suspended in a **CIP Reaction Mixture** that contains this type of phosphatase.

Lab Protocol: Phosphatase Treatment

1. Re-suspend the precipitated and dried plasmid DNA sample (from Step #1) in a 20 µL CIP Reaction Mixture, containing a phosphatase.
2. Incubate at 37ºC for 1 hour.
3. Run the phosphatase-treated plasmid sample in a 1% agarose gel and purify.

CIP Reaction Mixture

- 1.0 µL of Calf Intestinal Alkaline Phosphatase/CIP (1 unit)
- 2.0 µL of 10X CIP buffer* (1X final)
- 17.0 µL of distilled H20

*a 10X CIP buffer is supplied by the manufacturer. However, many restriction enzyme buffers can be used in this reaction cocktail (e.g., NEB Buffer #3)

Step #3: Gel Purification of Plasmid and Insert DNA

Another method of removing impurities from a DNA sample is to run the sample on an agarose gel, cut the DNA fragment out of the gel, and then purify the DNA from the excised piece of agarose (known as a "gene clean"). A protocol for preparing and running an agarose gel can be found in Lab Technique: Agarose Gel Electrophoresis. Many commercial kits are available for purification of DNA (or RNA) from agarose. Each of them require the "melting" of the excised piece of agarose in a buffer prior to purification. From this point, the purification can be done by binding to a spin column (see protocol below). In the protocol below, both the CIP-treated plasmid and the digested insert (from Step #1) are run out on 1% agarose gels and the fragments purified from the gel. The purified samples should then be used immediately to set-up the ligation reactions.

Lab Protocol: Purification of DNA from Gel

The protocol given below is typical of many gel extraction protocols. This protocol summarizes the steps of the Qiaquick® Gel Extraction kit from Qiagen.

1. Run the plasmid and insert DNA samples in a 1% agarose gel, containing ethidium bromide as a DNA stain.
2. Cut out (i.e., excise) each DNA fragment from the agarose gel with a clean, sharp scalpel. For best results, minimize the amount of excess agarose excised with each band.
3. Weigh each gel slice in a colorless 1.5 mL microfuge tube.
4. For each gel slice, add 3 volumes of Buffer QG to 1 volume of gel. For example, add 300 µL of Buffer QG to each 100 mg of gel.
5. Incubate each gel slice at 50°C for 10 min or until the gel slice has completely dissolved. To help dissolve the agarose, mix by vortexing the tube every 2–3 min during the incubation. It is important to dissolve the agarose completely.
6. After the gel slice has dissolved completely, check that the color of the mixture is yellow (i.e., similar to the color of the Buffer QG without dissolved agarose). The adsorption of DNA to QIAquick columns is efficient only at pH ≤7.5. The Buffer QG used for dissolving the agarose contains a pH indicator that is yellow at pH ≤7.5 and orange or violet at higher pH. Therefore, if the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.0, and mix.
7. To prepare the sample for column binding, add 1 gel volume of isopropanol to the sample and mix. For example, if the agarose gel slice is 100 mg, add 100 µL isopropanol.
8. Place a QIAquick spin column in a provided 2 mL collection tube.
9. To bind the DNA to the column, apply each sample to the column, and then centrifuge at 14,000 rpm for 1 min in a benchtop micro-centrifuge at room temperature.
10. Discard the flow-through and place the column back into the same collection tube.
11. To remove all traces of agarose from the column, add 500 µL of Buffer QG to the column and centrifuge for 1 min at 14,000 rpm.

12. To wash the column, add 750 µL of Buffer PE to the column and let the column stand 2–5 min. Centrifuge for 1 min at 14,000 rpm.
13. Discard the flow-through and centrifuge the column for an additional 1 min at 14,000 rpm. Residual Buffer PE will not be completely removed unless the flow-through is discarded before this centrifugation step.
14. Place the column into a clean 1.5 ml microfuge tube.
15. To elute the DNA, add 30 µL of Buffer EB (10 mM Tris·Cl, pH 8.5) or nuclease-free water (pH 7.0–8.5) to the center of the membrane and let stand for 4 minutes. The smaller volume will concentrate the DNA in the sample. The incubation step is required due to this small volume of Buffer EB
16. Centrifuge the column for 1 min at 14,000 rpm.
17. To increase chances of successful ligation, use the purified DNA samples immediately.

Step #4: Ligation of Plasmid and Insert DNA

At this point in the process, the plasmid and insert DNA samples have been digested, impurities removed, and precipitated into dried pellets. The plasmid DNA has then been treated with a phosphatase to remove any 5' phosphate groups. Finally, both the plasmid and insert DNA have been subject to agarose gel electrophoresis and purification from the agarose gel. The protocol given below outlines the ligation between the DNA insert and the prepared plasmid. This ligation is done using a DNA ligase from T4 bacteriophage called **T4 DNA ligase**. Successful ligation into a plasmid relies upon on a excess of insert DNA versus plasmid DNA. Since it can be difficult to determine the amount of DNA in each sample following gel purification, the protocol below sets up several ligation reactions of various insert:plasmid ratios. In all reactions, the T4 DNA ligase is added last. Ligation incubation times can be short and at room temperature or can be performed overnight at a slightly cooler temperature. Completed ligation reactions should be immediately used to transform bacteria.

Lab Protocol: Ligation

1. Label four 1.5 mL microfuge tubes as #1 through #4
2. Add the following components *in the order shown in Table* **13.5.1** to create a final ligation reaction volume of 20 µL.
3. Gently mix the reactions by pipetting up and down and flick spin briefly to collect the contents at the bottom of the tube.
4. For sticky ends, incubate at 16°C overnight or at room temperature for 10 minutes.
5. For blunt ends, incubate at 16°C overnight or at room temperature for 2 hours.
6. Heat inactivate the T4 DNA ligase at 65°C for 10 minutes.
7. Chill on ice and immediately transform competent bacteria with the four ligation reactions.

Table **13.5.1**: Ligation Reaction Set-Up

Ligation Components*	Reaction #1	Reaction #2	Reaction #3	Reaction #4
1. 10X T4 Ligase Buffer	2.0 µL	2.0 µL	2.0 µL	2.0 µL
2. Plasmid DNA	0.5 µL	0.5 µL	0.5 µL	0.5 µL
3. Insert DNA	0.5 µL	1.0 µL	1.5 µL	2.0 µL
4. Nuclease-free water	17.0 µL	16.5 µL	16.0 µL	15.5 µL
5. T4 DNA Ligase	1.0 µL	1.0 µL	1.0 µL	1.0 µL
* add components in the order given				

13.5: Lab Technique - Ligation of Insert DNA into a Plasmid is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.6: Lab Technique - Bacterial Transformation

Transforming Bacteria with Plasmid DNA

Bacterial transformation is a foundational technique in molecular cloning for the introduction of DNA into bacterial cells. Bacterial transformation involves two basic steps

1. Preparation of Competent Bacteria
2. Transformation of Competent Bacteria: can be performed either through chemical transformation or through electrical transformation **(i.e. electroporation)**

Step #1: Preparation of Competent Bacteria

The most common bacteria used for transformation is the DH5α strain of *E.coli*. However, other *E.coli* strains are also used. Because most bacterial strains used for bacterial transformation have a low level of "natural competency", they must be made competent. **Competent bacterial cells** are bacteria that have been "weakened" or made competent to allow for the entrance of the DNA. The preparation of competent cells for chemical transformation (i.e., chemically competent cells) is done using ice-cold salt solutions, whereas competent cells for electroporation (i.e., electrically competent cells) are prepared with ice-cold water. Both procedures require two days to complete. On the first day, a starter culture is grown overnight in a suitable liquid medium that does not contain any selective agents (e.g. antibiotics). On day 2, this starter culture is used to inoculate a larger volume of the same liquid medium. This subculture is grown until it reaches an **optical density (OD)** at 600 nm (i.e., OD600) of 0.4 to 0.9. The cells are then harvested and made competent. This optical density ensures that the bacteria are at the midpoint of their logarithmic growth phase. It is crucial that the bacterial cells are in logarithmic phase before they are made competent. The protocol given below uses ice-cold, sterile solutions. Once the cells are exposed to an ice-cold solution, they are in a weakened state and should be treated with care. Re-suspensions should be done slowly and gently and centrifugation should be done at a low enough speed to not damage the cells, but allow for pelleting.

Lab Protocol: Preparation of Competent Cells

1. Inoculate 2.0 mL of liquid medium (e.g. LB) with a single fresh bacterial colony, using sterile technique, and grow overnight at 37°C with shaking at 200 to 250 rpm. Do not include antibiotics in this liquid culture.
2. Using sterile technique, subculture the 2.0 mL starter culture by adding it to 1.0 L of the same liquid medium. Grow at 37°C with shaking at 200 to 250 rpm. At regular intervals, remove a small aliquot of this subculture using sterile technique and measure its absorbance in a spectrophotometer at 600 nm. Discard the aliquot. Culture until the medium reaches an optical density of 0.4 to 0.9.
3. Centrifuge the cells for 10 minutes at 5,000 rpm at 4°C to pellet.
4. Remove the supernatant completely.
5. Place the cells on ice.
6. To make chemically-competent cells:
    1. Re-suspend each pellet in 5.0 mL of sterile, ice-cold 10 mM $CaCl_2$ and combine the two re-suspensions into one tube. Place on ice.
    2. Centrifuge the cells for 5 minutes at 2,500 rpm at 4°C to pellet.
    3. Remove the supernatant completely.
    4. Gently re-suspend the pellet in 5.0 mL of sterile, ice-cold 10 mM $CaCl_2$ and incubate on ice for 30 minutes.
    5. Centrifuge the cells for 5 minutes at 2,500 rpm at 4°C to pellet.
    6. Remove the supernatant completely.
    7. Gently re-suspend the pellet in 2.0 mL of sterile, ice-cold 10 mM $CaCl_2$.
7. To make electrically-competent cells
    1. Re-suspend each pellet in 5.0 mL of sterile, ice-cold distilled water and combine the two re-suspensions into one tube. Place on ice.
    2. Centrifuge the cells for 5 minutes at 2,500 rpm at 4°C to pellet.
    3. Remove the supernatant completely.

4. Gently re-suspend the pellet in 5.0 mL of sterile, ice-cold distilled water and incubate on ice for 30 minutes.
5. Centrifuge the cells for 5 minutes at 2,500 rpm at 4°C to pellet.
6. Remove the supernatant completely.
7. Gently re-suspend the pellet in 2.0 mL of sterile, ice-cold distilled water. Place cells on ice.
8. Aliquot the cells into 50 µL aliquots.
9. Store aliquots at -80°C.

Competency Reagents

Transformation Medium (10 mM $CaCl_2$)

- dissolve 1.47 g of $CaCl_2$ in 1000 mL of water
- filter through a 10 µm filter to sterilize
- store at 4°C

Sterile Water

- autoclave 1.0 L of distilled water to sterilize
- store at 4°C

Step #2: Transformation of Bacteria

Transformation can be done chemically, through the use of ice-cold salt solutions and a rapid "heat shock", or electrically, using an **electroporator**. Once the bacteria have been transformed, the bacterial samples are plated onto the desired selective media for colony growth. How well the bacterial cells take up DNA can be measured by calculating **transformation efficiency**. Transformation efficiency is defined as the number of **colony forming units (cfu)** produced by transforming 1 µg of plasmid into a given volume of competent cells. It is calculated using the formula:

- **Transformation efficiency (# transformants per µg DNA) = (# of colonies/µg DNA) x (mL of recovery volume used/mL of volume plated)**

Chemical transformation relies upon a "heat shock" of ice-cold bacteria to weaken the cell membrane, permitting DNA entry. This heat shock is performed for a short period of time at 42°C. Electrical transformation through electroporation introduces DNA into bacterial cells by means of a short pulse of electricity that decreases exponentially over the course of the electroporation. This short pulse introduces tiny pores in the cell membrane, allowing the entrance of DNA into the cell. Because of this electric field, it is vital that all salt contamination from the competent cells and DNA preparations are absent. Excess salt in the competent cell aliquot will cause "arcing" upon electroporation, killing the cells. DNA ligations with plasmid DNA are not likely to have significant salt contamination. However, purified plasmid DNA samples should be re-suspended in nuclease-free water following their purification if electroporation is desired at some point. Electroporation requires the use of electroporation cuvettes and an **electroporator** (Figure **13.6.1**).

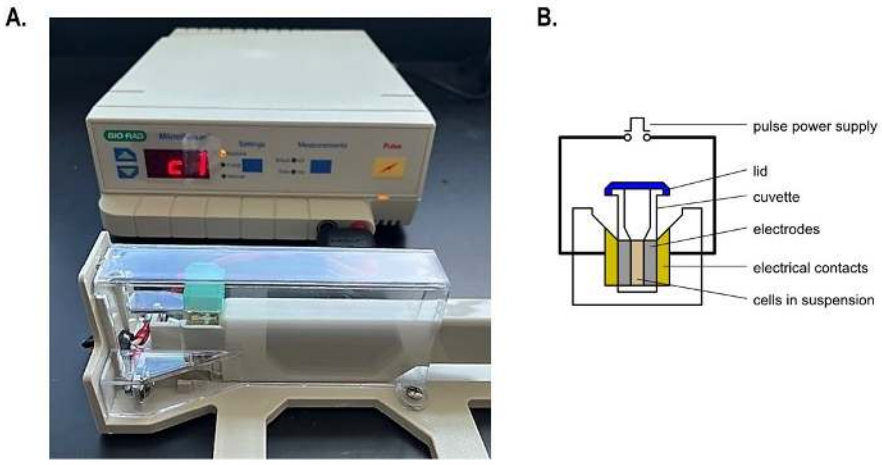


Figure **13.6.1**: The electroporator electrically transforms cells. (A) The electroporator is a piece of lab equipment that delivers an electrical pulse to a cell suspension, causing the entrance of DNA into the cells. (B) The disposable electroporation cuvette contains a chamber for the cell suspension and makes electrical contact with the electroporator through embedded electrodes. (Electroporation by Patricia Zuk, CC BY 4.0)

Following chemical transformation or electroporation, the transformed cells are allowed to "recover" in a nutrient-rich medium at 37°C for a short period of time with gentle shaking before plating onto the appropriate growth medium. **SOC media**, containing glucose and $MgCl_2$, is the preferred nutrient-rich medium for this recovery as it is known to increase the transformation efficiency of transformed cells more than LB broth. However, LB broth may be used.

The protocols given below describes the transformation of competent bacteria with plasmid ligations. However, any type of plasmid DNA preparation can be introduced into competent bacteria. The amount of DNA to be added to a competent cell aliquot should range from 1 ng to 10 ng per aliquot.

Lab Protocol: Chemical Transformation of Bacteria

1. Prior to transformation, remove the desired number of competent cell aliquots from the –80°C and thaw the cells slowly on ice. Pre-warm the required number of agar plates to 37°C.
2. As a positive control for transformation, dilute a plasmid, such as pUC19, to 10 pg/µL using sterile water.
3. Once thawed, immediately add 1.0 µL of each ligation (or plasmid DNA sample) to a 50 µL aliquot of chemically-competent cells.
4. Place the aliquot on ice for 5 minutes.
5. Heat shock cells at 42°C for exactly 30 seconds.
6. Immediately place the cells back on ice for 5 minutes.
7. Add 950 µl of room temperature SOC media (or LB Broth) to each cuvette, gently mix up and down twice to mix, then transfer to sterile round-bottom culture tubes (e.g. 17 mm x 100 mm).
8. Place the transformed cells onto a shaking incubator platform and incubate for 1 hour at 37°C while shaking at 200 to 250 rpm.
9. Plate 100 µL of the transformed cells onto an LB agar plate containing the appropriate antibiotic. Allow to "soak" into the agar. Label the plates accordingly.
10. Incubate the plates overnight in an inverted position in a 37°C bacterial incubator.

Lab Protocol: Electroporation of Bacteria

1. Prior to electroporation, place sterile SOC media (or LB broth) in a 37°C water bath. Place the desired number of electroporation cuvettes (0.1 cm) and 1.5 mL microfuge tubes on ice. Pre-warm the required number of agar plates to 37°C.
2. As a positive control for transformation, dilute the control plasmid pUC19 to 10 pg/µL using sterile water.
3. Thaw the desired number of aliquots of electrically-competent cells on ice.
4. For each ligation (or plasmid DNA sample), transfer 50 µL of competent cells into a chilled electroporation cuvette. Add 1 µL of DNA into each cuvette.
5. Carefully transfer the cell/DNA mix into a chilled cuvette without introducing bubbles and make sure that the cells are spread across the bottom of the cuvette.

6. Electroporate using the following conditions: 2.1 kV, 100 Ω, and 25 µF. The typical time constant is ~2.6 milliseconds.
7. Immediately add 950 µl of pre-warmed SOC media (or LB Broth) to each cuvette, gently mix up and down twice to mix, then transfer to sterile round-bottom culture tubes (e.g. 17 mm x 100 mm).
8. Place the transformed cells onto a shaking incubator platform and incubate for 1 hour at 37°C while shaking at 200 to 250 rpm.
9. Plate 100 µL of the transformed cells onto an LB agar plate containing the appropriate antibiotic. Allow to "soak" into the agar. Label the plates accordingly.
10. Incubate the plates overnight in an inverted position in a 37°C bacterial incubator.

Transformation Reagents

SOC Media

- dissolve the following in 500 mL distilled water
  - 10 g of tryptone (2% final)
  - 2.5 g of yeast extract (0.5% final)
  - 0.29 g of NaCl (10 mM final)
  - 0.095 g of KCl (2.5 mM final)
  - 1.015 g of $MgCl_2$ (10 mM final)
  - 0.6 g of $MgSO_4$ (10 mM final)
  - 1.8 g of glucose (20 mM final)
- autoclave to sterilize
- store at 4°C
- final concentration:
  - 2% tryptone
  - 0.5% Yeast Extract
  - 10 mM NaCl
  - 2.5 mM KCl
  - 10 mM $MgCl_2$
  - 10 mM $MgSO_4$
  - 20 mM glucose

LB Broth (Miller)

- dissolve the following in 1 L distilled water:
  - 10 g of tryptone
  - 5 g of yeast extract
  - 10 g of NaCl
- autoclave to sterilize
- store at 4°C
- final concentration:
  - 1% tryptone
  - 0.5% yeast extract
  - 1% NaCl

LB amp agar plates

- dissolve the following in 1 L distilled water:
  - 10 g of tryptone
  - 5 g of yeast extract
  - 10 g of NaCl
  - 15 g of agar
- autoclave to sterilize

- cool to 55°C and add antibiotic to the desired concentration (e.g. 100 mg ampicillin; 100 µg/ml final)
- once poured, store at 4°C
- final concentration:
  - 1% tryptone
  - 0.5% yeast extract
  - 1% NaCl
  - 1.5% agar
  - 100 µg/ml ampicillin

13.6: Lab Technique - Bacterial Transformation is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")

Isolation of DNA from Bacteria

The isolation of plasmid DNA starts with a re-suspension of the bacteria in a **re-suspension solution,** followed by the lysis of the bacterial cells in a **lysis solution** and precipitation of proteins using a **neutralization solution**. A description of each solution is given below. Because of the lysis solution, this lab technique is often called an **alkaline lysis isolation**. Beyond the neutralization step, the DNA can then be purified further using a wide variety of commercial kits (e.g. Qiagen Plasmid kit, Promega Wizard kit) or by a phenol:chloroform extraction step, followed by precipitation with ethanol. Commercial kits bind the DNA to a column comprised of a charged membrane (usually silica), allowing any contaminants to be washed out of the column. Commercial columns will use either gravity-fed or centrifugation "spin-columns" to apply the sample to the silica membrane of the column and wash away contaminants (Figure \(\PageIndex{1}\)). The DNA can then be eluted from the membrane using a small volume of Tris-EDTA (TE) buffer or nuclease-free water for additional downstream applications.

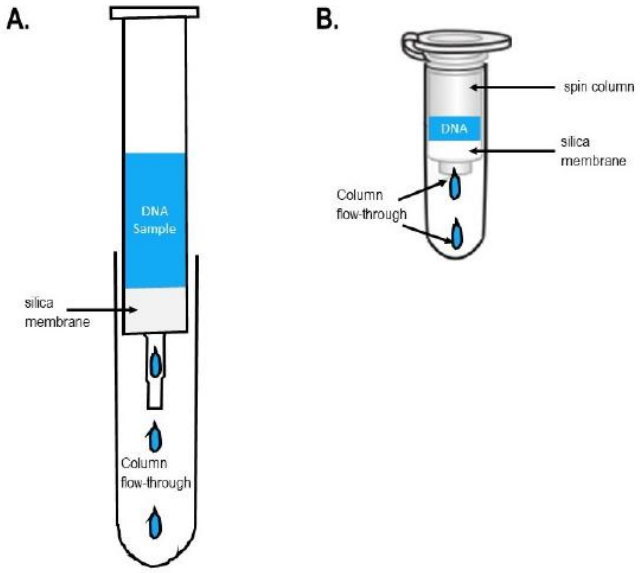


Figure \(\PageIndex{1}\): Columns for DNA isolation (A) Gravity-fed columns use gravity to flow the DNA sample through the silica membrane. (B) Spin columns use centrifugation to flow the DNA sample through the silica membrane. (Columns for DNA Isolation by Patricia Zuk, CC BY 4.0)

DNA isolation Buffers

- **Re-suspension Buffer:** a solution containing Tris, EDTA and RNase A
  - provides a stable salt solution for re-suspension of the bacterial pellet
  - the RNAse enzymatically removes contaminating RNA
- **Lysis Buffer**: a mixture of SDS and NaOH
  - the SDS solubilizes the lipid and protein components of the cell membrane, lysing the bacterial cells and releasing the DNA into the solution
  - the NaOH increases the pH of the solution, denaturing proteins and chromosomal DNA
  - short incubation time prevents denaturation of plasmid DNA
  - avoid vigorous mixing to prevent mixing of plasmid and chromosomal DNA
- **Neutralization Buffer**: a solution of potassium acetate
  - acidity of solution is designed to neutralize the alkaline lysis solution
  - high salt concentration precipitates proteins, chromosomal DNA (bound to the cell wall), cellular debris, and SDS
  - precipitation forms a white, fluffy precipitate that is easily removed through centrifugation
  - smaller plasmid DNA is renatured and remains in solution

Mini-Prep Isolation Protocols

The protocols given below represent some typical **alkaline lysis isolation protocols.** The first protocol uses reagents found in the biotech lab. The next two protocols are commercial kits from Qiagen. The first Qiagen protocol given below is a "mini-prep" protocol using a **gravity-fed column** to bind DNA. This column-based kit is designed to isolate small amounts of plasmid DNA (up to 20 µg; the "mini-prep" kit). However, larger amounts of DNA can be isolated using other kits (e.g. up to 10 mg; the "giga-kit"). All of these column-based DNA isolation kits use the same protocol but use different volumes of reagents and larger columns for DNA binding. The second Qiagen protocol below summarizes the steps of the QIAPrep Spin Mini-prep kit that uses **spin columns** for the isolation of small amounts of plasmid DNA (up to 20 µg). For each of these Qiagen protocols, the composition of the buffers used can be found in the manufacturer's handbook. However, the re-suspension, lysis, and neutralization buffers are similar to the ones used in the alkaline lysis protocol.

Lab Protocol: Alkaline Lysis "Mini-Prep"

Prior to starting the protocol, add RNase A to an aliquot of Resuspension Buffer. Store this buffer at 4°C until needed.

1. Select individual bacterial colonies and use them to inoculate 2 mL aliquots of a liquid bacterial broth (e.g., LB broth, nutrient broth), containing the appropriate selection agent (e.g. antibiotic).
2. Incubate the bacteria samples for 16 to 18 hours at 37°C with shaking at 200 to 250 rpm. If desired, the bacterial cultures can be stored for several days at 4°C.
3. Transfer 1.5 mL of each bacterial to labeled microfuge tubes and centrifuge in a benchtop micro-centrifuge tube for 5 minutes at room temperature at 5,000 rpm.
4. Aspirate the supernatant.
5. Re-suspend each bacterial pellet in 200 µL of Resuspension Buffer and vortex to completely re-suspend.
6. Lyse the cells by adding 200 µL of Lysis Buffer and gently invert 4 to 5 times. Do not vortex the sample.
7. Neutralize each sample by adding 200 µL of chilled Neutralization Buffer and gently invert 9 to 10 times. Incubate on ice for 5 minutes. Do not vortex the sample.
8. Invert each sample 4 to 5 times and centrifuge for 10 minutes at 4°C (or room temperature) at 14,000 rpm.
9. Transfer each supernatant to a new 1.5 mL microfuge tube. If the supernatant is not clear, transfer the supernatant to a new microfuge tube, and repeat the centrifugation step for an additional 5 minutes. Transfer the cleared supernatant to a new tube. The supernatant volume will be approximately 500 µL
10. Add an equal volume of (i.e. 500 µL) of phenol:chloroform (i.e., 250 µL phenol and 250 µL chloroform). The phenol should be saturated to ensure neutral pH and prevent damage to the DNA sample.
11. Gently invert to mix.
12. Centrifuge for 5 minutes at 14,000 rpm at room temperature.
13. Remove the top aqueous layer and place into a new 1.5 mL microfuge tube. The top layer should be approximately 500 µL. Be careful not to remove any of the phenol:chloroform layer.
14. Add two (2) volumes of ice-cold 95% ethanol to the aqueous layer (i.e., 1000 uL).
15. Vortex and place on ice for 30 minutes to precipitate the DNA.
16. Centrifuge at 14,000 rpm for 30 minutes at 4°C (or room temperature).
17. Pour off (i.e., decant) the supernatant.
18. Wash the pellet with 125 µL of ice-cold 70% ethanol.
19. Centrifuge at 14,000 rpm for 5 minutes at 4°C (or room temperature) and decant the supernatant.
20. Air dry the pellet.
21. Re-suspend the pellet in 20 µL of TE buffer or nuclease-free water.
22. Confirm DNA and determine the DNA yield by UV spectroscopy.

Alkaline Lysis Mini-Prep Reagents

Resuspension Buffer

- dissolve the following in 800 mL distilled water:
  - 6.06 g Tris base

- 3.72 g EDTA-$2H_20$
- adjust the pH to 8.0 with HCl
- add distilled water to 1 L
- store at room temperature
- when needed, remove an aliquot and add RNAse A to a concentration of 100 µg/ml
- after RNase A addition, store buffer at 4°C
- final concentration:
  - 50 mM Tris-Cl, pH 8.0
  - 10 mM EDTA
  - 100 µg/ml RNase A

Lysis Buffer

- combine the following:
  - 80 µL of 10N NaOH
  - 400 µL of 10% SDS
  - 3520 µL distilled water
- store at room temperature
- final concentration:
  - 0.2N NaOH
  - 1% SDS (w/v)

Neutralization Buffer

- dissolve the following in 500 mL distilled water:
  - 294.5 g potassium acetate
- adjust the pH to 5.5 with glacial acetic acid
- add distilled water to 1 L
- store at room temperature
- final concentration:
  - 3 M potassium acetate, pH 5.5

TE Buffer (Tris-EDTA Buffer)

- 1 mL of 1 M Tris base (10 mM final)
- 200 µL of 0.5 M EDTA (1 mM final)
- pH to 8.0 with 10 N HCl and adjust volume to 100 mL with distilled water
- store at room temperature

Protocol: Qiagen Column "Mini-Prep"

Prior to starting the protocol, add RNase A to an aliquot of Resuspension Buffer. Store at 4°C until needed. A workflow for this protocol can be seen in Figure \(\PageIndex{2}\).

1. Select individual bacterial colonies and use them to inoculate 2 mL aliquots of a liquid bacterial broth (e.g., LB broth, nutrient broth), containing the appropriate selection agent (e.g. antibiotic).
2. Incubate the bacteria samples for 16 to 18 hours at 37°C with shaking at 200 to 250 rpm. If desired, the bacterial cultures can be stored for several days at 4°C.
3. Transfer 1.5 mL of each bacterial to labeled microfuge tubes and centrifuge for 5 minutes at room temperature at 5,000 rpm.
4. Aspirate the supernatant.
5. Re-suspend each bacterial pellet in 300 uL of Resuspension Buffer P1 and vortex to completely re-suspend.
6. Lyse the cells by adding 300 uL of Lysis Buffer P2 and gently invert 4 to 5 times. Do not vortex the sample. Incubate at room temperature for 5 minutes.

7. Neutralize each sample by adding 300 uL of chilled Neutralization Buffer P3 and gently invert 9 to 10 times. Incubate on ice for 5 minutes. Do not vortex the sample.
8. Invert each sample 4 to 5 times and centrifuge for 10 minutes at 4°C (or room temperature) at 14,000 rpm.
9. Transfer each supernatant to a new 1.5 mL microfuge tube. If the supernatant is not clear, transfer the supernatant to a new microfuge tube, and repeat the centrifugation step for an additional 5 minutes. Transfer the cleared supernatant.
10. For each DNA sample, prepare a Qiagen-Tip 20 column with 1.0 mL QBT Buffer and allow to drain through the column. Discard flow-through.
11. Add the supernatant from step 9 to the column and allow to flow through the column. Retain the flow-through in a new tube until DNA isolation is confirmed.
12. Wash the column with 2.0 mL QC Buffer (wash buffer containing ethanol) and allow to completely drain through the column. Discard flow-through.
13. Repeat the wash step.
14. Elute the DNA with 800 uL of QF Buffer and allow the flow through to collect into a new 1.5 mL microfuge tube.
15. Precipitate the DNA by added 0.7 volume (i.e., 560 uL for 800 uL supernatant) of room temperature isopropanol. Vortex or mix thoroughly.
16. Centrifuge at 14,000 rpm for 10 minutes at room temperature.
17. Pour off (i.e., decant) the supernatant carefully. Isopropanol-precipitated DNA pellets are more loosely attached to the tube than ethanol-precipitated pellets.
18. Wash the pellet with 1000 µL of 70% ethanol.
19. Centrifuge at 14,000 rpm for 5 minutes at or room temperature and carefully decant the supernatant.
20. Air dry the pellet.
21. Re-suspend the pellet in 20 µL of TE buffer or nuclease-free water.
22. Confirm DNA and determine the DNA yield by UV spectroscopy.

Note: The absence of DNA at the end of this protocol may be due to the failure of the DNA to bind the column prepared at step 11. The flow-through kept at step 12 will contain the DNA. This DNA can be reapplied to a new column prepared by QBT buffer. If DNA is present at the end of the protocol, the flow-through from step 12 can be safely discarded.

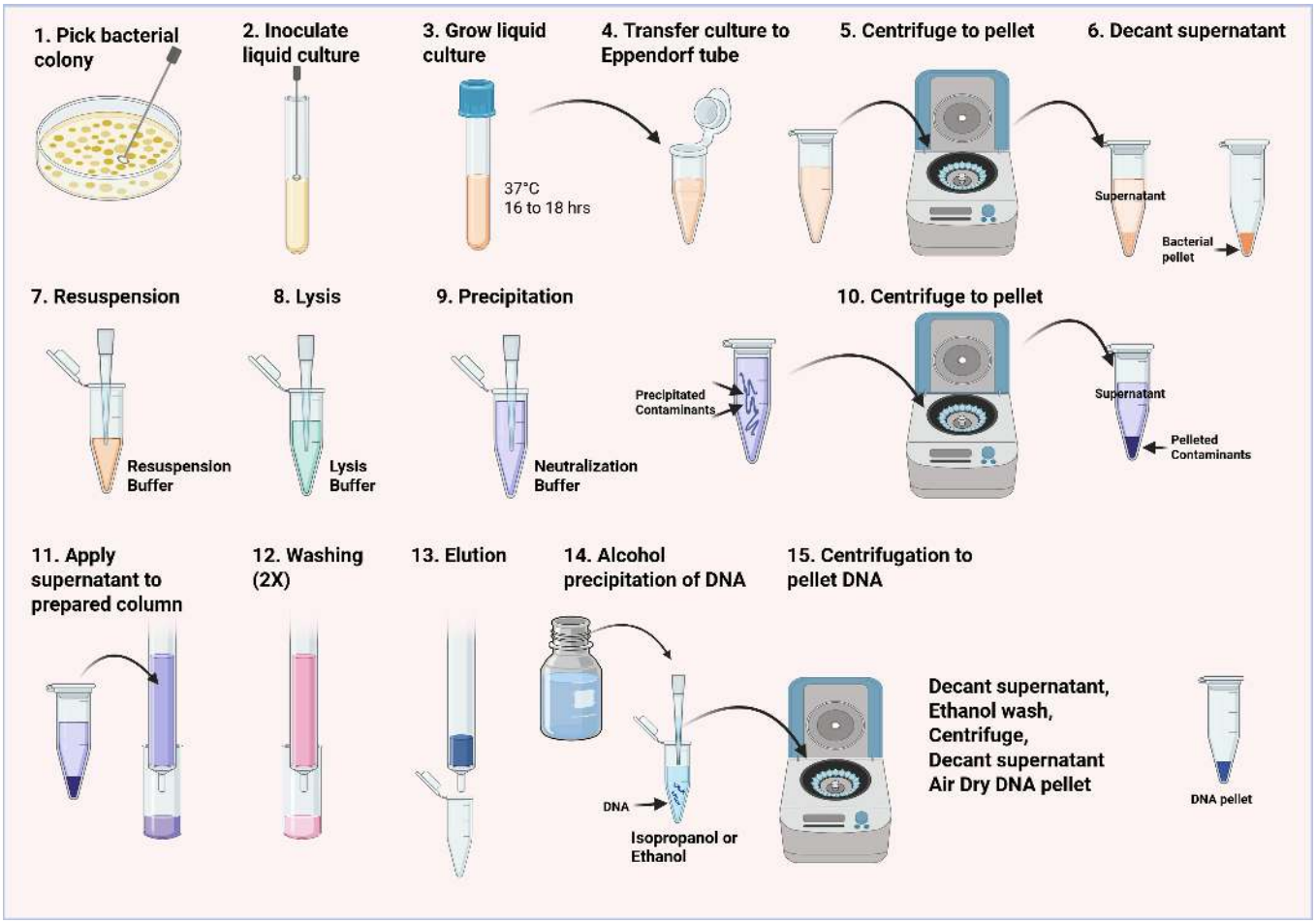


Figure \(\PageIndex{2}\): The Mini-Prep workflow for DNA isolation using a gravity-fed column. Plasmid DNA can be isolated from bacterial cells using a two-day column-based protocol. On day 1, bacterial colonies are selected and used to inoculate growth media. The cultures are grown "overnight" (i.e., 16 to 18 hours) at 37°C (steps 1 to 3). On day 2, the bacterial cells are pelleted through centrifugation and the supernatant decanted (steps 4 and 5). The pellet is re-suspended in a Resuspension Buffer (step 6). The cells are lyzed using an alkaline Lysis Buffer (step 7). The sample is neutralized and protein contaminants precipitated with a Neutralization buffer (step 8). The sample is centrifuged to pellet the contaminants (step 10) and the supernatant is applied to a prepared column that will bind the DNA (step 11). Contaminants are washed from the column using a Wash Buffer (step 12) and the bound DNA eluted from the column with an Elution Buffer (step 13). The eluted DNA is precipitated using either ice-cold 95% ethanol or room temperature isopropanol (step 14), followed by high-speed centrifugation (step 15). The supernatant is decanted and the precipitated DNA pellet washed with 70% ethanol. The sample is centrifuged again and the ethanol decanted. The resulting DNA pellet can be air dried. Spin-column protocols will use steps 1 through 10, followed by application to a spin column that uses centrifugation steps to bind, wash, and elute the DNA. Following elution, spin column protocols do not require ethanol precipitation steps (steps 14 and 15) and the DNA is ready to use for downstream applications. (The Mini-Prep Protocol by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Qiagen Mini-Prep Reagents

Resuspension Buffer P1

- dissolve the following in 800 mL distilled water:
  - 6.06g Tris base
  - 3.72g EDTA-2H_20
- adjust the pH to 8.0 with HCl
- add distilled water to 1 L
- store at room temperature
- when needed, remove an aliquot and add RNAse A to a concentration of 100 µg/ml
- after RNase A addition, store buffer at 4°C

- final concentration:
  - 50 mM Tris-Cl, pH 8.0
  - 10 mM EDTA
  - 100 µg/ml RNase A

Lysis Buffer P2

- combine the following:
  - 80 µL of 10N NaOH
  - 400 µL of 10% SDS
  - 3520 µL distilled water
- store at room temperature
- final concentration:
  - 0.2N NaOH
  - 1% SDS (w/v)

Neutralization Buffer P3

- dissolve the following in 500 mL distilled water:
  - 294.5g potassium acetate
- adjust the pH to 5.5 with glacial acetic acid
- add distilled water to 1 L
- store at room temperature
- final concentration:
  - 3 M potassium acetate, pH 5.5

QBT Buffer

- dissolve the following in 800 mL distilled water:
  - 43.83 g NaCl
  - 10.46 g MOPS (free acid)
- adjust the pH to 7.0 with NaOH
- add 150 ml pure isopropanol
- add 15 ml 10% Triton X-100 solution
- add distilled water to 1 L with distilled water
- store at 15–25°C
- final concentration:
  - 750 mM NaCl
  - 50 mM MOPS, pH 7.0
  - 15% isopropanol
  - 0.15% Triton X-100

QC Buffer

- dissolve the following in 800 mL distilled water:
  - 58.44 g NaCl
  - 10.46 g MOPS (free acid)
- adjust the pH to 7.0 with NaOH
- add 150 ml pure isopropanol
- add distilled water to 1 L with distilled water
- store at 15–25°C
- final concentration:

- 1.0 M NaCl
- 50 mM MOPS, pH 7.0
- 15% isopropanol

QF Buffer

- dissolve the following in 800 mL distilled water:
  - 73.05 g NaCl
  - 6.06 g Tris base in 800 ml distilled water
- adjust the pH to 8.5 with HCl.
- add 150 ml pure isopropanol
- add distilled water to 1 L with distilled water
- store at 15–25°C
- final concentration:
  - 1.25 M NaCl
  - 50 mM Tris-Cl, pH 8
  - 15% isopropanol

Protocol: Qiagen Spin-Column "Mini-Prep"

Prior to starting the protocol, add RNase A to an aliquot of Resuspension Buffer. Store at 4°C until needed. Add 95% ethanol to the PE wash buffer per the manufacturer's instructions. Store at room temperature until needed. The Neutralization Buffer N3 in this protocol cannot be used interchangeably with other Neutralization buffers such as P3.

1. Select individual bacterial colonies and use them to inoculate 2 mL aliquots of a liquid bacterial broth (e.g., LB broth, nutrient broth), containing the appropriate selection agent (e.g. antibiotic).
2. Incubate the bacteria samples for 16 to 18 hours at 37°C with shaking at 200 to 250 rpm. If desired, the bacterial cultures can be stored for several days at 4°C.
3. Transfer 1.5 mL of each bacterial to labeled microfuge tubes and centrifuge for 5 minutes at room temperature at 14,000 rpm.
4. Aspirate the supernatant.
5. Re-suspend each bacterial pellet in 250 µL of Re-suspension Buffer P1 (with RNase A) and vortex to completely re-suspend.
6. Lyse the cells by adding 250 µLof Lysis Buffer P2 and gently invert 4 to 5 times. Do not vortex the sample.
7. Neutralize each sample by adding 350 µL of Neutralization Buffer N3 and gently invert 4 to 5 times. Do not vortex the sample.
8. Centrifuge at room temperature for 10 minutes at 14,000 rpm.
9. If the supernatant is not clear, transfer the supernatant to a new microfuge tube, and repeat the centrifugation step for an additional 5 minutes.
10. For each DNA sample, place a QIAPrep spin column into a 2 mL collection tube.
11. Add the supernatant from step 8 to the spin column and centrifuge at room temperature for 60 seconds at 14,000 rpm. Retain flow through until DNA isolation is confirmed.
12. Wash the column with 500 µL Wash Buffer PE (containing ethanol) and centrifuge at room temperature for 60 seconds at 14,000 rpm.
13. Wash the column with 750 µL Wash Buffer PE and centrifuge at room temperature for 60 seconds at 14,000 rpm.
14. Discard flow through and centrifuge at room temperature for an additional 60 seconds at 14,000 rpm to remove residual wash buffer. The flow through must be removed prior to this centrifugation step or residual wash buffer will not be removed during centrifugation.
15. Place the washed spin column into a clean 1.5 mL microfuge tube.
16. Elute the DNA with 50 µL of EB Buffer (10 mM Tris-Cl, pH 8.5) or 50 uL of nuclease-free water. Let column stand for 1 min. Centrifuge at room temperature for 60 seconds at 14,000 rpm. TE buffer may also be used for elution.
17. Confirm DNA within eluate and determine the DNA yield by UV spectroscopy.

Qiagen Spin-Column Mini-Prep Reagents

Resuspension Buffer P1

- dissolve the following in 800 mL distilled water:
  - 6.06g Tris base
  - 3.72g EDTA-$2H_20$
- adjust the pH to 8.0 with HCl
- add distilled water to 1 L
- store at room temperature
- when needed, remove an aliquot and add RNAse A to a concentration of 100 µg/ml
- after RNase A addition, store buffer at 4°C
- final concentration:
  - 50 mM Tris-Cl, pH 8.0
  - 10 mM EDTA
  - 100 µg/ml RNase A

Lysis Buffer P2

- combine the following:
  - 80 µL of 10N NaOH
  - 400 µL of 10% SDS
  - 3520 µL distilled water
- store at room temperature
- final concentration:
  - 0.2N NaOH
  - 1% SDS (w/v)

Neutralization Buffer N3

- cannot be used interchangeably with Neutralization Buffer P3
- must be purchased from Qiagen

Wash Buffer PE

- must be purchased from Qiagen

Elution Buffer EB

- 10 mM Tris-Cl, pH 8.5

13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep") is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.8: Lab Technique - Quantitation of DNA

Quantifying DNA

The protocol given below outlines how to determine DNA yield using UV spectroscopy. For this protocol, a spectrophotometer equipped with a UV lamp and UV-transparent cuvettes are needed. Absorbance readings are performed at 260 nm (A260) where DNA absorbs light most strongly, and the absorbance will estimate the concentration of the solution. DNA concentration is calculated by measuring the absorbance at 260 nm, multiplying by the dilution factor, and using the relationship that an A260 of 1.0 is 50 µg/ml pure dsDNA.

- **Concentration (µg/ml) = (A260 reading) × dilution factor × 50 µg/ml**
- **Total yield of DNA in sample = concentration × volume of DNA sample**

Purity of the sample can be determined by measuring the absorbance at 280 nm (A280) and calculating the A260/A280 ratio.

- **Purity = (A260 reading) ÷ (A280 reading)**

An A260/A280 ratio between 1.7 and 2.0 indicates a good quality DNA sample, relatively free of contaminants, such as salt and proteins. Finally, if desired, the DNA sample can also be measured at 320 nm and that value subtracted from the A260 and A280 values in the equations given above.

Lab Protocol:

- Prepare a 1:100 or 1:200 dilution of DNA in nuclease free water. The volume of this dilution will depend upon the volume capacity of the cuvette.
- Transfer the dilution into a UV-transparent cuvette.
- To prepare the blank, add the sample volume of nuclease-free water into an optically matched UV-transparent cuvette.
- Set the absorbance value of the spectrophotometer to 260 nm
- Blank the spectrophotometer with the water only cuvette.
- Read the A260 reading for the DNA sample.
- Set the spectrophotometer to 280 nm and blank the spectrophotometer with the water only cuvette.
- Read the A280 reading for the DNA sample.
- If desired, set the spectrophotometer to 320 nm and blank he spectrophotometer with the water only cuvette.
- Read the A320 reading for the DNA sample.
- Calculate the concentration, total yield, and purity of the DNA sample using the equations above.

13.8: Lab Technique - Quantitation of DNA is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.9: Lab Technique - DNA Sequencing

Determining a DNA Sequence by Sanger Sequencing

Numerous scientific disciplines rely on the precise determination of DNA sequences. DNA sequencing is based on the DNA replication mechanism used by cells. During sequencing, a DNA polymerase extends from a sequencing primer bound to the anti-sense "parental" DNA strand and links nucleotides to create a "daughter" DNA strand that has the same sequence as the sense strand. Today, most DNA sequencing techniques are based on the Sanger "chain termination" approach in which modified nucleotides, called **dideoxynucleotides (ddNTPs)**, become incorporated into the growing daughter DNA strand and stop its extension. Dideoxynucleotides lack the hydroxyl functional group at the 3rd carbon of the nucleotide's sugar that is required to form a phosphodiester bond between the existing DNA chain and the incoming nucleotide. Once incorporated, the DNA polymerase can no longer extend from this ddNTP and the extension of the daughter DNA chain stops. Each ddNTP (i.e., ddATP, ddTTP, ddCTP, ddGTP) used in the Sanger sequencing reaction is coupled to a detectable marker. Original ddNTPs were radioactively labelled. Today, ddNTPs used in sequencing are coupled to unique fluorochromes that the DNA sequencer can detect and use to compile the sequence. For more information on ddNTPs and DNA sequencing, refer to Chapter 5.3: Genome Sequencing.

Preparation of the DNA template for sequencing

The DNA region to be sequenced must be prepared prior to sequencing. In the most common scenario, the DNA is amplified through PCR to add unique restriction sites to the ends of the DNA. This DNA is then cloned into a **sequencing plasmid** (Figure **13.9.1**). Sequencing plasmids have a multi-cloning site (or polylinker) made up of multiple restriction sites and allow for directional cloning into the plasmid. In many sequencing primers, the multi-cloning site is flanked by several sequencing primer sites, giving the researcher flexibility in what sequencing primer will be used in the sequencing reaction. Examples of common sequencing primer sites include sequences from the M13 and T7 bacteriophage. For more information about cloning into a plasmid, refer to Chapter 13.5 Lab Technique: Ligation of a DNA Insert into a Plasmid.

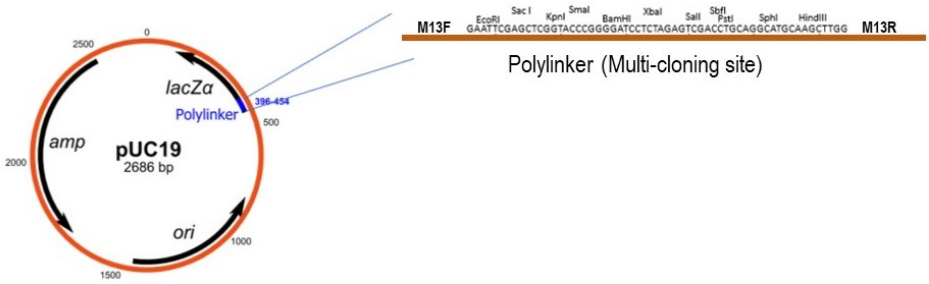


Figure **13.9.1**: The DNA sequencing plasmid pUC19. The pUC plasmid has a multi-cloning site (or polylinker) for the insertion of DNA. The multi-cloning site is flanked by sequences from the M13 bacteriophage (M13F/forward and M13R/reverse). Sequencing primers that bind these regions can be purchased from numerous companies (The Sequencing Plasmid by Patricia Zuk, CC BY 4.0)

Once cloning is complete, the recombinant sequencing plasmid must be purified to remove contaminants, like salt and cloning enzymes, that can interfere with the sequencing reaction. Numerous companies offer kits that will remove these contaminants and produce a recombinant sequencing plasmid of high purity. Purification of the sample after the sequencing reaction has been completed can also be performed with commercial kits in order to improve output by the DNA sequencer.

Components of the Sanger sequencing reaction (i.e., the Sanger reaction)

1. **Template DNA:**
    - cloned into a sequencing primer
    - purified to remove contaminants that could interfere with sequencing
2. **A DNA polymerase:**
    - usually the large subunit of the DNA polymerase I complex called the **Klenow fragment**
    - Klenow fragment contains the polymerization site and the site for proofreading
    - Klenow ensures the DNA region is sequenced without major errors

- Taq polymerase may lead to the sequencing outside of the desired DNA region

3. **A sequencing primer:**
   - a short piece of single-stranded DNA that binds to the template DNA
   - acts as a "starter" for polymerase extension
   - often sold in a set as a **forward sequencing primer** that binds the "anti-sense" DNA strand and a **"reverse" sequencing primer** that binds the "sense" DNA strand
   - sequencing using two primers ensures coverage of the DNA region you wish to sequence
4. **A dNTP mix:**
   - dATP, dTTP, dCTP, dGTP
   - added to the sequencing reaction in excess amounts
5. **A ddNTP mix:**
   - a mixture of dideoxy, or chain-terminating, versions of all four nucleotides (ddATP, ddTTP, ddCTP, ddGTP)
   - added to the sequencing reaction in small amounts
   - each labeled with a different color of dye
6. **Other components:**
   - polymerase buffer
   - $MgCl_2$

Today, most companies will sell a sequencing "master mix" containing each of these components with the exception of the sequencing primer and the DNA template.

Overview of the Sanger Reaction

The DNA sample to be sequenced is added to a PCR tube along with the other components of the sequencing reaction and subject to a PCR program made up of multiple cycles of denaturing, annealing, and extension. For more information about the PCR protocol, refer to Chapter 13.2 Lab Technique: Polymerase Chain Reaction (PCR). The first temperature of the cycle is a denaturing step in which the sample is heated to high temperatures (i.e., 95°C) in order to denature the double-stranded template DNA. The second step of the cycle is an annealing step where the reaction temperature is lowered to an "annealing" temperature (usually between 50°C and 55°C) so that the sequencing primer can bind to the single-stranded "anti-sense" template. The temperature is raised again, in the last step of the cycle, to an extension temperature (i.e., 60°C) in which the DNA polymerase extends from the sequencing primer and synthesizes the daughter DNA strand. Because the dNTPs are added in excess in a sequencing reaction, the DNA polymerase will synthesize the majority of the daughter DNA strand using these nucleotides. However, the DNA polymerase will eventually incorporate a ddNTP into the growing DNA strand. When this happens, the extension of the daughter DNA strand stops. This PCR cycle is repeated in a number of times and by time the PCR program is complete, it is virtually guaranteed that a ddNTP will have been incorporated at every single position of the daughter DNA strand. The DNA sample that results from this PCR program will contain fragments of different lengths, ending at each of the nucleotide positions in the original DNA (Figure **13.9.2**).

While individual laboratories often prepare the DNA sample for sequencing and perform the Sanger reaction, it is unusual for them to run the DNA sample through the DNA sequencer. This is because DNA sequencers are very expensive. As a result, sequence analysis of the sample is usually done in a sequencing facility by trained laboratory technicians. These facilities will run the DNA sample through their sequencer once the Sanger reaction has been completed and will forward a compiled sequence to the laboratory. Many of these facilities also offer a "full-service" option where Sanger reaction is performed, along with purification of the sample, subsequent analysis by the sequencer. Laboratories can also purchase sequencing and purification kits from the sequencing facility that are compatible with the facility's sequencers.

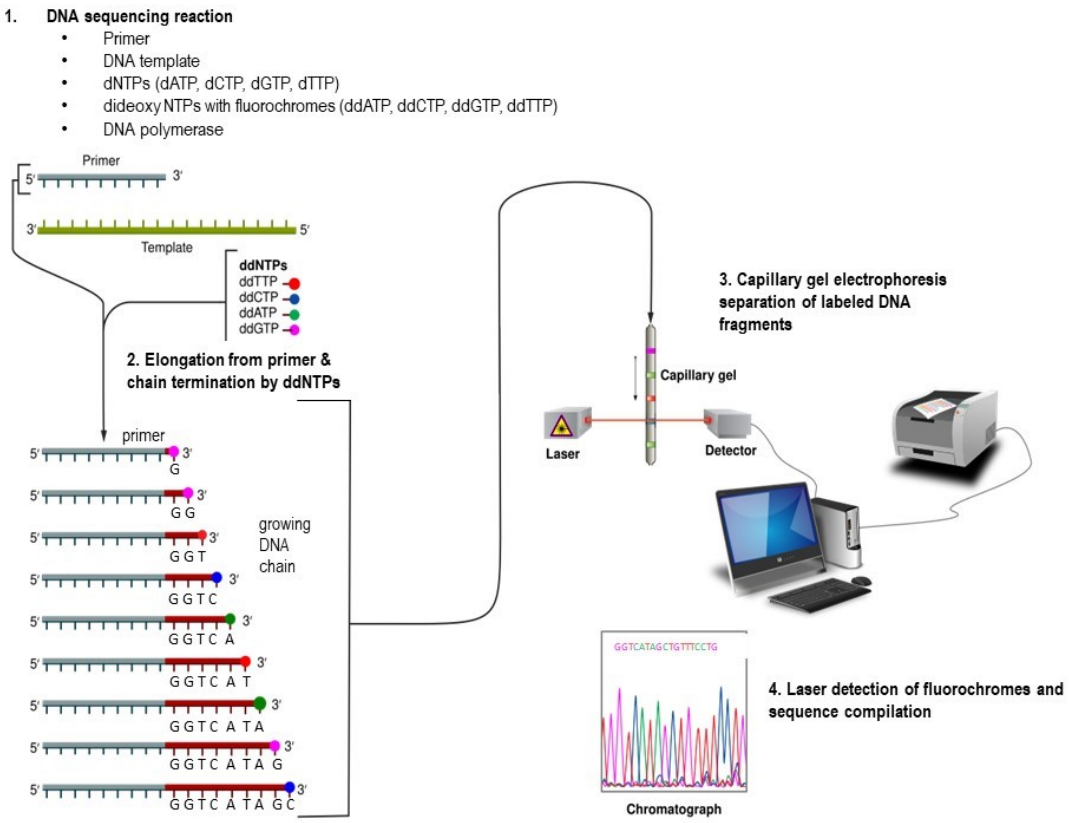


Figure **13.9.2**: Sanger sequencing of DNA. The sequencing of DNA is made of the following steps that are described in the text above:
(1) DNA sequencing reaction, (2) Elongation from primer & chain termination by ddNTPs, (3) Capillary gel electrophoresis separation of labeled DNA fragments, and (4) Laser detection of fluorochromes and sequence compilation. (Sanger DNA sequencing by Patricia Zuk, CC BY 4.0; adapted from The Sanger Method for DNA Sequencing by Estevezj, CC BY-SA 3.0)

Lab Protocol: Sanger Sequencing

The protocol given below is for the sequencing of double-stranded DNA (i.e., a sequencing plasmid) using the commercial kit ProDye™ Terminator Sequencing System from Promega. This commercial kit is compatible with a variety of DNA templates, such as sequencing plasmids and PCR products. The sequencing reaction can be analyzed by numerous DNA sequencers, including those from Applied Biosystems (a division of Thermo Fisher). It is important to confirm compatibility with other DNA sequencers. Similar commercial kits from other companies may be used for other DNA sequencers.

1. Clone the DNA region to be sequenced into a suitable sequencing plasmid, such as pUC19.
2. Purify the resulting plasmid to remove contaminants.
3. Determine the concentration of the plasmid and its purity using a UV spectrophotometer.
4. Add 300 to 500 ng of plasmid DNA to a PCR tube. Note that different DNA quantities will be added if sequencing single-stranded DNA or PCR products.
5. Add 2.5 to 5.0 pmole of sequencing primer to the tube.
6. Add 8.0 µL of the ProDye™ 2.5X Master Mix to the tube. Beyond this point, protect the tube from light exposure.
7. Add nuclease-free water to a final volume of 20 µL.
8. Place the tube in thermocycler
9. Run the following protocol
    1. 96°C for 1 minute
    2. 96°C for 10 seconds
    3. 50°C for 5 seconds
    4. 60°C for 4 minutes

5. Repeat Steps 2 through 4 for a total of 25 cycles
6. Hold at 4°C

10. Place the reaction at 4°C overnight or -20°C for longer-term storage. Protect from light exposure.

Protocol: Ethanol Purification of Sequencing Reaction

Once the Sanger sequencing reaction has been completed, the sample will need to be purified to remove any remaining reaction components that will interfere with the DNA sequencer. Purification can be done by sequencing facilities (for an additional cost) or can be done by the researcher. Numerous commercial kits are available for clean-up of PCR products (e.g., QIAquick PCR purification kit from Qiagen). If cost is a concern, the ethanol purification method given below is easy to perform. However, care must be taken in this procedure not to lose the DNA pellet.

1. Add 5.0 µL of 125 mM EDTA (pH 8.0) and mix well.
2. Add 60 µL of 100% ethanol.
3. Vortex briefly
4. Incubate for 15 minutes at room temperature, protected from the light.
5. Centrifuge at 4°C for 45 minutes at 2000 xg.
6. Decant off the liquid.
7. Add 60 µL of 70% ethanol.
8. Centrifuge at 4°C for 15 minutes at 2000 xg.
9. Decant off the liquid.
10. Centrifuge at 4°C for 1 minute at 2000 xg.
11. Carefully aspirate any remaining liquid and air dry the pellet.
12. Analyze the sample in a DNA sequencer.

13.9: Lab Technique - DNA Sequencing is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells

Introduction of DNA into Cells Using Liposomes

Liposomal-mediated transfection is used to deliver DNA or RNA into cells by encapsulating these nucleic acids into spherical, lipid-based vesicles called **liposomes**. Because these liposomes are phospholipid bilayers, they pass easily through the plasma membrane, transporting their nucleic acids into the cells. In the transfection protocol, nucleic acids are mixed with commercial preparations of liposomes (e.g. Lipofectamine, from Invitrogen). The liposome mixture is added to cell cultures where the positively-charged liposomes are attracted to the negatively-charged cell membrane. This interaction allows the liposome to bind and fuse with the cell membrane. The liposome is then taken up into the cell through endocytosis, forming an endocytic vesicle inside the cell. Once inside the cell, the nucleic acid is released into the cytoplasm. Liposomal-mediated transfection is relatively safe, as liposomes are biocompatible with cells. It can deliver DNA or RNA to a wide range of cell types and is a non-viral alternative to introducing genetic material into a cell.

Considerations

The protocol given below uses Lipofectamine® 2000, the most popular formulation of transfection liposomes. The Lipofectamine® 2000 reagent is a proprietary formulation for transfecting nucleic acids into a wide range of eukaryotic cells. The protocol is for transfection of cells in a 6-well dish. Other culture dishes may be used. However, the amount of Lipofectamine® 2000 reagent will need to be adjusted accordingly. Refer to the Lipofectamine® 2000 product insert for important information about cell confluence and DNA concentration.

- DNA-Lipofectamine® 2000 complexes must be made in serum-free medium. However, the complexes can be added directly to cells in culture medium, in the presence or absence of serum/antibiotic
- It is not necessary to remove the complexes or change/add medium after transfection
- The amount of Lipofectamine® 2000 reagent required for successful transfection will vary depending on the cell type and passage number
- It is recommended to start any new transfection by testing different concentrations of Lipofectamine® 2000 Reagent to determine the optimum amount
- The DNA concentration for transfection should be between 0.5 and 5.0 µg/µL
- Cells should be at 70 to 90% confluence at the time of transfection

Lab Protocol: Liposomal Transfection

A workflow for this protocol is given in Figure **13.10.1**.

1. Prior to transfection, seed cells in a 6-well dish so that they will be 70 to 90% confluent in each well. This will result in a cell number of 250,000 to 1,000,000 cells per well.
2. Prepare four concentrations of Lipofectamine® 2000 reagent in serum-free medium (e.g. DMEM or Opti-MEM®).
    - To four sterile 1.5 mL microfuge tubes, add 150 uL of serum-free medium to each tube
    - Add 6, 9, 12, and 15 µL of Lipofectamine® 2000 to these tubes, respectively
3. Pipette 700 µL of serum-free media into a sterile 1.5 mL microfuge tube and add 14 µg of DNA.
4. Add 150 uL of the diluted DNA to each Lipofectamine® 2000 tube.
5. Incubate for 5 minutes at room temperature.
6. Add 250 µL of the DNA-Lipofectamine® 2000 mixture to each well. The culture medium does not need to be removed.
7. Culture cells for 1 to 3 days.
8. Analyze cells for transfection.

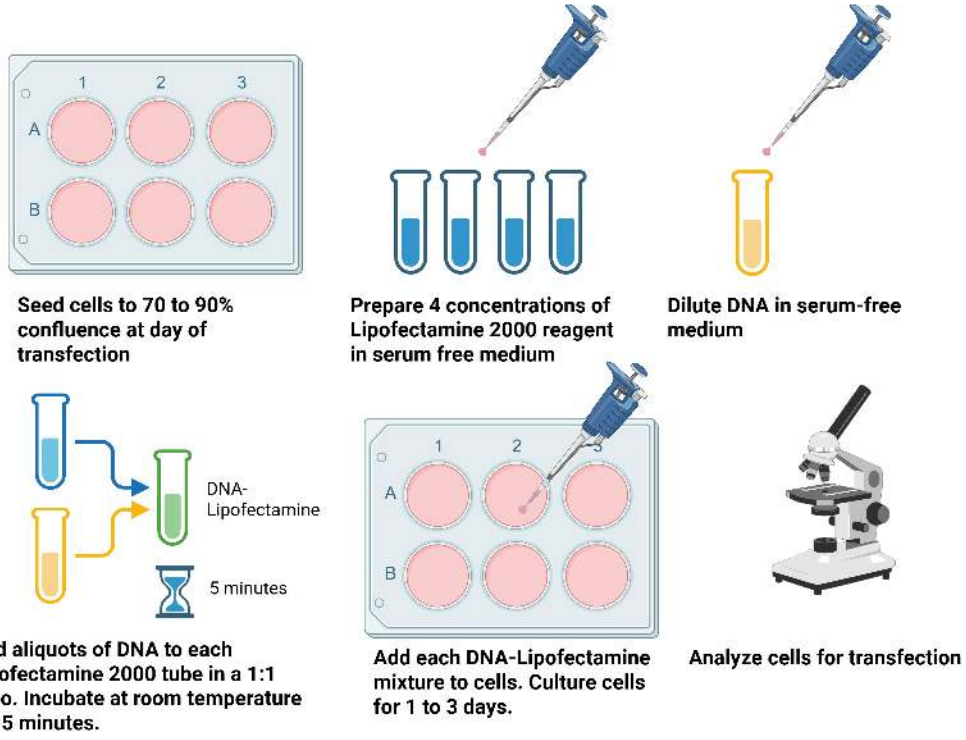


Figure **13.10.1**: Transfection using liposomes. Cells should be seeded so that they are 70 to 90% confluent the day of the transfection. In order to optimize transfection, four concentrations of the Lipofectamine® 2000 should be prepared in serum-free medium. DNA for transfection is also diluted in serum-free medium. Aliquots or DNA are added to each Lipofectamine® 2000 tube in a 1:1 ratio and then added to the cultured cells for 1 to 3 days. Cells can then be analyzed and transfection efficiency determined. (Transfection Workflow by Patricia Zuk, PhD, CC BY 4.0; figure created in BioRender. Zuk, P. (2025)

13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.11: Lab Technique - SDS-PAGE

Separating Proteins by Denaturing SDS-PAGE Electrophoresis

Electrophoresis (meaning to “carry with electricity”) separates molecules based on their size using an electric field. Like DNA, electrophoresis can separate proteins based on their charge and size. While agarose can be used in this separation, the preferred polymer is polyacrylamide. **Polyacrylamide** is a water-soluble polymer made up of linear chains of acrylamide. It can be cross-linked and polymerized to form a soft gel comprised of small pores capable of separating proteins ranging from 5 to 250 kDa. The separation of proteins using polyacrylamide is known as **Polyacrylamide Gel Electrophoresis or PAGE**. Because most PAGE techniques use a detergent, such as **sodium dodecyl sulfate (SDS)**, to denature the proteins being separated, the separation of proteins by electrophoresis is often called **SDS-PAGE or denaturing SDS-PAGE**. PAGE in the absence of detergents is called **native-PAGE**. SDS-PAGE separates proteins primarily by their size because the ionic detergent SDS denatures proteins into their individual subunits and binds to each subunit to make them uniformly negatively charged. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel toward the positively charged electrode. The smaller protein subunits will migrate faster through the gel, while the larger proteins will take longer to migrate towards the positive electrode. Therefore, *the rate at which a protein subunit passes through the gel is inversely proportional to its size*.

Preparation of the Polyacrylamide Gel

Polyacrylamide gels are prepared by mixing acrylamide with bis-acrylamide in a defined ratio. The amount of polyacrylamide in the mixture produces gels of specific percentages (i.e., concentrations). The higher percentage of acrylamide in the gel translates to smaller the pores within the gel and better resolution of small proteins (5 to 60 kDa). For example, a 7% acrylamide gel will have larger pores in comparison to a 10% gel. Low percentage gels are used to separate larger proteins (e.g., from 60 to 200 kDa). The standard polyacrylamide gel used is a 10% gel which can separate proteins ranging from 20 to 150 KDa. However, "gradient" gels can be prepared with higher percentages of acrylamide (i.e., 20% with smaller pores) at the bottom of the gel and lower percentages (i.e., 4% with larger pores) at the top. This enables the user to separate a larger range of proteins using a single gel. The polyacrylamide-bis-acrylamide mixture is crosslinked to form a gel through the addition of a polymerizing agent, **ammonium persulfate (APS)** and the catalyst **TEMED** (N,N,N’,N'-tetramethylenediamine). The gels are often prepared in a buffer that is similar to the one that will be used to run the gel. Because of this, this type of SDS-PAGE is called **discontinuous**.

Once prepared, the acrylamide/bis-acrylamide solution is poured between into a thin space found between two glass or plastic plates. These plates are assembled with spacers of specific size to form a gel with a desired thickness. The assembled plates are called a "cassette" and the process is referred to as "casting" a gel (Figure 13.11.1). To obtain optimal resolution of proteins, two types of gels are made and polymerized in the cassette: a **separating or resolving gel** with a specific concentration and a **stacking gel** with a set concentration of 7%. The separating gel makes up the majority of the gel's total volume (i.e., 80%) and separates the proteins according to their size. The stacking gel has a lower pH and a different ionic concentration than the separating gel. This, together with the discontinuous nature of the system, ensures that all proteins, irregardless of their size, flow through the stacking gel at the same rate and enter into the separating gel at the same time. A stacking gel is not necessary when using a gradient gel, as the gradient itself performs this function. The separation gel is poured into the cassette first and allowed to polymerize. The stacking gel is then poured on top of the resolving gel. A "comb" is inserted into the stacking gel and allowed to polymerize. When the comb is removed, wells are created for individual protein samples.

Users now have the option of buying "pre-cast" gels for SDS-PAGE. Precast gels are a more convenient way of analyzing proteins and give researchers a level of gel consistency that hand cast gels may not have. Precast gels are available in a range of percentages and include difficult-to-pour gradient gels. Precast gels are also available in the different buffer formulations (e.g., Tris-glycine, Bis-Tris, Tris-acetate), which are designed to optimize shelf life, run time, and/or protein resolution.

Preparing the Samples

In denaturing SDS-PAGE, samples are prepared in a concentrated **Sample Buffer** containing the anionic detergent SDS and a reducing agent. The presence of SDS denatures proteins into their subunits and surrounds them with a uniform negative charge. Reducing agents, like dithiothreitol (DTT) or β-mercaptoethanol, break disulfide bonds found between cysteine amino acids, denaturing the protein subunits into their linear conformations. Denaturing is assisted by heating the protein sample 70-100°C.

Finally, typical Sample Buffers contain some type of tracking dye (usually bromophenol blue) used to assess the rate of sample migration through the separating gel.

Running the Gel

Once the gels polymerize, it is ready to be "run" (Figure **13.11.1**). To run the gel, the cassette is mounted (usually vertically) into an **electrophoresis tank** containing an upper cathode and a lower anode. Once the cassette is placed in the tank, an upper and lower buffer chamber will form and the top and bottom edges of the gel cassette will come into contact with the cathode and anode, respectively. The comb is removed from the stacking gel, the wells rinsed, and the two buffer chambers in the electrophoresis tank are filled with a **Running Buffer.** The most common Running Buffer is a combination of Tris base and Glycine. The Tris-Glycine buffer allows for the efficient flow of electricity through the gel and permits the separation of a large range of protein sizes. Alternate buffers include Tris-Acetate, Tris-Tricine, or Bis-Tris. The Tris-Tricine buffer is a modification of the Tris-Glycine buffer. It is often used with gels that resolve low molecular weight proteins in the range of 2–20 kDa. Tris-Acetate and Bis-Tris buffers, with their neutral pH, decrease protein degradation in the gel and are often used in the analysis of high molecular weight proteins or native-PAGE applications where the more neutral pH is beneficial. However, gels run with Bis-Tris buffer must be run with alternative reducing agents, such as sodium bisulfite. The samples are loaded alongside a "protein ladder" of known molecular weights in order to assess the size of the separated proteins. The gel is run at a constant voltage (e.g. 120V to 150V) until the tracking dye found in the Sample Buffer is reaches off the bottom of the plate.

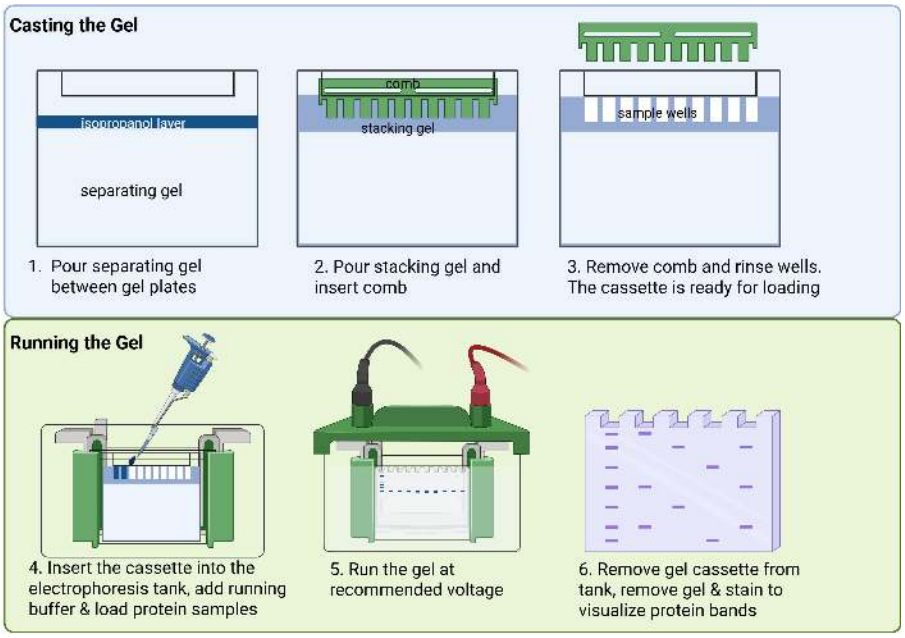


Figure **13.11.1**: Denaturing SDS PAGE workflow. (1) The separating gel is poured between two glass plates and allowed to polymerize. Isopropanol is layered on top of the separating gel to create a smooth interface between separating and stacking gel. (2) The isopropanol is removed and the stacking gel is poured. A comb is inserted to create wells. The gel is allowed to polymerize. (3) The comb is gently removed and the wells rinsed with PBS or water. (4) The prepared electrophoresis "cassette" is loaded into the electrophoresis tank and the chambers of the tank are filled with Running buffer. The protein samples are loaded into the wells. (5) The gel is run. (6) The cassette is removed from the tank. The gel is removed from the cassette and can be stained to visualize protein bands. If moving on to a Western blot, the gel is not stained. (SDS PAGE by Patricia Zuk by CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

The protocol given below outlines gel electrophoresis using precast or hand-cast denaturing SDS-PAGE gels.

Lab Protocol: Gel Preparation

1. For a precast gel:
    1. remove the gel from its packaging, remove the comb and rinse wells with deionized water.
2. For a hand cast gel:
    1. Assemble the plates to form the gel cassette.

2. Prepare a separating gel and pour into the cassette. The volume of this gel should be approximately 80% of the total volume of the cassette.
3. Add a thin layer of isopropanol to prevent evaporation and create a smooth interface with the stacking gel.
4. Allow the gel to fully polymerize.
5. Pour off the isopropanol and rinse with deionized water.
6. Prepare the stacking gel and pour into the cassette until the volume reaches the top of the notched plate.
7. Insert the desired comb size.
8. Allow the gel to fully polymerize.
9. Gently remove the comb and rinse the wells with deionized water.

Lab Protocol: Sample Preparation

1. Prepare the proteins samples for analysis by mixing with a 4X Sample Buffer (i.e. Laemelli Buffer) in a 3:1 ratio.
2. Boil the samples for 95°C for 5 minutes.
3. Allow to cool.

Lab Protocol: Electrophoresis

1. Secure the gel cassette in the electrophoresis tank with the notched plate facing inward.
2. Fill the upper and lower buffer chambers with 1X SDS Running Buffer.
3. Using protein loading tips, carefully pipette the samples into the appropriate wells. Ensure one of the wells is a protein ladder for reference.
4. Connect the electrophoresis tank to the power supply.
5. Run the gel at 120V until the tracking dye reaches the bottom of the gel (~1-2 hours).
6. Remove the cassette from the tank.
7. Carefully separate the two plates of the cassette.
8. Scrape off the stacking gel. and discard.
9. For visualization of protein bands, stain the separation gel. Do not stain if Western blotting. Refer to Western Blotting protocol for additional steps.

Lab Protocol: Gel Staining and Visualization

1. Carefully remove the separating gel from the cassette plate and immerse in Coomassie Brilliant Blue staining solution.
2. Incubate for 30 minutes with gentle agitation.
3. Destain the gel by washing in Destaining solution with gentle agitation. Several changes of this solution may be necessary. (e.g., methanol/acetic acid/water mixture). Destain until protein bands are visible and the background is clear.
4. Compare the migration of protein bands to the molecular weight marker to estimate protein sizes.

Safety Considerations

- Handle acrylamide with care as it is a neurotoxin in its liquid form. Use gloves and work in a well-ventilated area.
- Dispose of buffers and gels according to institutional waste disposal guidelines.

PAGE Reagents

30% Acrylamide/0.8% bis-acrylamide stock

- add 150 g of acrylamide to 250 mL distilled water and mix
- add 4.0 g bis-acrylamide and mix
- add distilled water to 500 mL and mix well
- filter through a 0.2 µm filter
- store at 4ºC in a foil-wrapped bottle

10% Separation gel (pH 8.7)

- combine the following:
  - 5.0 mL 30% acrylamide/0.8% bis-acrylamide solution (10%/0.3% final)
  - 3.75 mL 1.5M Tris-HCl, pH 8.7 (0.375M final)

- 150 µL 10% SDS
- 75 µL 10% APS
- 7.5 µL TEMED

- add distilled water to 15 mL
- pour gel immediately

4% Stacking gel (pH 6.8)

- combine the following:
  - 2.0 mL 30% acrylamide/0.8% bis-acrylamide solution (4%/0.1% final)
  - 3.75 mL 0.5M Tris-HCl, pH 6.8 (0.125M final)
  - 150 µL 10% SDS
  - 75 µL 10% APS
  - 15 µL TEMED
- add water to 15 mL
- pour gel and insert comb immediately

5X SDS Running Buffer (pH 8.3)

- combine the following in 800 mL distilled water:
  - 15.1 g Tris base
  - 72.0 g glycine
  - 5.0 g SDS
- add water to 1 L (pH will be ~8.3)
- store at room temperature
- dilute to 1X before use
- 1X final concentration:
  - 25 mM Tris
  - 208 mM glycine
  - 0.1% SDS

4X Sample Buffer

- combine the following:
  - 250 µL 0.5M Tris-Cl pH 6.8
  - 400 µL 10% SDS
  - 250 µL 80% glycerol
  - 100 µL 1M DTT or 25 µL β-mercaptoethanol
  - 50 µL 1% bromophenol blue
- store at room temperature
- 1X final concentration:
  - 31.25 mM Tris-Cl pH 6.8
  - 1% SDS
  - 5% glycerol
  - 30 mM DTT or 0.005% β-mercaptoethanol
  - 0.0125% bromophenol blue

Coomassie Brilliant Blue Staining Solution

- dissolve 1.0 g Coomassie Brilliant Blue R250 in 300 mL methanol
- add:
  - 50 mL glacial acetic acid
  - 650 mL distilled water
- filter through Whatman paper

- store at room temperature protected from light
- final concentration:
  - 0.1% Coomassie Brilliant Blue R250
  - 30% methanol
  - 5% acetic acid

Destaining Solution

- to 650 mL of distilled water add:
  - 300 mL of methanol
  - 50 mL of glacial acetic acid
- store at room temperature
- final concentration:
  - 30% methanol
  - 5% acetic acid

13.11: Lab Technique - SDS-PAGE is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.12: Lab Technique - Western Blotting

Analyzing Proteins Through Western Blotting

The **Western blot** is a widely used laboratory technique that uses antibodies to identify a specific protein within a sample, quantify the expression of a protein as a result of experimental treatment, and detect post-translational modifications, such as phosphorylation or glycosylation. **Western blotting** (also called **immunoblotting**) begins with the separation of proteins through SDS-PAGE. Because the acrylamide gel is delicate, the proteins are electrically transferred or "blotted" from the gel onto a positively-charged matrix to capture the proteins (usually nitrocellulose paper). This produces an exact replica of the proteins on the substrate. This "copy" is then incubated with antibodies to detect the target protein. In a direct western blot, a single antibody, called a **primary antibody** is used to detect a specific region of a target protein. This primary antibody is coupled to some sort of marker (e.g. fluorescent, chemiluminescent). When the marker is detected, the protein is identified. In the more common indirect western blot, a primary antibody and a **secondary antibody** is used. The primary antibody is detected using the secondary antibody that is coupled to a chemiluminescent or fluorescent marker (Figure **13.12.1**) The indirect method allows for amplification of the signal as multiple labeled secondary antibodies can bind a primary antibody. The most common type of marker is a chemiluminescent marker, such as **horseradish peroxidase (HRP)**, that develops a substrate to produce a luminescent signal. This signal can be detected using a documentation system or light-sensitive film. Once visualized, the luminescent protein band can be compared to protein markers of known size to confirm its size and compared to reference standards to quantify its expression level.

Key Components of the Western Blot

- **SDS-PAGE gel:** can be pre-cast or hand cast. For more information, see Laboratory Technique: SDS-PAGE
- **Transfer apparatus:** used to transfer the proteins in the acrylamide gel to a secondary matrix support
  - most common method is electrophoretic
  - can be done under wet, semi-dry or dry conditions
  - wet transfer:
    - most common method
    - requires use of a transfer tank, transfer cassette, and nitrocellulose membrane
    - transferred using a **Transfer Buffer** containing methanol
    - alternatives to nitrocellulose can be used, such as **polyvinylidene di-fluoride (PVDF) membrane**
- **Antibodies:** used for detection of a specific protein
  - primary antibody: specific to target protein
  - secondary antibody: used to detect the primary antibody; conjugated to a signal marker (enzyme or fluorochrome)
  - reduction of non-specific antibody binding to the nitrocellulose can be achieved using a **Blocking Buffer** containing non-fat dry milk and a non-ionic detergent to block non-specific sites in the protein
  - Blocking buffers with serum in lieu of non-fat milk can be used
- **Chemiluminescence detection system**: used to process the enzyme linked to the secondary antibody
  - most sensitive detection method
  - the most common system uses a secondary antibody is coupled to the enzyme horseradish peroxidase/HRP and an **Enhanced Chemiluminescent (ECL) Reagent**
  - the ECL reagent is comprised of a stable **peroxide** and an enhanced **luminol** reagent
- **Imaging system:** used to detect and quantify chemiluminescence or fluorescence
  - chemiluminescent signal detected either by X-ray film or a charged-coupled device (CCD) camera
  - fluorescence detected by digital camera

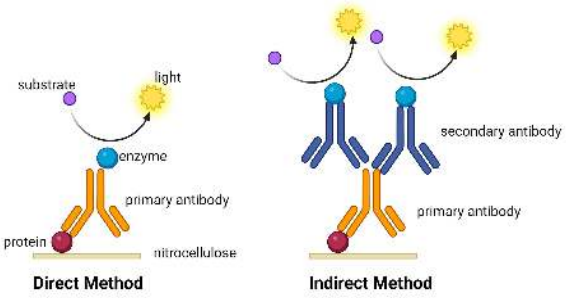


Figure **13.12.1**: Indirect vs. Direct Western Blotting. In the direct method of Western blotting (left image), a "labeled" primary antibody, coupled to a marker, such as an enzyme, is used to detect a specific protein. Development of a substrate by the enzyme produces a detectable signal. In the indirect method (right image), an uncoupled primary antibody detects the protein and a labeled secondary antibody is used to detect the primary antibody. As with the direct method, the marker on the secondary antibody produces a detectable signal. The use of a secondary antibody allows for signal amplification as more than one secondary antibody can bind a primary antibody. (Indirect vs. Direct Western Blotting by Patricia Zuk; figure created in BioRender. Zuk, P. (2025))

The protocols given below outline the transfer of proteins under wet conditions using a nitrocellulose membrane, followed by protein detection using the indirect blotting method (Figure **13.12.2**).

Lab Protocol: Wet Transfer of Proteins to Nitrocellulose

1. Separate proteins using SDS-PAGE and carefully open the gel cassette.
2. Remove the stacking gel by scraping it away.
3. Place a piece of Whatman filter/blotting paper on top of the separating gel.
4. Invert the gel plate and carefully lift it from the gel, leaving the separating gel adhered to the filter paper.
5. Trim off the excess blotting paper.
6. Cut another piece of filter paper and a piece of nitrocellulose membrane to the same size.
7. Prepare the transfer cassette:
    1. Fill a glass container with enough Transfer Buffer ensure coverage of the nitrocellulose membrane.
    2. Open the plastic transfer cassette and place in the container.
    3. Add a transfer sponge to one half of the transfer cassette.
    4. Place the gel on the sponge with the filter paper side down.
    5. Wet the piece of nitrocellulose in Transfer Buffer. Carefully place on top of gel, ensuring no air bubbles form between the gel and the membrane.
    6. Add the second piece of filter paper and the second transfer sponge.
    7. Close the transfer cassette.
8. Fill the transfer tank with Transfer Buffer.
9. Carefully insert the Transfer cassette into the transfer tank with the hinge facing up. Ensure that the acrylamide gel faces the black/negative electrode and the nitrocellulose membrane faces the red/positive electrode.
10. Transfer at 100V for 90-120 minutes. If done at 4°C, the transfer can be left in the transfer tank overnight.

Lab Protocol: Membrane Probing

1. Following transfer, remove the transfer cassette and disassemble it.
2. Using forceps, remove the nitrocellulose membrane and place it into a plastic staining container.
    1. Optional: To confirm transfer, stain the nitrocellulose membrane with a Ponceau S solution for 5 minutes with gentle shaking.
    2. Pour off the excess stain.
    3. Wash the membrane 3 times with Wash Buffer for 5 minutes each.
3. Block non-specific sites (and remove the Ponceau S stain) by incubating the membrane in Blocking Buffer for 1 hour at room temperature or overnight at 4°C with gentle agitation.
4. Dilute the primary antibody in Blocking Buffer to the desired concentration. Incubate the membrane in primary antibody for 1-2 hours at room temperature or overnight at 4°C with gentle shaking.

5. Wash the membrane 3 times with Wash Buffer for 5-10 minutes each.
6. Dilute the secondary antibody in Blocking Buffer to the desired concentration. Incubate the membrane in secondary antibody for 1-2 hours at room temperature with gentle shaking.
7. Wash the membrane 3 times with Wash Buffer for 5-10 minutes each.

Lab Protocol: Protein Detection

1. Decant the wash buffer.
2. Add the chemiluminescent substrate to the membrane and incubate for the time recommended by the substrate manufacturer.
3. Place the membrane in an imaging system or expose to X-ray film to visualize protein bands.
4. Compare the visualized protein band to the protein ladder.

Safety Considerations

- Handle methanol, chemiluminescent substrates, and any hazardous reagents with appropriate PPE.
- Dispose of waste according to institutional guidelines.

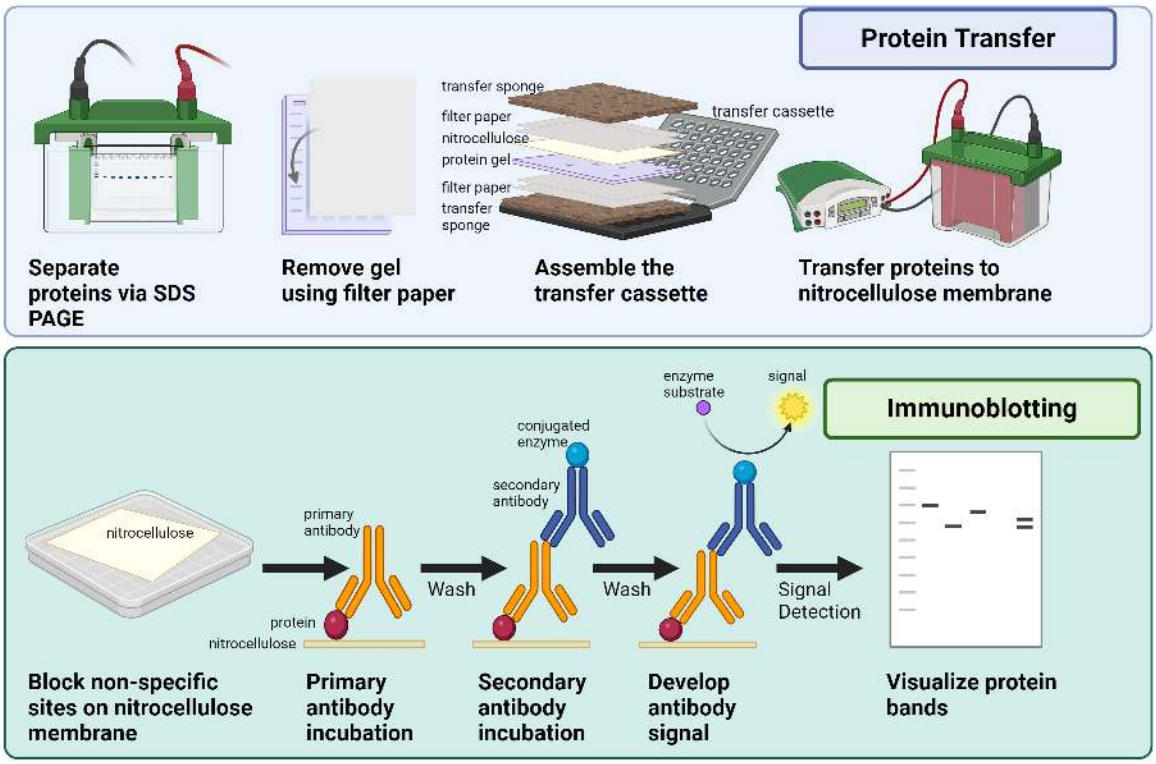


Figure **13.12.2**: The Western Blotting workflow. Protein Transfer (top box): Proteins are separated by size through acrylamide electrophoresis (SDS PAGE). The acrylamide gel is transferred to filter paper for increased support and then assembled into a transfer cassette, comprised of two transfer sponges surrounding filter paper, the gel, and a membrane made of nitrocellulose (or PVDF). The proteins are electrically transferred from the acrylamide gel onto the nitrocellulose. Immunoblotting (bottom box): The transfer cassette is disassembled and non-specific sites on the nitrocellulose blocked for several hours (usually with buffer containing non-fat milk or animal serum). The nitrocellulose is incubated with a buffer containing the desired primary antibody, followed by several short wash steps. The membrane is incubated with buffer containing the appropriate secondary antibody and then washed again. The secondary antibody is conjugated to a marker that can be detected. The most common marker is an enzyme like horseradish peroxidase (HRP). Protein bands in the nitrocellulose membrane bound to the primary-secondary antibody complex are visualized by developing the conjugated marker and producing a detectable signal. For HRP, the membrane is incubated with a luminol/peroxide solution that results in a light signal that can be visualized using X-ray film or a camera-based detection system. (Western Blotting by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Western Blot Reagents

Western Transfer buffer

- combine the following in 3800 mL distilled water:
  - 115.31 g Glycine
  - 24.23 g Tris base
  - 800 mL methanol
- add distilled water to 4 L
- final concentration:
  - 384 mM Glycine
  - 50 mM Tris
  - 20% methanol

Western Blocking buffer

- combine the following in 400 mL distilled water:
  - 25.0 g non-fat dry milk or 15.0 g bovine serum albumin (BSA)
  - 50 mL 10X Phospho-buffered Saline (PBS)
  - 500 uL Tween-20
- add water to 500 mL
- final concentration:
  - 5% non-fat milk or 3% BSA
  - 1X PBS
  - 0.1% Tween-20

Western Wash Buffer

- combine the following in 800 mL distilled water:
  - 100 mL 10X Tris-buffered Saline (TBS)
  - 1.0 mL Tween-20
- add distilled water to 1 L
- final concentration:
  - 1X TBS
  - 0.1% Tween-20

10X Phospho-buffered Saline (PBS)

- combine the following in 900 mL distilled water:
  - 80.0 g NaCl
  - 2.0 g KCl
  - 17.8 g Na_2HPO_4
  - 2.4 g KH_2PO_4
- add distilled water to 1 L
- 10X final concentration:
  - 1.37 M NaCl
  - 27 mM KCl
  - 100 mM Na_2HPO_4
  - 18 mM KH_2PO_4

10X Tris-buffered Saline (TBS)

- combine the following in 900 mL distilled water:
  - 24.0 g Tris

- 88.0 g NaCl
- adjust the pH to 7.6 with 10N HCl
- add distilled water to 1 L

- 10X final concentration:
  - 200 mM Tris-Cl
  - 1.5 M NaCl

Ponceau S Solution

- dissolve 0.5 g of Ponceau S in 1.0 mL glacial acetic acid
- bring to 100 mL with distilled water
- final concentration:
  - 0.5% (w/v) Ponceau S
  - 1.0% (v/v) glacial acetic acid

13.12: Lab Technique - Western Blotting is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.13: Lab Technique - Cell Culture

In Vitro Cell Culture

Culturing of mammalian cells *in vitro* requires a specific set of physical and chemical conditions to ensure their viability and growth. The physical conditions are provided by the culture incubator. The chemical conditions are provided through the culture reagents.

Culture Conditions

- **Culture temperature:** depends on the type of cells being cultured (i.e., animal vs. plant)
  - mammalian cells - typically cultured at 37°C; mimics the normal body temperature of humans and other mammals
  - insect cells - usually cultured at 27–28°C
  - plant cells - typically cultured at 25°C; mimics the ambient temperature of their natural environment
  - yeast cells - commonly cultured at 30°C; can grow between 25°C and 37°C depending on the experiment
  - molds - usually cultured at 25–30°C; mimics their natural environmental conditions.
  - pathogenic fungi (e.g., Candida albicans) - often cultured at 35–37°C; reflects human body temperature to study their pathogenic behavior
- **Culture CO2 concentration:** ranges from 5% to 10% depending on the cell type
  - 5% CO2 mimics the *in vivo* CO2 concentration found in many tissues
  - addition of CO2 is to the incubator is required to provide cells with the proper physiologic pH when grown in media
  - provided to the incubator through a compressed gas cylinder
- **Culture pH:** should range between 7.2 to 7.4
- **Culture humidity:** ranges from 95% to 98%
  - prevents evaporation of the culture medium and a reduction of medium volume;
  - evaporation could lead to increased osmolarity (higher salt concentration) and changes to nutrient and pH levels
  - adequate humidity can be provided by using a pan of water at the bottom of the incubator
  - some incubators can more precisely control humidity levels

Culture Media & Reagents

The culture media and reagents used in cell culture are designed to provide chemical support of the cells under sterile conditions. More information can be found in Chapter 6.1: Principles of *In Vitro* Cell Culture. Commonly used media and reagents include:

- **Basal Medium:** a defined mixture of amino acids, vitamins, minerals, and glucose
  - numerous formulations available commercially
- **Serum:** the plasma fraction of blood with clotting factors removed
  - isolated from animal sources - associated with ethical concerns
  - source of essential growth factors, hormones, and other key factors like plasma proteins, attachment proteins, anti-proteases, and lipids
  - most commonly used - Fetal Bovine Serum (FBS)
  - can be a source of contamination - e.g. mycoplasma
    - heat-inactivation can mitigate risk of contamination
    - can be sold as heat-inactivated
  - batch-to-batch variations may occur - new batches should be tested before use
- **Salt solutions:**
  - Phosphate-buffered Saline/Phospho-buffered Saline (PBS)
    - contains sodium phosphate and sodium chloride
    - can be sold as powder, tablets, or a sterile, concentration solution (i.e., 10X),
    - cell culture solutions adjusted to pH 7.2
  - Hank's Balanced Salt Solution (HBSS)

    - modified to contain salts such as sodium bicarbonate, calcium, and magnesium
    - can be as a sterile, concentration solution (i.e., 10X)
- **Trypsin/EDTA:** used for the harvesting of culture cells by releasing them from the culture surface
    - trypsin - enzymatically digests integrin complexes
    - EDTA - calcium chelator; inhibits function of cadherins
    - sold in various concentrations
    - working concentrations of 0.25% trypsin and 0.02% EDTA
- **Penicillin & Streptomycin:** the most commonly used antibiotics in cell culture
    - penicillin - effective against gram-positive bacteria such as *Streptococcus sp.* and *Staphylococcus sp.*
    - streptomycin - effective against gram-negative bacteria, mycoplasma, and certain gram-positive bacteria
    - sold as a concentrated (i.e. 100X) "pen/strep" solution of 10,000 units/mL penicillin, 10,000 ug/mL streptomycin
    - 1X working concentrations are penicillin - 100 units/mL; streptomycin – 100 ug/mL.
- **Amphotericin B:** commonly used anti-fungal
    - used to prevent fungal infections, including yeast
    - often sold as a concentrated solution (i.e., 100X) together with pen/strep (e.g. pen/strep/ampho)
    - 1X working concentration is 25 mg/mL
    - generally not used - can harm sensitive cells

Aseptic Technique Fundamentals & Checklists

Maintaining sterile cultures depends on proper **aseptic technique**. One key aspect of aseptic technique is the prevention of contamination. The use of disinfectants can prevent the start of a contamination, in addition to preventing its spread within the culture facility. Commonly used disinfectants are **70% ethanol** (or **isopropanol**) placed into spray or squeeze bottles. These spray bottles can be used to clean the inside of the BSC, the incubator, and the bottles and flasks placed in the BSC. In addition to ethanol, culture laboratories should have a dilute solution of household bleach (e.g. 10%) that can be used to clean and disinfect surfaces where a spill or contamination has occurred. This bleach solution is also used to clean and decontaminate the aspiration flask used in the BSC.

The checklist given below can be used to ensure the safety of the user and sterility of the equipment and reagents used for cell culture.

Personal Safety Checklist

- Did you wash your hands?
- Are you wearing appropriate personal protective equipment (e.g. gloves, lab coat)?
- Are you wearing close toed shoes and long pants?
- If you have long hair, is it tied in the back?

Biosafety Cabinet (BSC) Checklist

- Is the BSC located in an enclosed area free from drafts and through traffic?
- Is the BSC sash placed at the correct level?
- Is the blower motor functioning properly?
- Is the UV light off?
- Is the air flowing through the front and back grill of the BSC unimpeded?
- Is the work surface inside BSC uncluttered - does it contain only the items required for your experiment?
- Did you wipe the work surface of the BSC with 70% ethanol before work?

Culture Equipment Checklist

- Are you routinely cleaning and sterilizing your incubator(s) on a regular basis?
- Are you regularly cleaning the culture refrigerator(s), freezer(s), water bath(s) on a regular basis?
- Does the water inside the water bath contain a disinfectant solution to prevent microbe growth?
- Do you certify your BSC on a regular basis?

- Does the UV light in the BSC work?
- Does the blower motor in the BSC work?

Reagents, Media, and Material Checklist

- Have you sterilized any newly prepared reagents, media, and solutions using the appropriate procedure?
- Can you aliquot any reagents into smaller volumes to minimize the chances of contamination?
- Did you wipe the outside of all reagent containers with 70% ethanol before placing them on your work surface?
- Are all reagent and media containers capped when not being used in the BSC?
- Are all reagent and media containers stored properly to ensure their sterility when not in use?
- Is all culture plastic-ware stored in sterile re-sealable bags?
- Do any reagents look cloudy? Do they contain floating particles? Is there a foul smell? Unusual color? If yes, have you decontaminates and discarded those items?

Aseptic Handling

- Are you working slowly and deliberately, mindful of aseptic technique?
- Did you wipe the surfaces of all culture items (e.g. pipette-aids, bottles, tip boxes, centrifuge tubes etc....) with 70% ethanol before placing them in the cell culture hood?
- Are you placing container caps or dish covers face up on the work area?
- Are you using sterile glass pipettes or sterile disposable plastic pipettes to manipulate all liquids?
- Are you using a sterile pipette only once to avoid cross contamination?
- Are you careful not to touch the pipette tip to anything non-sterile, including the outside edge of the bottle threads?
- Did you mop up any spills immediately, and wipe the area with 70% ethanol?

BSL-2 Lab Regulations

Cell culture facilities have a minimum classification of BSL-2. The following safety precautions are to be observed at all times when in a BSL lab:

1. Wash hands with a disinfectant soap before and after working with cells.
2. Wear appropriate PPE, such as gloves.
3. Do not eat or drink in the lab. Never store food or drink in the lab.
4. Treat all cells as if they were pathogenic – even if they are not.
5. When handling tubes, pipette tips, and culture dishes that have come in contact with cells, dispose of them in the appropriate biohazard bag or container.
6. Do not put liquid into a biohazard bag. Remove liquid waste (e.g. culture medium) into the appropriate container (e.g. aspiration flask).
7. Make sure that all cultures and reagents are properly labeled with names and dates. If they are hazardous, include proper hazard information.
8. Know the location of the lab's nearest eyewash station, shower and sink.
9. Disinfect all benches and work areas before and after working using a disinfectant solution.
10. In case of a spill, soak several paper towels with a dilute bleach solution (10% bleach) and cover the spill. To prevent spread of potential contamination, do not spray onto the spill. Let sit for 5 minutes. Carefully gather up the paper towels and place them into a biohazard bag.

Culture Medium Preparation

The protocol given below details how to make a common medium for cell growth and viability. This medium is made using sterile basal medium, FBS, penicillin, and streptomycin. Several types of basal medium may be used.

Lab Protocol: Medium Preparation

1. Warm all culture reagents in a 37°C water bath.
2. Prepare the BSC for use.
3. Wipe all culture supplies, reagent bottles, and tubes with 70% ethanol before placing in the BSC.

4. To 500 mL of basal medium (e.g. DMEM), add the following using proper aseptic technique:
    1. 50 mL FBS (final concentration 10%).
    2. 5 mL of 100X Pen/Strep (final concentration 1X).
5. Label the prepared growth medium with the name of the user and the date.
6. Store growth medium at 4°C.

Changing Culture Medium

This protocol outlines how to aseptically remove culture medium from a culture dish or flask. The used medium should be completely vacuum aspirated from the culture dish into an aspiration flask. To prevent contamination, this aspiration flask should be emptied after use and placed back into the BSC with a small amount of dilute bleach solution (e.g. 10%).

Lab Protocol: Changing Culture Medium

1. Warm the culture medium in a 37°C water bath.
2. Prepare the BSC for use.
3. Wipe all culture supplies, reagent bottles, and tubes with 70% ethanol before placing in the BSC.
4. Turn on the vacuum line and insert a sterile, glass Pasteur pipette into the tubing of the aspirator flask.
5. .Open the culture dish, tilt it slightly to one side to collect the culture medium, and slowly aspirate the medium into the aspiration flask
    - If changing the media in multiple dishes of the same cell type grown in the same media, the same pipette can be used for all dishes if proper aseptic technique is used.
6. Discard the used glass pipette in a glass waste container.
7. .Using a sterile serological pipette and pipette-aid, pipette the appropriate volume of new culture medium onto the dish
    - The amount of medium added depends on the size of the culture dish.
    - 2 to 3 mm of medium is sufficient to completely cover the cultured cells.
8. Close and dish and return the cells to the incubator.

Harvesting & Sub-culturing Cells

This protocol outlines how to harvest and sub-culture (i.e., passage) cells from one culture dish to another using a solution of trypsin/EDTA (Figure **13.13.1**). The harvested cells may be placed into a new culture dish (i.e., sub-cultured) or divided up across several new dishes to increase their numbers (i.e., sub-cultured and "split"). It is important to keep track of the number of times the cells are sub-cultured as sub-culturing can stress the cells. Each new sub-culture should be indicated with a new passage number (e.g., P1, P2 etc....).

Lab Protocol: Harvesting Cells

1. Warm the culture medium and trypsin/EDTA in a 37°C water bath.
2. Prepare the BSC for use.
3. Wipe all culture supplies, reagent bottles, and tubes with 70% ethanol before placing in the BSC.
4. Turn on the vacuum line and insert a sterile, glass Pasteur pipette into the tubing of the aspirator flask.
5. Open the culture dish, tilt it slightly to one side to collect the culture medium and slowly aspirate the medium into the aspiration flask.
6. Discard the used glass pipette in a glass waste container.
7. Add the appropriate volume of trypsin/EDTA to the culture dish.
    - the amount of trypsin/EDTA added will depend on the size of culture dish.
    - add only enough trypsin/EDTA to cover the dish surface.
8. Close the dish and let incubate in the BSC for 5 to 10 minutes.
9. To harvest the cells from the dish surface, open the culture dish, tilt it slightly to one side, and slowly and gently pipette the trypsin/EDTA solution over the dish surface multiple times using a sterile serological pipette and pipette-aid to release the cells from the dish surface.
    - the release of the cells may be visible to the eye.

- to enhance the harvesting of the cells, the dishes containing trypsin/EDTA can be placed in the incubator for 5 to 10 minutes.

10. Pipette the harvested cells into a sterile, plastic centrifuge tube.
11. To deactivate the trypsin/EDTA, add an equal volume of serum-containing culture medium to the centrifuge tube.
12. Centrifuge the cells at low speed (e.g. 1,000 rpm) for 5 minutes to pellet the cells.
13. Wipe the outside of the centrifuge tube with 70% ethanol and return to the BSC.
14. Using a sterile, glass Pasteur pipette, slowly and carefully aspirate the supernatant into an aspiration flask. Avoid placing the pipette tip near the pellet.
    - a sterile micropipette tip can be placed onto the Pasteur pipette to decrease the strength of the vacuum and slow the rate of aspiration.
15. Re-suspend the pellet in the desired volume of culture medium.
    - the amount of culture medium used will depend on the number and size of the culture dish(es) being used for subsequent culture.
    - to expand the number of cells in culture, the pellet can be divided over multiple new culture dishes.
16. Pipette the cell suspension into the new culture dish(es).
17. Return the cells to the incubator.

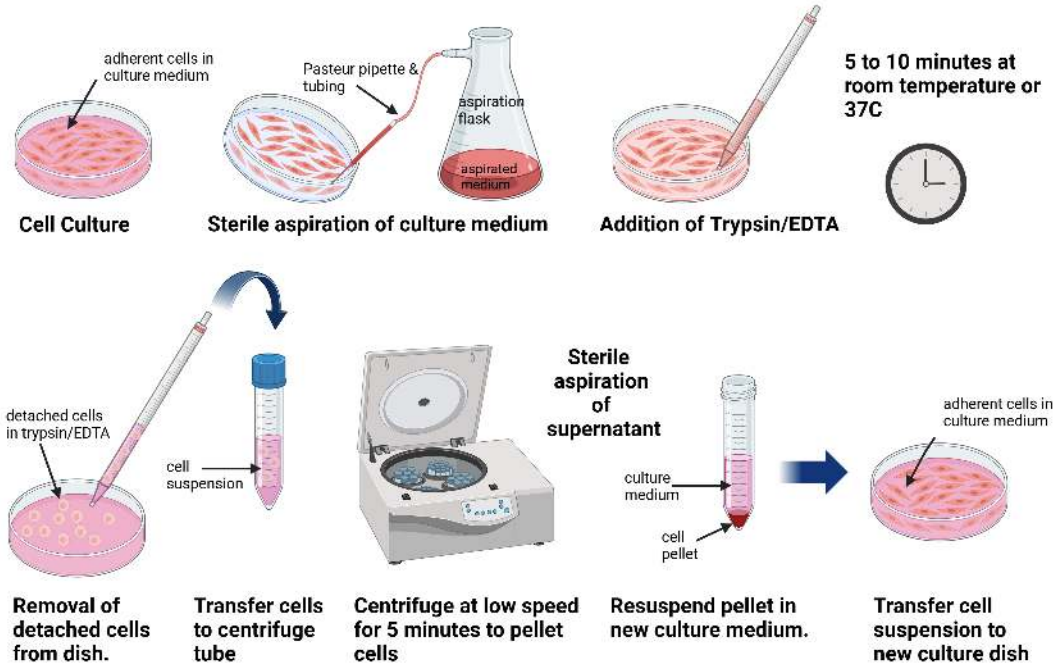


Figure **13.13.1**: Harvesting and sub-culturing of adherent cells. Culture medium is aspirated from the culture dish using a Pasteur pipette connected to a vacuum-driven aspiration flask. A volume of trypsin/EDTA is pipetted onto the cells and incubated for 5 to 10 minutes at room temperature (or 37°C). The cells are then collected by pipetting up and down several times and the cell suspension transferred to a centrifuge tube. The cells are pelleted through centrifugation for 5 minutes at low speed. The supernatant is aspirated (not shown) and the pellet is re-suspended in a volume of new culture medium. The cell suspension is transferred to a new culture dish. The cell suspension can also be "split" across several new dishes to increase the number of cultured cells (not shown). (Cell Culture by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Counting cells

The protocol below outlines the steps involved in counting cells using a manual hemacytometer (Figure **13.13.2**). Alternatively, the cells may be counted using an automated counting system according to the manufacturer's instructions. For more information about cell counting, refer to Chapter 6.3 Cell Culture Equipment.

Lab Protocol: Cell Counting wiht a Hemacytometer

1. Harvest the cells using steps 1 through 14 of the Harvesting & Sub-culturing protocol above.
2. Re-suspend the pellet in 1.0 mL of culture medium to produce a cell suspension.
    - a larger pellet may require a larger volume of culture medium in order to prevent a cell suspension that is too concentrated.
3. Using a sterile micropipette and pipette tip, remove 10 uL of the cell suspension and transfer to a sterile microfuge tube.
    - If Trypan blue exclusion staining is desired, add an equal volume (i.e., 10 uL) of 0.4% Trypan Blue to these cells.
4. The following steps can be performed outside of the BSC:
    1. Pipette 10 uL of the cell suspension (with or without Trypan Blue) onto the edge of the hemacytometer coverslip and allow the liquid to be drawn into the counting chamber through capillary action.
    2. Place the hemacytometer onto the stage of an inverted microscope and focus onto the cells in the counting grid.
        - If the cells are too numerous, dilution will be necessary.
        - To dilute:
            1. Pipette 1.0 uL of the cell suspension into a new microfuge tube and add 9 uL of 1X PBS for a 10-fold dilution.
            2. Pipette this diluted cell suspension onto the hemacytometer.
    3. Count the number of cells in the four 'corner' grids and the center grid
        - If using Trypan Blue, count the unstained cells that have excluded the Trypan Blue stain (i.e., clear and bright).
    4. Calculate the average number of cells.
    5. Multiply the average number of cells by 10,000 to obtain the number of cells per mL cell suspension
        - If the cells were stained with Trypan Blue, multiply the average number of cells by 2 (i.e., the total dilution factor) and then by 10,000 to obtain the number of cells per mL cell suspension
        - Additional dilutions will require multiplying the cell concentration by the dilution factor(s)

The formula for **cell concentration (# cells/mL) is (the average number of cells per square) X (10,000) X (any dilution factor(s))**

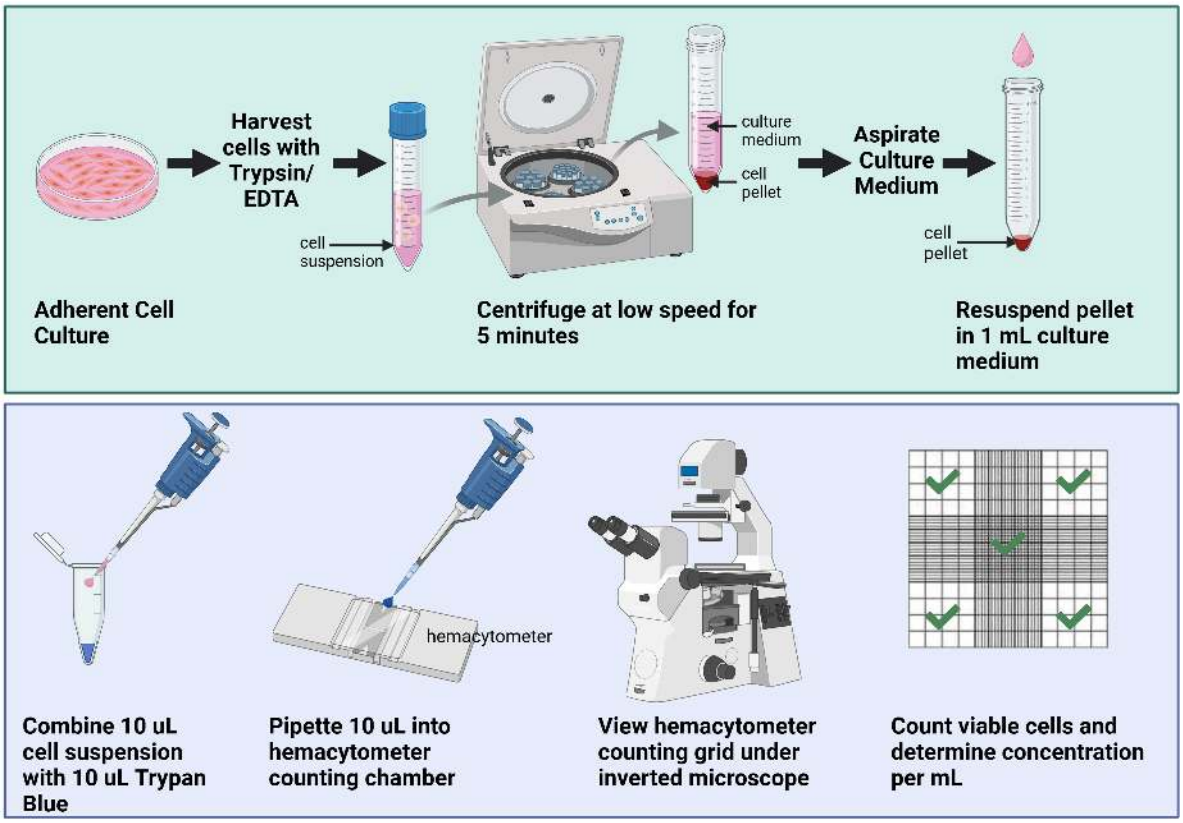


Figure **13.13.2**: Cell counting with a hemacytometer. Adherent cells are harvested using trypsin/EDTA and centrifuged at low speed into a cell pellet. The cell pellet is re-suspended in 1 mL of culture medium. To count viable cells, a small aliquot of cell suspension (e.g. 10 uL) is mixed with an equal volume of 0.4% Trypan Blue. This is considered a 2-fold dilution of the cell suspension.The hemacytometer is loaded with 10 uL of the mixture through capillary action. The hemacytometer counting grid is viewed under an inverted microscope. The unstained cells are counted in the four outside corner grids and the center grid (green checkmarks) and the average number of cells determined. The concentration of the cell suspension (i.e., number of cells per mL) is determined by multiplying the average number of cells by 10,000 and any dilution factor(s) applied (e.g., two-fold). The steps shown at the top box should be performed aseptically in the biosafety cabinet. The steps in the bottom box can be performed outside of the biosafety cabinet. (Cell Counting by Patricia Zuk; figure created in BioRender. Zuk, P. (2025))

Cryostorage of Cultured Cells

The protocol given below outlines how harvested cells can be placed into cryostorage for the long-term using liquid nitrogen (Figure **13.13.3**). In this protocol, the cell pellet is re-suspended in a **cryostorage medium** that prevents the formation of ice crystals within the cells during the freezing process. As the cells thaw, these crystals can expand and kill the cells. Most cryopreservation media use **DMSO (dimethylsulfoxide)** to prevent ice crystal formation. Cryoprotectants, like DMSO, lower the melting point of ice and reduce the amount of ice that forms within cells at any given temperature. New versions of cryopreservation media use compounds other than DMSO (e.g. glycerol) to prevent ice crystal formation. Cryopreservation medium can also contain a serum like FBS. Cryostorage is done using **cryostorage vials** that are designed to ensure the safe and long-term preservation of biological samples by minimizing contamination, leakage, and damage during freezing and thawing. Finally, successful cryostorage requires a slow and controlled "step-down" of temperature from room temperature to at least -80°C before the cells can be placed under liquid nitrogen. Controlled rate cooling chambers (e.g. "Mr Frosty) containing isopropanol are used for this purpose.

Lab Protocol: Crystoring Cells

1. Harvest the cells using steps 1 through 14 of the Harvesting & Sub-culturing protocol above.
2. Resuspend the cell pellet in an appropriate volume of Cryostorage Medium to give a cell concentration of 1-10 x 10^6 cells/mL.
3. Aliquot this cryostorage cell suspension into cryostorage vials.
4. Label the vials with the cell type, the passage number, and the date of cryopreservation.
5. Place the vials into a Mr. Frosty container filled with isopropanol.
6. Place the container at -80°C for at least 12 hours.

7. Transfer the vials to liquid nitrogen.

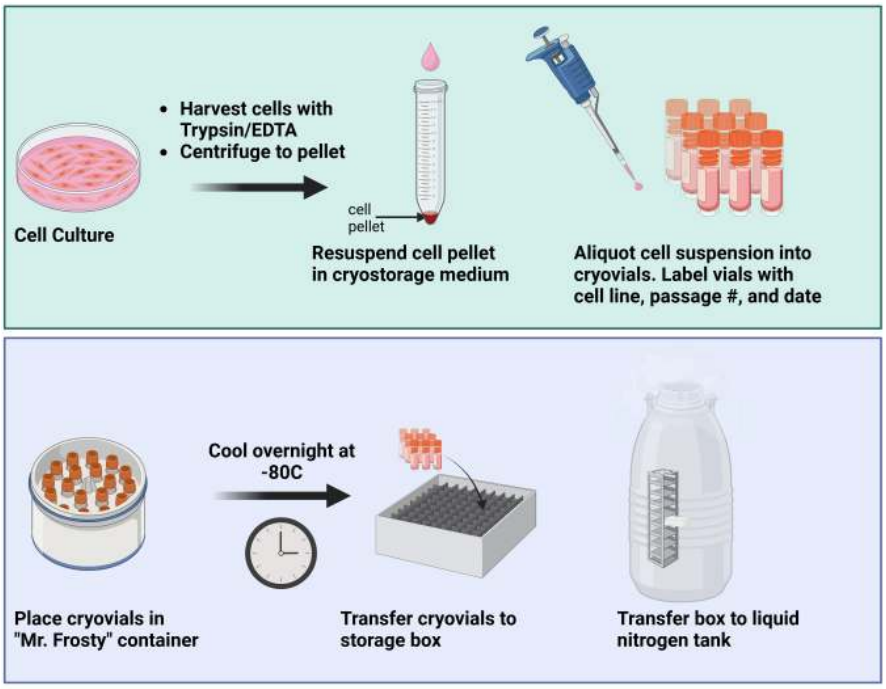


Figure **13.13.3**: Cryostorage of cultured cells. Cell cultures are harvested with trypsin/EDTA and pelleted through centrifugation. The cell pellet is re-suspended in a cryostorage medium and aliquoted into cryovials. The cryovials are labeled appropriately and placed into a "Mr. Frosty" container filled with isopropanol for controlled rate cooling overnight at -80°C. The vials are transferred into cryostorage boxes and placed into liquid nitrogen storage. The steps shown at the top box should be performed aseptically in the biosafety cabinet. The steps in the bottom box can be performed outside of the biosafety cabinet. (Cryostorage by Patricia Zuk; figure created in BioRender. Zuk, P. (2025))

Introduction of Cryostored Cells to Culture

Successful introduction of cells into culture requires the complete removal of the cryostorage medium, as DMSO can cause some cell types to differentiate.

Protocol: Thawing Cryostored Cells

1. Warm all culture reagents in a 37°C water bath.
2. Remove the cryostorage vial from liquid nitrogen and slowly thaw in a 37°C water bath. Ensure the contents are completely thawed.
3. Wipe all reagent bottles, culture supplies and cryostorage vials with 70% ethanol before placing in the BSC.
4. Using a micropipette, transfer the contents of the vial to a 15 mL conical tube containing 5 mL of pre-warmed medium.
5. Centrifuge at low speed (e.g. 200xg) for 5 minutes to collect the cells.
6. Aspirate the supernatant and resuspend the pellet in the appropriate amount of culture medium.
    - the amount of culture medium will depend on the type and size of the culture dish being used.
7. Transfer the cell suspension to a culture dish and label the dish accordingly.
8. Place the culture in the incubator.

Cell Culture Reagents

0.4% Trypan Blue

- dissolve 400 mg Trypan Blue in 100 mL phospho-buffered saline (PBS), pH 7.2 to 7.4
- filter to remove particulates
- store at room temperature

Cryostorage Medium

- Combine the following in a sterile container:
  - 10% DMSO
  - 90% sterile FBS
- store at -20°C

Alternate Cryostorage Medium

- Combine the following in a sterile container:
  - 70% sterile basal medium such as DMEM
  - 20% sterile FBS
  - 10% DMSO
- store at -20°C

13.13: Lab Technique - Cell Culture is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

13.14: Lab Technique - Light Microscopy

The Light Microscope

The microscope is the single most important tool in biology, extending our ability to view both very small organisms and the internal structure of cells. The **compound light microscope** operates by passing visible light through a very thin, typically stained specimen to enhance contrast. It uses two sets of lenses, an objective and an ocular lenses, to focus and magnify the image. **The objective lens** usually comes in different powers which could be switched from one to another (4X very lower power or scanning lens, 10X low power, 40X high power dry, 100X high power oil). **The ocular lens** usually has a power of 10X. A light microscope has many advantages: it is easy to use, it is relatively cheap, and allows to visualize live specimen. However, it has its limitations. Its magnification range only extends from 10X to 1000X, with a resolving power of the order of 0.2 µm. This means it does not permit to view smaller objects such as ribosomes or viruses or details of a structure. In this case, other types of microscopes could be used such as an electron microscope. To learn more about microscopy, go to Chapter 1.4: Advanced Biotechnology Equipment.

Properties of Microscopy

- **Magnification or Magnifying power:** indicates how many times (X) larger an object appears to its actual size
  - expressed as **total magnification**
  - total magnification is calculated as the combined power of the ocular lens and the objective lens (i.e., ocular lens power x objective lens power)
  - e.g. with an ocular of 10X and the 4X objective lens, the total magnifying power or magnification is 40X (4 x 10)
- **Resolution or Resolving power:** the ability of a microscope to distinguish small objects
  - indicates the sharpness of the image
  - given as a value representing the smallest distance between two points at which they are still distinguishable from each other
  - the higher the resolution, the smaller the resolution distance
  - 0.2 um apart for a compound light microscope
- **Depth of Focus:** the distance or depth that is in focus at any one time (Figure **13.14.1**)
  - sometimes also called "depth of field"
  - objects that are closer or farther away from the lens will go out of focus
  - indicates how far the stage can be moved and still keep the object in focus
  - the higher the total magnification, the thinner the depth of focus
- **Field of View (FOV):** the illuminated circular area visible under a microscope (Figure **13.14.1**)
  - the FOV diameter represents the distance from one edge of the circle to the opposite edge
  - the greater the magnification, the smaller the diameter of the field of view

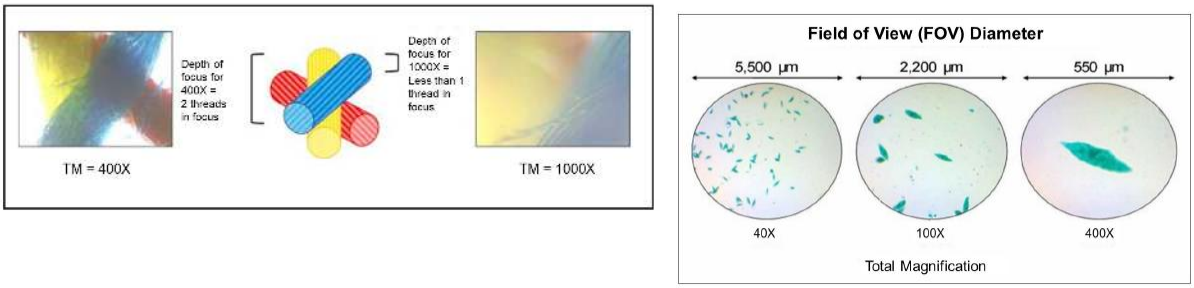


Figure **13.14.1**: Depth of Focus (DOF) and Field of View (FOV) at different magnifications. Right image: the DOF is dependent upon the total magnification being used. Under 400X total magnification (TM = 400X), only two of the three threads are in focus. Under 1000X total magnificant (TM = 1000X) less than one thread is in focus. (Depth of Focus by Kareen Martin, CC BY 4.0)
Left image: Single celled organisms called Euglena, viewed under a total magnification of 40X, with a FOV diameter of 5,500 microns (left), viewed under 100X, with a FOV diameter of 2,200 microns (middle) and viewed under 400X, with a FOV diameter of 550 microns (right). (Diameter of the field of view by Kareen Martin, CC BY 4.0)

Light Microscopy Preparations

Light microscopy uses two basic types of slide preparations: **prepared slides** and **wet mounts.**

Prepared Slides

Prepared slides contain organisms, cells, or thin sections of tissues that have been fixed and stained with special dyes (or antibodies) to highlight specific structures. These slides can be prepared in the laboratory prior to viewing or purchased from a manufacturer. Prepared slides that are purchased from companies are typically labeled with the following information:

- **specimen name:** identifies what is on the slide
  - can contain the species name and/or the common name of the specimen
  - e.g. onion root tip, *Euglena sp.*
- **stain or dye used:** identifies the stain or dye used in the preparation (if any)
  - specific stains are used to highlight specific structures
  - common stains:
    - **Hematoxylin and Eosin** or **H&E:** hematoxylin (blue/purple stain for nuclei), eosin (pink stain for positively-charged structures like the cytoplasm)
    - **Methylene Blue:** blue stain for nuclei
    - **Iodine:** blue/black stain for the cytoplasm
    - **Crystal violet:** purple stain for Gram-positive bacteria
    - **Safranin:** red/pink stain for Gram-negative bacteria
- **preparation type:** explains how the specimen was prepared (Figure **13.14.2**)
  - **smear** - the specimen is spread thinly across a slide to allow for viewing of individual cells
  - **squash** - the specimen is pressed (i.e., squashed) between the slide and coverslip to spread out the specimen for easier viewing
  - **whole mount ('w.m') -** the entire organism or structure
  - **section** - a slice of the organism or tissue that has been cut
    - '**x.s**' or '**c.s**' for cross section

Wet Mounts

Wet mount are prepared by placing the sample in a drop of liquid onto a clean microscope slide. The liquid can be water, isotonic saline or another type of fluid appropriate for the organism or tissue. A coverslip is then placed over the sample, creating a little "fluid sandwich". Air bubbles commonly become trapped under the plastic coverslip. These can be distinguished from the sample since they are usually perfect circles with very dark borders. Wet mounts are easy to prepare and allow for the observation of live tissues, cells, or organisms (Figure **13.14.2**).

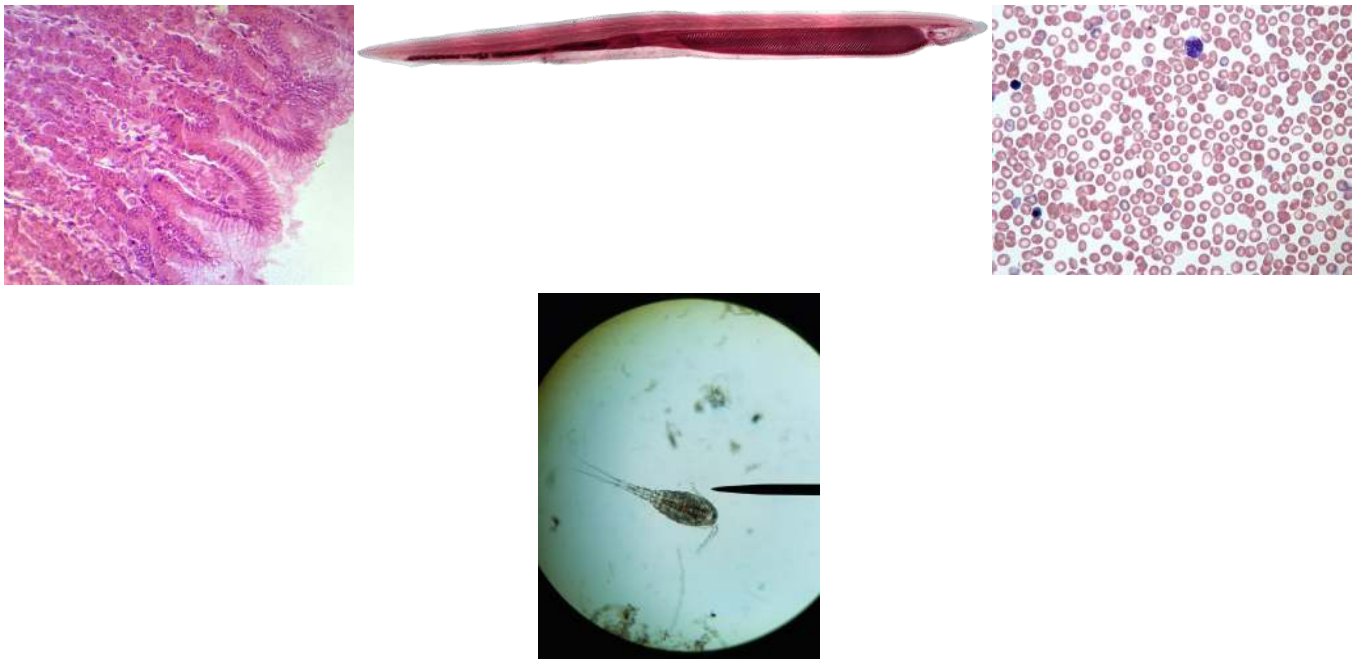


Figure **13.14.2**: Microscopy preparations. Top left image - a thin section of the stomach mucosa taken from a dog and stained with Hematoxylin and Eosin. (H&E dog stomach by Kareen Martin, CC BY 4.0)
Top middle image - a whole mount preparation of an amphioxus (Branchiostoma by Thomas Gyselinck, CC0 1.0)
Top right image - a blood smear (Neonate Blood Smear by Keith Chambers, CC BY-SA 3.0)
Bottom image - a wet mount of a microscopic fresh water crustacean, called a cyclop. (Cyclop by Kareen Martin, CC BY 4.0)

Microscope Maintenance

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Since lenses are carefully designed and manufactured to refract light with a high degree of precision, even a slightly dirty or scratched lens will refract light in unintended ways, degrading the image of the specimen. In addition, microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces. Among other things, proper care of a microscope includes the following:

- cleaning the lenses with lens paper
- not allowing lenses to contact the slide (e.g., by rapidly changing the focus)
- protecting the bulb (if there is one) from breakage
- not pushing an objective into a slide
- not using the coarse focusing knob when using the 40X or greater objective lenses
- only using immersion oil with a specialized oil objective, usually the 100X objective
- cleaning oil from immersion lenses after using the microscope
- cleaning any oil accidentally transferred from other lenses
- covering the microscope or placing it in a cabinet when not in use

Light Microscopy Protocols

The protocols below are to be used with a compound light microscope. Refer to Chapter 1.4: Advanced Biotechnology Equipment to learn the major components of the compound light microscope.

Basic Protocol:

1. Place the microscope on a flat, clean surface
2. Plug the microscope in and turn on the light course
3. Select the lowest magnifying lens (i.e., 4X or 5X) by rotating the revolving nosepiece
4. Place the slide on the stage and secure it with the stage clips
5. Use the mechanical stage knobs to center the specimen in the slide over the light source

6. While looking through the eyepiece (i.e., the ocular lens), use the coarse focus knob to lift the stage to bring the object into the field of view
    - adjust the light level if needed by using the diaphragm (i.e., the iris)
7. Use the fine focus knob to sharpen the focus
8. Rotate the objective lens nosepiece to change to a higher magnification lens
9. Re-center and re-focus on the specimen
    - higher magnifications will require increasing light levels

Wet Mount Protocol:

1. Place a drop of liquid (e.g., water, saline) on a clean microscope slide using an eyedropper
2. Place the specimen in the drop of liquid
    - alternatively, the specimen may be placed onto the slide first and then the drop of liquid added
3. Place one edge of a coverslip on the microscope slide so that it touches the drop and slowly lower the cover glass onto the slide

Smear Protocol:

1. Place a small drop of the sample on a clean microscope slide
2. Spread the sample evenly across the slide using the edge of another slide (i.e., the spreader slide)
3. Air-dry the smear completely
4. Fix the sample to preserve and kill cells
5. Stain using an appropriate dye
6. Rinse, dry, and view under the microscope

Squash Protocol:

1. Place a small tissue piece on a clean microscope slide
2. Add a drop of stain (if desired) or water
3. Cover with a coverslip
4. Gently press down (or tap) the coverslip with a blunt object to squash the tissue
5. Observe under the microscope

Oil Immersion

The protocol below decribes how to use **immersion oil,** in combination with a compound light microscope, to increase the resolving power of an objective lens. The **oil immersion lens,** also called the 100X oil lens, is often used when viewing very small objects, such as bacteria. Because the end of this high power lens has such a small opening, a drop of optically clear immersion oil is dropped onto the surface of the slide and the immersion lens is lowered into it. The immersion oil reduces light refraction, allowing more light rays to enter the lens, resulting in a sharper and clearer image. To read more about oil immersion, go to Chapter 1.4: Advanced Biotechnology Equipment.

Oil Immersion Protocol:

1. Attempt to focus on the object using the highest power “air” medium lens (i.e. the 40X lens).
2. Lower the stage slowly and carefully.
    - Do not move the stage side to side or front and back or the object will move in the field of view
    - If this is the case, return to the 40X lens and find and focus on the object again
3. With the stage in a lowered position, place a small drop of immersion oil on the area of the slide where the specimen is located.
4. Put the 100X oil immersion lens into place.
5. Slowly raise the stage until the bottom of the lens touches the oil. The moment the lens touches the oil, stop moving the stage.
6. View the specimen object. Use the fine focus to sharpen the focus if needed.
7. When done, spray some lens paper with a lens cleaner solution and gently dab it onto the bottom of the oil immersion lens to remove the oil. To prevent scratching the lens, use only lens paper and do not rub the lens.
8. Clean the prepared slide with dilute soap and water.

Indirect Immunofluorescence

The protocol and workflow outlined below describe how to perform indirect immunofluorescence on cells or tissue sections using a fluorescent microscope (Figure **13.14.4**). Cells can be cultured in a "chamber slide" (e.g., Nunc Lab-Tek Chamber Slides) made up individual chambers sealed off from one another. Because of this, cells in each chamber can be cultured under different conditions prior to analysis. In addition, each chamber can be incubated with different antibodies in order to detect different proteins. For tissue sections, the sample should be sectioned onto a glass microscope slide. For both cells and tissues, the sample is fixed using paraformaldehyde. However, ice-cold methanol (at -20°C) can be used as an alternative fixative. The sample is then incubated with a primary antibody, followed by incubation with a secondary antibody that is coupled to a fluorophore such as FITC, RITC, Alexa Fluor. The appropriate dilution of both primary and secondary antibody should be empirically determined. The appropriate filter, corresponding to the secondary antibody's fluorophore, must be used when imaging. A duplicate sample incubated only with secondary antibody should also be processed in order to determine the level of non-specific binding by the secondary antibody. To minimize non-specific binding, antibodies should be diluted in a blocking buffer containing BSA and serum.

Immunofluorescence Protocol:

1. Fix the sample (i.e., cells or tissue section) for 10–15 min at room temperature in Fixative Solution.
2. Wash the sample 3 times for 5 minutes each with Wash Buffer at room temperature.
3. If detecting intracellular targets, permeabilize with Permeabilization Solution for 5–10 min at room temperature and then wash 3 times for 5 minutes each with Wash Buffer
    - if detecting cell surface targets, skip this step.
4. Incubate the sample in Blocking Buffer for 30–60 min at room temperature to reduce non-specific binding.
5. Incubate the sample in Blocking Buffer containing the diluted primary antibody for 1–2 hours at room temperature
    - incubation can be done overnight at 4°C in a humidified chamber (to prevent buffer evaporation).
6. Wash 3 times for 5 minutes each with Wash Buffer at room temperature.
7. Incubate the sample in Blocking Buffer containing the diluted secondary antibody for 1–2 hours at room temperature in the dark.
8. Wash the sample 3 times for 5 minutes each with Wash Buffer at room temperature in the dark.
    - during the final wash, a nuclear stain like DAPI can be added to counterstain the cells.
9. If using a chamber slide, carefully remove the upper chamber and the gasket to yield the glass slide.
10. Mount a coverslip onto the glass slide.
    - mounting can be done using a commercial mounting medium.
    - if using a mounting medium containing a counterstain like DAPI (e.g. Vectashield), do not use DAPI in step #8.
11. Image the cells using a fluorescence microscope with the appropriate filters for the fluorophores used (e.g., FITC, RITC, Alexa Fluor, DAPI).

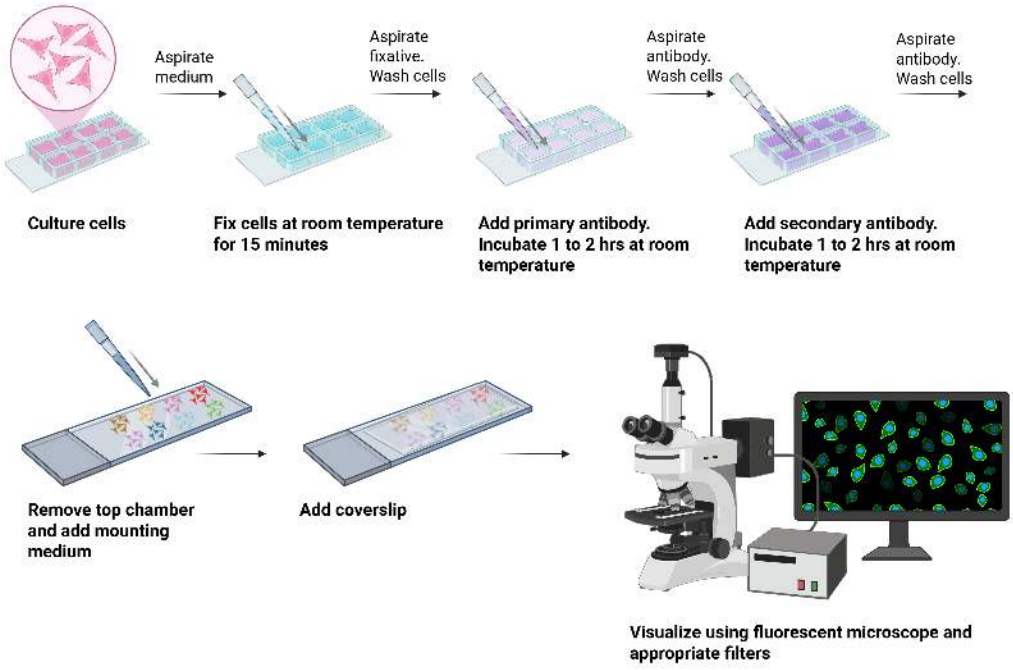


Figure **13.14.4**: Immunofluorescence workflow for cells. Prior to analysis, cells can be cultured in chamber slides. To process the cells, the media is removed and a fixative added for 15 minutes at room temperature. The fixative is aspirated and each chamber is washed multiple times. A primary antibody is added for 1 to 2 hrs at room temperature, followed by aspiration and washing steps. A secondary antibody is then added for 1 to 2 hrs and then removed. The cells are washed a final time before the upper chamber is carefully removed from the glass slide. A mounting medium and a coverslip is added to complete the process. The slide is visualized using a fluorescent microscope and the appropriate filter. (Immunofluorescence by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Immunofluorescence Reagents

Fixative Solution

- combine the following:
  - 250 µL of 16% paraformaldehyde
  - 200 µL of 0.5M sodium phosphate buffer (pH 7.0) or 1X PBS
  - 550 µL water
- prepare fresh before use
- final concentration:
  - 4% paraformaldehyde
  - 0.1M sodium phosphate buffer (pH 7.0)

0.5M Sodium Phosphate Buffer (pH 7.0)

- for 10 mL, add:
  - 6.1 mL of 0.5M Na_2HPO_4
  - 3.9 mL of 0.5M NaH_2PO_4
- store at room temperature

10X PBS

- dissolve the following in 800 mL water:
  - 80 g NaCl
  - 2 g KCl
  - 11.5 g Na_2HPO_4

- 2 g KH_2PO_4
- adjust volume to 1 L with water
- store at room temperature

Permeabilization Solution

- to 40 mL, add:
  - 5 mL 10X PBS
  - 50 uL Triton X-100
  - adjust volume to 50 mL with water
- store at room temperature
- final concentration:
  - 0.1% Triton X-100
  - 1X PBS

Wash Buffer

- dissolve the following in 40 mL water
  - 5 mL 10X PBS
  - 0.375 g glycine
- add water to 50 mL
- store at room temperature
- final concentration:
  - 0.1M glycine
  - 1X PBS

Blocking Buffer

- add the following to 40 mL water:
  - 5.0 mL fetal bovine serum (FBS)
  - 1.0 mL bovine serum albumin (BSA)
  - 5.0 mL of 10X PBS
  - 50 uL Triton X-100
  - adjust volume to 50 mL with water
- store at 4°C
- final concentration:
  - 10% FBS
  - 2% BSA
  - 0.1% Triton X-100
  - 1X PBS

13.14: Lab Technique - Light Microscopy is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

- **2.3: Instruments of Microscopy** by OpenStax is licensed CC BY 4.0. Original source: https://openstax.org/details/books/microbiology.

13.15: Bacterial Identification Methods

Bacterial Smear Preparation

Cells, such as bacteria, are colorless (and small) which means they are difficult to observe microscopically. The cytoplasm is essentially transparent and specimens need to contrast with the background of the field. It is then necessary to use a **stain** (i.e., a dye) to be able to visualize them. The staining methods outlined in this page are used to observe and study the size, morphology, arrangement, and cellular structures of bacteria. The quality of the staining depends on the preparation of a suitable smear. Its thickness or concentration can affect the stain, cells should also adhere to the slide to resist one or more washings.

A **bacterial smear**, also known as an **emulsion**, is a thin layer of bacteria spread on the surface of a clean microscope slide. Proper smear preparation is essential to ensure effective staining and accurate observation of bacterial cells. The *thickness of the smear* is a critical factor. A smear that is too thick contains a high concentration of cells, which reduces light transmission through the specimen and makes individual cells difficult to observe. Excessive thickness can also interfere with proper staining by trapping the stain, potentially leading to false-positive results. A thin bacterial smear does not have enough cells making it difficult to find cells, the staining might not be clear and the cells too interspersed to assess their arrangement. The bacterial load in a smear depends on the source of the sample. In liquid media, bacteria are suspended throughout the broth, typically resulting in a more diluted sample. In contrast, bacteria grown on solid media form dense colonies on the agar surface, leading to a higher concentration of cells.

Another factor to consider when preparing a smear is **adherence**. Adherence of the cells to the slide is critical, as they must withstand the application of stains and the wash steps. Before fixing it is essential to air-dry the smear. This allows excess moisture to evaporate, preparing the sample for proper heat-fixation. Doing so will prevent the sample from boiling when passed over the heat, which results in the breakage of the cells. Once air-dried, the slide is quickly passed over the flame 3 to 4 times, without exposing the cells directly to the flame. Heat-fixation not only firmly attaches the cells but it also kills the cells, making them safer to handle. It also prevents **autolysis**, the self digestion of cells, by denaturing enzymes. Finally, heat-fixation coagulates cytoplasmic proteins, improving cell visibility.

The two protocols below outline how to prepare a smear from a liquid broth and from a solid medium. In addition, Figure **13.15.1** illustrates the major steps of preparing a bacterial smear.

Lab Protocol: Smear Preparation from a Broth

1. Obtain a clean microscope slide and mark an area in the center of the slide with a grease/wax pencil.
2. Open the tube containing the inoculated liquid broth and pass the mouth through the flame several times.
3. Sterilize an inoculating loop by passing it several times through the flame. Allow it to cool down.
4. Aseptically collect a loopful of broth and deposit it into the marked area on the slide. Carefully disperse the broth in this area using the loop.
5. Repeat step 4 two more times.
6. Air dry the slide. Do not use the flame to dry the smear.
7. Once dry, heat-fix the slide by running it through the flame back and forth 2 times.
8. Allow the slide to cool.

Lab Protocol: Smear Preparation from Agar

1. Obtain a clean microscope slide and mark an area in the center of the slide with a grease/wax pencil.
2. Sterilize an inoculating loop by passing it several times through the flame. Allow it to cool down.
3. Aseptically transfer 3 to 4 loopful of water into the marked area on the slide.
4. Sterilize the inoculating loop by passing it several times through the flame. Allow it to cool down.
5. Aseptically lift a bacterial colony from the plate with the inoculation loop.
6. Disperse the specimen in the water drop on the glass slide.
7. Air dry the slide. Do not use the flame to dry the smear.
8. Once dry, heat-fix the slide by running it through the flame back and forth 2 times.
9. Allow the slide to cool.

Common Staining Methods

There are several different stains that can be used to stain bacterial smears. Some of the most common are:

- **Methylene blue (simple stain)**
- **Gram staining (differential stain)**
- **Acid Fast staining (differential stain)**
- **Endospore staining (differential stain)**

Lab Protocol: Simple Staining with Methylene Blue

The protocol below outlines how to stain bacterial cells with **methylene blue**, a positively-charged dye that binds to the negatively charged molecules of a cell, causing them to stain blue. This protocol is an example of a **simple staining method** (Figure **13.15.1**, in which only a primary stain is used.

1. Prepare a bacterial smear from a liquid broth or a colony.
2. Stain the smear with a 1% methylene blue solution for 1 minute.
3. Gently rinse the stain away with distilled water.
4. Place the slide between two sheets of bibulous paper (or paper towel) and carefully blot dry.
5. Observe the slide.

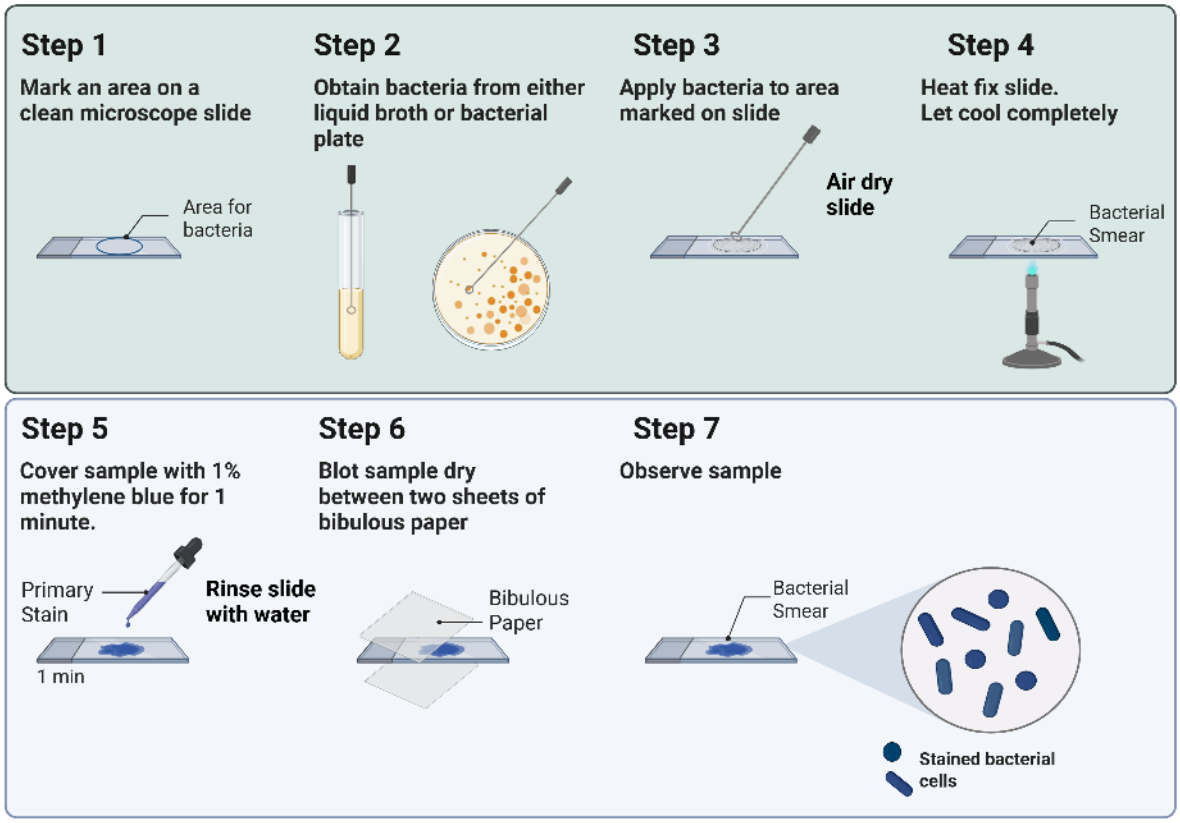


Figure **13.15.1**: Simple staining. Steps 1 to 4 (top box) show the production of a bacterial smear. Step 1: An area is outlined on a clean microscope slide with a wax pencil (outlined in blue). Step 2: A sample of bacterial broth culture, or bacterial colony, is obtained using a sterile inoculating loop. Step 3: The sample is applied to the area on the slide and then the smear is air dried. Steps 2 and 3 are repeated until sufficient bacteria is added to the slide. Step 4: The sample is quickly heat fixed and then allowed to cool completely before stained. Steps 5 through 7 (bottom box) show the simple staining procedure with methylene blue. Step 5: The primary stain (i.e., 1% methylene blue) is added to the area and allowed to sit on the cells for 1 minute. The dye is rinsed well with water. Step 6: The slide is gently dried by placing between two sheets of bibulous paper. Step 7: The stained bacterial cells (rods and circles) are observed under the microscope. (Simple Staining by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Methylene Blue Staining Reagents

1% Methylene Blue

- add 1.0 g methylene blue powder to 80 mL distilled water
- mix well
- adjust volume to 100 mL with distilled water
- store at room temperature

Gram Staining

Gram staining is an important staining method universally used in clinical microbiology. It differentiates between two main types of bacteria cells, Gram-positive or Gram-negative, so named based on whether they stain with the crystal violet component of the Gram stain. Gram staining is an example of a **differential staining method**, meaning it uses more than one dye. To learn more about Gram staining, go to Chapter 7.2: Microbial Identification.

Lab Protocol: Gram Staining

1. Prepare a bacterial smear from a liquid broth or a colony.
2. Stain the smear with a 2% crystal violet solution for 1 minute.
3. Gently rinse the stain away with distilled water.
4. Add the Gram's iodine stain to the slide and let sit for 1 minute.
5. Gently rinse the stain away with distilled water.
6. Decolorize the slide using 95% ethanol for ten to fifteen seconds.
    - alternate decolorizer solutions include acetone or an acetone:ethanol solution
7. Rinse the slide of decolorizer with distilled water.
8. Apply the 0.25% safranin O counterstain for 1 minute.
9. Gently rinse the stain away with distilled water.
10. Pat dry with bibulous paper.
11. Observe the slide.

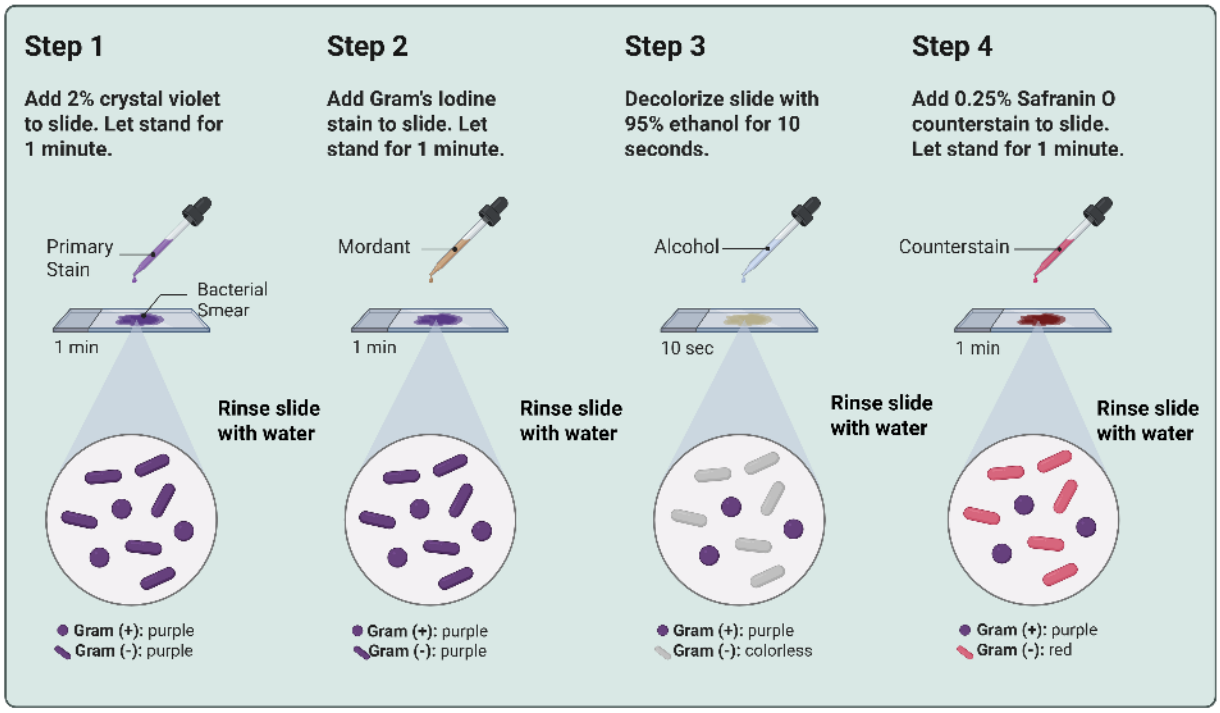


Figure **13.15.2**: Gram Staining. Step 1: A 2% crystal violet solution is added to a bacterial smear and allowed to stain the sample for 1 minute. The slide is rinsed of stain with distilled water. At the end of this step, both Gram positive (+) and Gram negative (-) cells will stain purple. Step 2: An iodine solution is added to the slide for 1 minute to act as a mordant. The slide is rinsed with distilled water. At the end of this step, both Gram positive (+) and Gram negative (-) cells be stained purple. Step 3: The sample is quickly decolorized of crystal violet stain for 10 to 15 seconds using 95% ethanol. The slide is rinsed with distilled water. After this step, only the Gram (+) cells will be stained purple (purple circles). Gram (-) cells will be decolorized and will be colorless (gray rods). Step 4: A 0.25% safranin O solution is added for 1 minute to counterstain the sample. The slide is rinsed of counterstain with distilled water. At the end of this step, the Gram positive (+) cells will be purple (purple circles) and the Gram negative (-) cells will be pink (pink rods). (Gram Staining by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Gram Staining Reagents

2% Crystal Violet

- dissolve 2 g of crystal violet in 20 mL of 95% ethanol
- add 80 mL of distilled water and mix well
- store at room temperature in a light-protected bottle

Gram's Iodine

- dissolve 2.0 g of KI (potassium iodide) powder in 100 mL of distilled water
- add 1.0 g of iodine crystals and mix until fully dissolved
- adjust volume to 300 mL with distilled water
- store at room temperature in a light-protected bottle

0.25% Safranin O

- dissolve 0.25 g of safranin O powder in 10 mL of 95% ethanol
- add 90 mL of distilled water and mix well
- store at room temperature in a light-protected bottle

Acetone-Ethanol Decolorizer

- combine 50 mL of acetone with 50 mL of 95% ethanol
- store at room temperature

The quality of the Gram stain depends on several key factors. One of the most critical is the age of the bacterial culture. Fresh cultures typically have intact, rigid cell walls that respond well to Gram staining, while older cultures may contain dying cells with

compromised cell wall integrity, leading to inconsistent or weak staining results. The quality of the smear itself also plays a significant role. Thick smears tend to retain more dye, and areas of high cell density may appear overly saturated with crystal violet, potentially obscuring the counterstain and leading to false interpretation. Another major variable is the decolorization step, which is often the most sensitive part of the process. Both over-decolorization and under-decolorization can occur easily, even within the recommended time window of a few seconds. The optimal duration of this step depends on the smear's thickness—dense smears typically require more time, while thinner smears need less. Optimal decolorizing time will require empirical determination and experience.

Acid-Fast Staining

An **acid-fast stain** is a differential staining technique that identifies organisms that have a waxy, lipid-rich cell wall (rich in mycolic acid) that is resistant to standard stains. Under treatment with acid-alcohol, the organisms will retain certain dyes. Acid-fast staining is typically used to stain Mycobacterium species like *M. tuberculosis*. Acid-fast staining can be performed using a "hot" method (i.e., the **Ziehl–Neelsen Method**) that uses heat to help the primary stain penetrate the cells, and a "cold" method (i.e., the **Kinyoun Method**) in which the heating step is eliminated but uses higher concentrations of the primary stain. To learn more about acid-fast staining, go to Chapter 7.2: Microbial Identification.

Lab Protocol: Ziehl–Neelsen Acid-Fast Staining

1. Prepare a bacterial smear from a liquid broth or a colony.
2. Cover the smear with Ziehl-Neeson carbol fuchsin stain.
3. Gently heat the slide until steam appears from the smear. Keep steaming the slide for 5 minutes.
    - Do not boil the bacterial smear or allow it to dry out
    - Reapply stain if the smear dries out
4. Let the slide cool for 1 minute.
5. Gently rinse the stain away with distilled water.
6. Apply the acid-alcohol stain until the runoff is clear (about 10 to 30 seconds)
7. Immediately rinse the slide with distilled water.
8. Cover the smear with 1% methylene blue for 1 minute.
9. Gently rinse the stain away with distilled water.
10. Pat dry with bibulous paper.
11. Observe the slide.

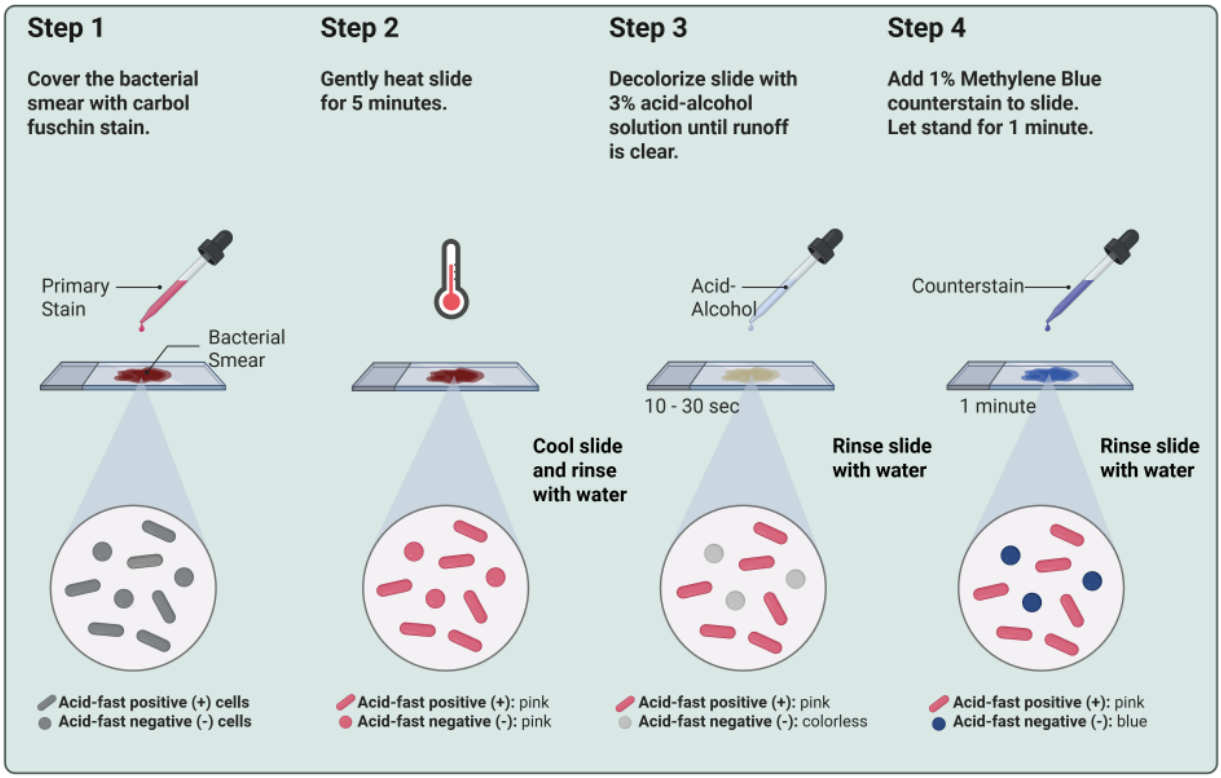


Figure **13.15.4**: Ziehl-Neeson Acid-Fast Staining. Step 1: A carbol fuschin solution is added to a bacterial smear. At the end of this step both acid-fast positive (+) and acid-fast negative (-) cells are stained pink. Step 2: The slide is gently heated for approximately 5 minutes. The slide is allowed to cool and rinsed with distilled water. At the end of this step, both acid-fast (+) and acid-fast (-) cells are stained pink. Step 3: The sample is quickly decolorized of crystal violet stain for 10 to 30 seconds with a 3% acid-alcohol solution. The slide is immediately rinsed with distilled water. After this step, only the acid-fast (+) cells will be stained pink (pink rods). Acid-fast (-) cells will be decolorized and will be colorless (gray circles). Step 4: A 1% methylene blue solution is added for 1 minute to counterstain the sample. The slide is rinsed of counterstain with distilled water. At the end of this step, the Acid-fast (+) cells will be pink (pink rods) and the acid-fast negative (-) cells will be blue (blue rods). (Ziehl-Neeson Staining by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Ziehl-Neeson Acid-Fast Staining Reagents

Ziehl-Neeson Carbol Fuschin

- dissolve 5.0 g of phenol crystals (i.e., carbolic acid) in 90 mL of warm distilled water
- dissolve 0.3 g of basic fuchsin power in 10 mL of 95% ethanol
- combine the two solutions and mix thoroughly.
- store in a dark, tightly sealed bottle at room temperature
- use caution - phenol is toxic and corrosive

3% Acid-Alcohol Decolorizer

- slowly add 3.0 mL of concentrated HCl to 97 mL of 95% ethanol and mix well
- store in a labeled, sealed bottle
- caution - solution is flammable and corrosive

1% Methylene Blue

- dissolve 1.0 g of methylene blue powder in 100 mL distilled water
- filter if necessary
- store at room temperature

Lab Protocol: Kinyoun Acid-Fast Staining

1. Prepare a bacterial smear from a liquid broth or a colony.
2. Cover the smear with Kinyoun carbol fuchsin stain and let stand for 5 to 10 minutes.
3. Gently rinse the stain away with distilled water.
4. Apply the acid-alcohol stain until the runoff is clear (about 10 to 30 seconds)
5. Immediately rinse the slide with distilled water.
6. Cover the smear with 1% methylene blue for 1 minute.
7. Gently rinse the stain away with distilled water.
8. Pat dry with bibulous paper.
9. Observe the slide.

Kinyoun Acid-Fast Staining Reagents

Kinyoun Carbol Fuschin

- dissolve 8.0 g of phenol crystals (i.e., carbolic acid) in 80 mL of warm distilled water
- dissolve 4.0 g of basic fuchsin power in 20 mL of 95% ethanol
- combine the two solutions and mix thoroughly.
- store in a dark, tightly sealed bottle at room temperature
- use caution - phenol is toxic and corrosive

Acid-Alcohol Decolorizer

- slowly add 3.0 mL of concentrated HCl to 97 mL of 95% ethanol and mix well
- store in a labeled, sealed bottle
- caution - decolorizer solution is flammable and corrosive

1% Methylene Blue

- dissolve 1.0 g of methylene blue powder in 100 mL distilled water
- filter if necessary
- store at room temperature

Endospore Staining

The **endospore stain** is a differential staining technique that detects bacterial endospores, which are highly resistant, dormant structures formed by some bacterial genera like *Bacillus* and *Clostridium*. The most common method is the **Schaeffer–Fulton method**. In this method, heat, in the form of steam, is used to help the primary stain, malachite green, penetrate the spore coat. Water is used to rinse the primary stain away and decolorize the specimen. This method will distinguish endospores from the vegetative cells of the bacterial specimen. To learn more about endospore staining, go to Chapter 7.2: Microbial Identification.

Lab Protocol: Schaeffer-Fulton Endospore Staining

1. Prepare a bacterial smear from a liquid broth or a colony.
2. Cover the smear with 5% malachite green.
3. Place the slide over boiling water or a steam bath and let stand for 5–10 minutes.
    - Keep the stain from drying out by adding more if needed.
4. Let the slide cool for 1–2 minutes.
5. Gently rinse the stain away with distilled water.
    - this step will also decolorize the sample
6. Cover the smear with safranin O for 30–60 seconds.
7. Gently rinse the stain away with distilled water.
8. Pat dry with bibulous paper.
9. Observe the slide.

Lab Protocol: Quick Endospore Staining

1. Prepare a bacterial smear from a liquid broth or a colony by heat-fixing 20 times over the flame
2. Cover the smear with 5% malachite green and let stand for 15 minutes.
   - Keep the stain from drying out by adding more if needed.
3. Gently rinse the stain away with distilled water.
   - this step will also decolorize the sample
4. Cover the smear with safranin O for 30–60 seconds.
5. Gently rinse the stain away with distilled water.
6. Pat dry with bibulous paper.
7. Observe the slide.

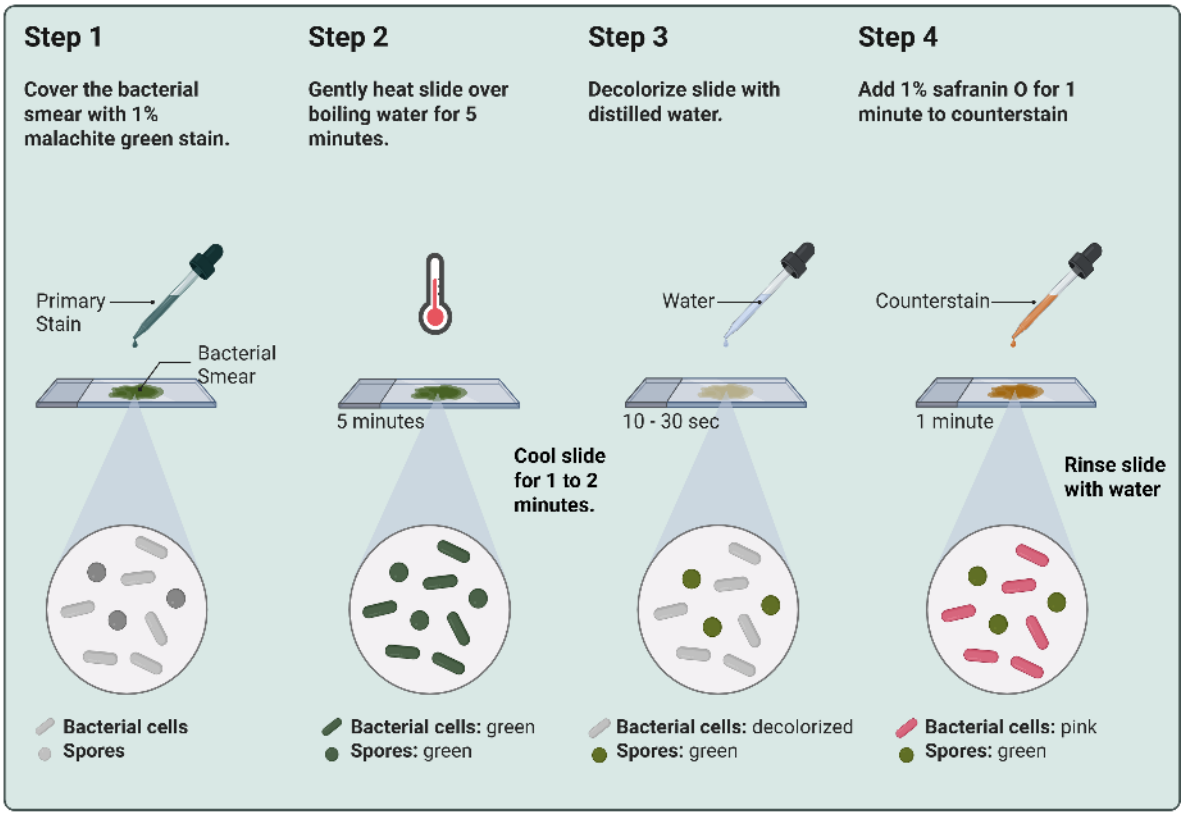


Figure **13.15.5**: Endospore staining. Step 1: A 1% malachite green solution is added to a bacterial smear. Step 2: The slide is gently heated over boiling water for 5 to 10 minutes and then allowed to cool for 1 to 2 minutes. At the end of this step, both the bacterial cells (rods) and spores (circles) are stained green. Step 3: The sample is quickly decolorized of the stain for 10 to 30 seconds with distilled water. After this step, only the spores will be stained green (green circles) and the bacterial cells will be colorless (grey rods). Step 4: A 1% safranin O solution is added for 1 minute to counterstain the sample. The slide is rinsed of counterstain with distilled water. At the end of this step, the bacterial cells will be pink (pink rods) and the spores will be green (green rods). (Spore Staining by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Endospore Staining Reagents

5% Malachite Green

- add 5.0 g malachite green (or malachite oxide) powder in 100 mL distilled water
- use gentle heat to fully dissolve the powder
- filter the solution if needed

- store at room temperature in a light-protected bottle

1% Safranin O

- dissolve 1.0 g of safranin O powder in 10 mL of 95% ethanol
- add 90 mL of distilled water and mix well
- store at room temperature in a light-protected bottle

Carbohydrate Fermentation Testing

A **carbohydrate fermentation test** (i.e., a fermentation test) is a type of biochemical test that is used to determine whether a bacteria (or yeast) can ferment specific sugars. Bacteria, or yeast, are grown in a Carbohydrate Broth containing nutrients, a specific sugar for fermentation, and a pH indicator. Common sugars used in this test include glucose, lactose, sucrose, maltose, mannitol, galactose, cellulose, and starch. To detect gas production, a small, thin glass tube, called a **Durham tube**, is inverted into the broth. The presence of air within the Durham tube at the end of the incubation period will be a positive indication of gas production. The use of a pH indicator will confirm the production of acids. The most common pH indicator used is Phenol Red which will turn the broth yellow with the production of acids. Other pH indicators include Andrade's indicator (colorless to pink) and Bromothymol Blue (blue to yellow). To learn more about carbohydrate testing, go to Chapter 7.2: Microbial Identification.

Lab Protocol: Carbohydrate Fermentation

1. Prepare a Carbohydrate Broth.
2. Aliquot 5 to 10 mL of broth into sterile test tubes.
3. Invert and submerge a sterile Durham tube into each test tube, ensuring that it fills completely and that there is no air in the Durham tube.
4. Loosely cap the tubes.
5. Autoclave to sterilize. Let cool.
6. Label each tube with the organism's name and type of sugar being used for fermentation.
7. Aseptically inoculate each tube with the test organism using a sterile loop.
8. Incubate the tubes at 37°C for 24 to 48 hours.
9. Observe and record results.

Fermentation Reagents

Carbohydrate Broth with 0.18% Phenol Red

- to 90 mL of distilled water, add the following
  - 1.0 g Peptone
  - 0.5 g NaCl
  - 1.8 mL of a 1% Phenol Red stock solution
  - 1.0 g of the specific carbohydrate to be tested
- mix until all components are dissolved (heat to boiling if required)
- adjust pH to 7.0
- adjust volume to 100 mL with distilled water
- autoclave at 121°C for 15 minutes to sterilize
- let cool
- store at 4°C

API Testing

In microbiology, API strips refers to the **Analytical Profile Index (API)**, a standardized identification system used to identify bacteria and yeast based on their biochemical activities. Each API strip is composed of multiple chambers, called **cupules**, that are specific to a metabolic function or enzyme. A small aliquot of cellular suspension is added to each chamber and then incubated. The results are then analyzed and recorded. Based on the results, the microbe can be identified and classified. To read more about API testing, go to Chapter 7.2: Microbial Identification.

Lab Protocol: API Test Strips

1. Select a bacterial colony and prepare a bacterial suspension in sterile saline or sterile distilled water.
    - a suspension of yeast cells can also be prepared
2. Fill each cupule of the test strip with the cell suspension using a Pasteur pipette.
    - anaerobic cupules should be covered with mineral oil
3. Place the strip in its incubation tray and add a few drops of water to maintain humidity.
4. Incubate at 37°C for 18–24 hours
    - incubation times will vary by test panel and organism being tested
5. Observe color changes in each well
    - some cupules will require the addition of another reagents
6. Record the pattern of positive/negative results.

13.15: Bacterial Identification Methods is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

13.16: Basics of Bacterial Culture

Bacterial Cultures

Bacterial culture is a fundamental technique in microbiology that allows the growth and study of microbial species under controlled laboratory conditions. This page outlines the physical and chemical conditions required for bacterial growth, media preparation methods, and standard protocols for handling and isolating bacteria.

Culture Conditions

Optimal bacterial growth depends on various environmental parameters, including temperature, pH, oxygen availability, and pressure. These conditions vary among bacterial species and must be controlled to ensure successful cultivation.

- **Culture Temperature:** varies depending on the organism's optimal growth range
  - bacteria can be classified, based on temperature preferences, into several categories:
    - **Psychrophiles** grow best at 0–20°C
    - **Mesophiles** prefer 20–45°C (most human pathogens thrive at 37°C)
    - **Thermophiles** grow optimally at 45–80°C
    - **Hyperthermophiles** can tolerate temperatures >80°C
- **Oxygen Levels**: can vary among bacteria, depending on their metabolic needs and their ability to tolerate or neutralize reactive oxygen species
  - bacteria can be classified, based on their oxygen preferences, into several categories:
    - **Obligate aerobes** require oxygen
    - **Obligate anaerobes** are inhibited or killed by oxygen
    - **Facultative anaerobes** grow with or without oxygen
    - **Microaerophiles** require low oxygen concentrations
    - **Aerotolerant anaerobes** do not use oxygen but tolerate its presence
- **pH:** optimal pH depends on bacterial species
  - most bacteria prefer a **neutral pH** (6.5–7.5)
  - **Acidophiles** thrive in acidic conditions (pH < 5.5)
  - **Alkaliphiles** grow best in basic conditions (pH > 8.5)
- **Pressure:**
  - **Barotolerant** organisms can withstand increased pressure
  - **Barophiles** (also called piezophiles) require high-pressure environments (e.g., deep-sea organisms)

Culture Media

The culture media and reagents used in cell culture are designed to provide chemical support of the cells under sterile conditions. More information can be found in Chapter 7.2 Microbial Identification. Commonly used media and reagents include:

- **liquid medium:**
  - **broth**
- **solid medium, including:**
  - **slant**
  - **deep** (also called a stab)
  - **plates**

Liquid Medium (Broth)

Most bacteria can be grown in a basic **nutrient broth** under standard laboratory conditions. Nutrient broth is an example of a **non-selective medium** that provides essential nutrients to a wide range of bacteria. However, nutrient broth can be modified based on the specific requirements of the experiment. For example, antibiotics can be added to this medium to turn it into a **selective medium**. To read more about non-selective and selective media, go to Chapter 7.2: Microbial Identification. A basic nutrient broth can be prepared from individual components (see below) or from commercially available nutrient broth powders that simply require the addition of distilled water to reconstitute.

The components of a basic nutrient broth are:

- **Peptone:** source of organic nitrogen and growth factors
  - prepared from partially digested protein (typically from casein, meat, or soy)
  - used to cultivate a wide range of bacteria that do not require special nutrients
  - contains short peptides, amino acids, and some carbohydrates
  - can also buffer the liquid medium against pH changes by neutralizing acids made during microbial growth
- **Beef extract:** source of vitamins, minerals, and growth factors
  - complements the nutrients provided by peptone
  - contains water-soluble vitamins, especially B-complex vitamins, required for bacterial metabolism and enzyme function
  - contains phosphates, potassium, magnesium, iron, and sulfur compounds vital for enzyme function
  - contains a small amount of carbohydrates, organic acids, and lipids
- **NaCl:** maintains osmotic balance during bacterial growth
  - also stabilizes cell membranes and supports the function of membrane transport systems

Nutrient Broth Recipe

- to 900 mL of distilled water, add the following to a 2 L glass flask:
  - 5.0 g Peptone
  - 3.0 g Beef extract
  - 5.0 g NaCl
- place a magnetic stir bar in the flask and swirl gently on a stir plate until the components are fully dissolved
- adjust the pH to 7.0 ± 0.2 (if specified)
- adjust the volume to 1.0 L using distilled water
- autoclave at 121°C for 15 minutes
- store at 4°C

- if adding antibiotics, cool to room temperature (or 4°C) before adding.

Another equally popular non-selective liquid medium used in the growth of a wide range of bacteria are **Luria-Bertani (LB) broth**. LB broth is especially popular when growing *E.coli* bacteria that have been transformed with DNA. The recipe given below is sometimes called LB Miller broth. Modifications of this broth include LB Lennox broth (5.0 g NaCl) and LB Low Salt broth (up to 5.0 g NaCl).

The components of LB broth are:

- **Tryptone:** source of nitrogen and carbon compounds
  - partially digested casein that is produced using the enzyme, trypsin
  - provides short peptides and free amino acids
- **Yeast extract:** source of vitamins, growth factors, and minerals
  - contains essential B-complex vitamins (e.g., niacin, riboflavin, thiamine, and folic acid) needed for enzymatic and metabolic functions
  - contains nucleic acids, NAD, and other co-enzymes needed for DNA/RNA synthesis and energy metabolism
  - contains magnesium, iron, and phosphorus
- **NaCl:** maintains osmotic balance during bacterial growth
  - also stabilizes cell membranes and supports the function of membrane transport systems

LB Broth Recipe

- to 900 mL of distilled water, add the following to a 2 L glass flask:
  - 10.0 g Tryptone
  - 5.0 g Yeast extract
  - 10.0 g NaCl
- place a magnetic stir bar in the flask and swirl gently on a stir plate until the components are fully dissolved
- adjust the pH to 7.0 ± 0.2 (if specified)
- adjust the volume to 1.0 L using distilled water
- autoclave at 121°C for 15 minutes
- store at 4°C
- if adding antibiotics, cool to room temperature (or 4°C) before adding.

Solid Medium (slants, stabs, and plates)

Solid medium is a medium that has had **agar** added to it, resulting in solidification to a soft gel-like solid upon reaching room temperature. The three basic types of solid media used for bacteria are the:

- **plate**
- **deep (or stab)**
- **slant**

The recipe below outlines the ingredients needed to prepare a basic **nutrient agar** for slants, deeps, and plates. This recipe is the liquid nutrient medium altered through the addition of **agar**. Similarly, liquid LB broth can be modified to LB agar medium through the addition of the same amount of agar as nutrient agar. Both nutrient and LB agar can be prepared using commercially available nutrient agar powder. These powders are easy to use as they contain all necessary components in pre-measured form. To read more about these types of solid media, go to Chapter 7.2: Microbial Identification.

Lab Protocol: Preparing Agar Plates

1. Prepare the liquid media using the recipe below.
2. Autoclave to sterilize.
3. Allow to cool to to 50 to 55°C.
   - If required, add antibiotics upon reaching this temperature range.
4. Using aseptic technique, pour ~20 mL of media into sterile Petri dishes. Be careful to avoid introducing bubbles.
5. Let cool under aseptic conditions with the lid slightly ajar until fully solidified.
6. To prevent condensation dripping onto the agar surface, store the plates upside down at 4°C.

Lab Protocol: Preparing Agar Slants and Deeps

1. Prepare liquid media containing agar following Lab Protocol: Preparing Agar Plates.
2. To prepare a deep (also called a stab), dispense 5 to 7 mL of medium into sterile test tubes, using aseptic technique, cover, and allow to cool.
3. To prepare slants, dispense 5 to 7 mL of medium into sterile test tubes, using aseptic technique and cover.
   - Place tubes in a slanted position to form a slanted surface.
   - Allow to cool.

As an alternative to steps 2 and 3, liquid medium can be poured into non-sterile test tubes and then autoclaved to sterilize. The tubes can be allowed to solidify into stabs and slants. However, these preparations cannot contain antibiotics (or any other heat-sensitive compound) as autoclaving will inactivate it.

Agar Recipes

Nutrient Agar

- to 900 mL of distilled water, add the following to a 2 L glass flask:
  - 5.0 g Peptone
  - 3.0 g Beef extract
  - 5.0 g NaCl
  - 15.0 g Agar
- place a magnetic stir bar in the flask and swirl gently on a stir plate until the components are fully dissolved
- adjust the pH to 7.0 ± 0.2 (if specified)
- adjust the volume to 1.0 L using distilled water
- autoclave at 121°C for 15 minutes
- if adding antibiotics, cool the medium to 50 to 55°C before adding the antibiotic (note: higher temperature will inactivate the antibiotic; lower temperatures may allow for solidification before antibiotic addition)

LB Agar

- to 900 mL of distilled water, add the following to a 2 L glass flask:
  - 5.0 g Peptone
  - 3.0 g Beef extract
  - 5.0 g NaCl
  - 15.0 g Agar
- place a magnetic stir bar in the flask and swirl gently on a stir plate until the components are fully dissolved
- adjust the pH to 7.0 ± 0.2 (if specified)
- adjust the volume to 1.0 L using distilled water
- autoclave at 121°C for 15 minutes
- if adding antibiotics, cool the medium to 50 to 55°C before adding the antibiotic (note: higher temperature will inactivate the antibiotic; lower temperatures may allow for solidification before antibiotic addition)

Inoculating Bacterial Media

The protocols given below outline how several types of bacterial cultures can be established through inoculation. In the first protocol illustrated in Figure **13.16.1**., a liquid bacterial culture is used inoculate a solid bacterial medium (e.g., a slant or stab). The next protocol shown in Figure **13.16.2** outlines how a liquid culture can be used to "streak" an agar plate. This streak plate technique progressively thins out the bacterial load across the agar surface until individual colonies are produced from single bacterial cells (Figure **13.16.3**). To do this, the surface of the agar plate should be imagined as being divided into 4 equal quadrants. The bacterial sample will be successively streaked through each quadrant. The last protocol illustrated in Figure **13.16.4** explains how a solid bacterial culture (e.g., a slant or a plate) can be used to establish a liquid bacterial culture. The amount of time these preparations are cultured and their temperature will depend on the bacterial species and the type of bacterial preparation made. For example, liquid cultures may require a culture time of only 16 to 18 hours until sufficient cell growth is achieved, while a solid agar plate might require 24 to 36 hours bacterial colonies of significant size to appear. Because liquid cultures are often incubated "overnight" (i.e., 16 to 18 hours), they are often called an "overnight" culture.

Lab Protocol: Slant or Stab Inoculation Using a Liquid Bacterial Culture

The protocol below uses a small volume of liquid bacterial culture prepared in a test tube as the starting material. However, a larger volume of bacterial culture prepared in a flask can also be used.

1. Sterilize the loop in the flame of a Bunsen burner until red-hot. Allow to cool completely.
2. Open the test tube containing the liquid culture and sterilize the mouth of the test tube by passing it over the flame.
   - do not place the cap of the test tube on the work surface, but hold it between the little finger and the palm of the hand.
3. Dip the loop into the liquid culture.
4. Sterilize the mouth of the tube by passing it back over the flame and close the tube. Put it aside.
5. Remove the cap from the test tube containing the slant (or deep) and sterilize the mouth of the tube by passing it over the flame.
   - do not place the cap of the test tube on the work surface, hold the cap between the little finger and the palm of the hand.
6. Gently streak the loop across the top of the slant several times to transfer the bacteria from the loop to the agar.
   - if inoculating a deep (i.e., a stab), insert the inoculation loop halfway down the total depth the solid medium and pull out.
7. Incubate the prepared culture under the appropriate culture conditions.

Figure **13.16.1**: Slant inoculation. Step 1: An inoculation loop is sterilized by flaming it in a Bunsen burner and then cooled. Step 2: The opening of a liquid bacterial culture sterilized in the flame of a Bunsen burner. Step 3: The cooled inoculation loop is inserted into the liquid culture. Step 4: The opening of the liquid culture is re-flamed, the culture tube closed, and set aside. Step 5: The opening of a slant culture tube is sterilized in the flame of a Bunsen burner. Step 6a: To prepare a slant, the inoculation loop is gently streaked over the surface of the slant several times. Step 6b: To prepare a stab, the inoculation loop is inserted halfway into the solid medium and then pulled out. The inoculation loop is re-flamed to sterilize (not shown). Step 7: The prepared culture is incubated under the appropriate conditions (e.g., 37°C for 24 hours). (Slant Inoculation by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

Lab Protocol: Streaking an Agar Plate Using a Liquid Bacterial Culture

The protocol below uses a small volume of liquid bacterial culture prepared in a test tube as the starting material. However, a larger volume of bacterial culture prepared in a flask can also be used.

1. Sterilize an inoculation loop in the flame of a Bunsen burner until red-hot. Allow to cool completely.
2. Open the test tube containing the liquid culture and sterilize the mouth of the test tube by passing it over the flame.
   - do not place the cap of the test tube on the work surface, but hold it between the little finger and the palm of the hand.
3. Dip the inoculation loop into the bacterial sample.
4. Open the lid of the agar plate and place it facing up on the work surface.
5. Quickly but gently streak the loop 5 or 6 times across "quadrant 1" of the agar surface.
6. Re-flame the loop and allow it to cool completely.
7. Drag the loop from "quadrant 1" into "quadrant 2" and gently streak the loop 5 or 6 times across "quadrant 2" of the agar surface being sure to avoid the streaks made in "quadrant 1".
8. Re-flame the loop and allow it to cool completely.
9. Drag the loop from "quadrant 2" into "quadrant 3" and gently streak the loop 5 or 6 times across "quadrant 3" of the agar surface being sure to avoid the streaks made in "quadrant 2".

10. Re-flame the loop and allow it to cool completely.
11. Drag the loop from "quadrant 3" into "quadrant 4" and gently streak the loop 5 or 6 times across "quadrant 4" of the agar surface being sure to avoid the streaks made in "quadrant 3".
12. Re-flame the loop and allow it to cool completely.
13. Close the lid on the agar plate, invert it and incubate it under the appropriate culture conditions.

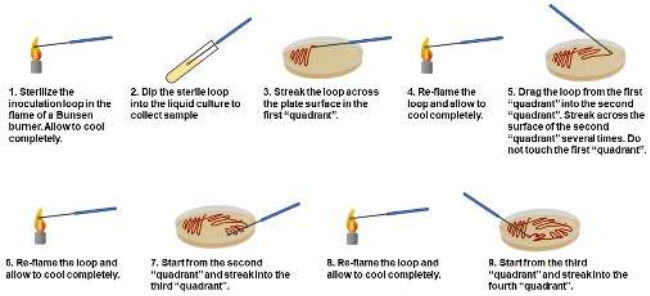


Figure **13.16.2**: Streaking an agar plate (1) Sterilize the loop in the flame until red-hot. Let it cool completely. (2) Dip the sterile loop into the bacterial sample. (3) Gently streak the loop across "quadrant" 1 of the agar surface. (4) Re-flame the loop and allow it to cool. (5) Drag the loop from "quadrant" 1 into "quadrant" 2, and streak several times across the surface being careful not to touch the bacteria in "quadrant 1" (6) Re-flame the loop and allow it to cool. (7) Drag the loop from "quadrant" 2 into "quadrant" 3, and streak as outlined in step 5. (8) Re-flame the loop and allow it to cool. 9. Drag the loop from "quadrant" 3 into "quadrant" 4, and streak as outlined in step 5. (Streak Plate Procedure by Kareen Martin, CC BY 4.0)

As shown in Figure **13.16.2**, the bacteria in "quadrant 4" have been sufficiently thinned out enough to form individual colonies.

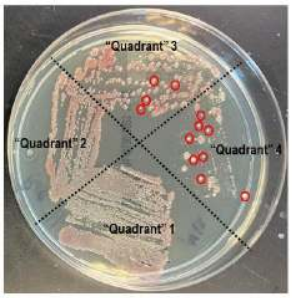


Figure **13.16.3**: An agar plate streaked with a mixed population of bacteria. The bacterial sample was streaked starting in "quadrant" 1 and carried over into "quadrants" 2 through 4. The location of these 4 "quadrants" is approximated with dashed black lines. The bacterial load decreases as the sample is carried into "quadrants" 3 and 4 and well-formed, individual colonies can be observed. Several individual colonies are shown in this figure (red circles). (Streak Plate by Raekwon Holden, CC BY 4.0)

Streak Plate Troubleshooting

A few common mistakes, listed below in Table **13.16.1**, can be made while streaking an agar plate.

Table **13.16.1**: Possible mistakes in streaked plates and their results

Mistake	Result	Reason
The inoculating loop is not sterilized in between streaking quadrants.	No isolated colonies can be seen.	Too much bacteria is added to the pl
The inoculating loop has not been cooled down and was too hot when streaking.	No growth on one or several quadrants.	Bacterial cells are dead.
The user dips the inoculation loop in the liquid culture after each quadrant streak.	No isolated colonies can be seen.	Too much bacteria is added to the pl

Lab Protocol: Inoculating a Broth Using a Solid Bacterial Culture

1. Sterilize the loop in the flame until red-hot. Allow it to cool completely.
2. Open the bacterial sample.
   - For a slant culture, hold the cap between the little finger and the palm of the hand. Sterilize the mouth of the tube by passing it over the flame.
   - For an agar plant, lift the lid up slightly from the plate.
3. Gently collect the bacterial sample.
   - Touch the loop to the bacteria growing on the surface of the slant. Close the tube.
   - Touch the loop to a bacterial colony on the agar plate. Close the plate.
4. Remove the cap from the test tube containing a small volume of culture broth and hold the cap between the little finger and the palm of the hand. Sterilize the mouth of the tube by passing it over the flame.
   - The volume of this culture broth can be a small (e.g., 2 to 5 mL) and prepared in a sterile test tube, or a larger volume prepared in a sterile flask (e.g. 5 mL to 1000 mL). Note that larger culture volumes will require longer incubation times in order to ensure the cells are in their growth phase.
5. Dip the loop into the culture broth and gently swirl to release the bacterial cells. Cap the tube.
6. Incubate the under the appropriate culture conditions.
   - Gentle shaking at 200 rpm during the incubation period can be used to increase bacterial yields.

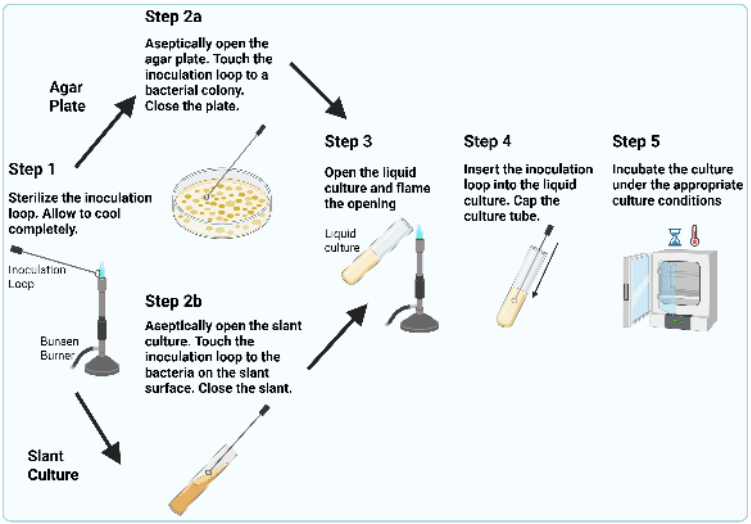


Figure **13.16.4**: Inoculating a liquid culture from solid media. Step 1: An inoculation loop is sterilized by flaming it in a Bunsen burner and then cooled. Step 2a: Using an agar plate, the plate is opened using aseptic techniques (not shown) and the inoculation loop is touched to a single colony. Step 2b: Using an bacterial slant, the tube is opened using aseptic techniques (not shown) and the inoculation loop is touched to the bacteria on the surface of the slant. Step 3: A liquid culture is opened an flamed using the Bunsen burner. Step 4: The inoculation loop is inserted into the liquid culture media. The tube is capped and the inoculation loop is re-flamed to sterilize (not shown). Step 5: The prepared liquid culture is incubated under the appropriate conditions (e.g., 37°C for 16 to 18 hours). (Bacterial Overnight Cultures by Patricia Zuk, CC BY 4.0; figure created in BioRender. Zuk, P. (2025))

13.16: Basics of Bacterial Culture is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

14: Metric System and Measurements

14.1: Metric System Fundamentals

14: Metric System and Measurements is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

14.1: Metric System Fundamentals

The History of Measuring

Scientific fields like biotechnology require a strong working knowledge of measurement systems. The two major measurement systems used today are the Imperial system and the metric system. The **Imperial system** is a system of weights and measures traditionally used in the United Kingdom and the United States (though the U.S. version has its own variations). The Imperial system is not decimal-based and uses non-uniform units, making conversions less straightforward than in the metric system. Examples of units used in the Imperial system are the gallon, the quart, the mile, and the inch.

Scientific measurement requires standards. In response to the non-uniformity of older measurement systems, like the Imperial system, the metric system was created. The **metric system** is a decimal-based system of measurement used worldwide in science, engineering, and medicine. It is *built on units of 10*, making it simple and consistent for converting between units. The metric system can trace its roots back to 18th century France. It was developed to replace the chaotic and inconsistent system of local measrement units used throughout Europe. The first adoption of the metric system was in 1795 and included standard units like the "metre" (meter) for length, kilogram for mass, and "litre" (liter) for volume. In 1875, the Metre Convention was signed by 17 nations, establishing the International Bureau of Weights and Measures (BIPM) in order to promote metric standardization worldwide. In 1960, the General Conference on Weights and Measures (CGPM) officially adopted the the metric system globally as base unit within the Système International d'Unités or the International System of Units (SI for short). As a restul, metric system measurements like kilogram and meter (an liter) joined other SI base units like second, ampere, and mole (Figure **14.1.1**).

SI Base Units			
Base quantity		Base unit	
Name	Typical symbol	Name	Symbol
time	t	second	s
length	l, x, r, etc.	meter	m
mass	m	kilogram	kg
electric current	I, i	ampere	A
thermodynamic temperature	T	kelvin	K
amount of substance	n	mole	mol
luminous intensity	I_v	candela	cd

Source: NIST Special Publication 330:2019, Table 2.

Figure **14.1.1**: International System of Units includes the metric system as one of its based units for measurement. (SI units from the US government, CC BY-SA 4.0)

The metric system is based on a single unit of measurement, called a **base unit**, for each type of quantity measured. The three most common base units in the metric system are the **meter**, **gram**, and **liter.** The meter is a unit of length equal to 3.28 feet; the gram is a unit of mass equal to approximately 0.0022 pounds; and the liter is a unit of volume equal to 1.05 quarts. Large and small objects are expressed as multiples of ten of the base unit using prefixes that indicate the specific multiple. For example, rather than saying that the Nile River is 6,650,000 meters long, it is easier to say that it is 6,650 kilometers long. The prefix "kilo" in front of meter means a thousand meters. Therefore, 6,650 kilometers is really 6,650 thousand-meters, which is 6,650,000 meters. Metric prefixes can be used with any base unit. Some common prefixes used in the metric system are listed in Table **14.1.1** below.

Table **14.1.1**: **Metric System Prefixes**

Common Metric Prefix	Symbol	Base Unit Multiple
Tera	T	1,000,000,000,000 (10^{12})
Giga	G	1,000,000,000 (10^{9})
Mega	M	1,000,000 (10^{6})
Kilo	k	1,000 (10^{3})
Hecto	h	100 (10^{2})
Deca	da	10 (10^{1})
Deci	d	0.1 (10^{-1})
centi	c	0.01 (10^{-2})
milli	m	0.001 (10^{-3})
micro	u	0.000001 (10^{-6})
nano	n	0.000000001 (10^{-9})
pico	p	0.000000000001 (10^{-12})
fempto	f	0.000000000000001 (10^{-15})

Changing metric units

The metric system is a called a decimal-based system because it is based on *multiples of ten*. Any measurement given in one metric unit (e.g., kilogram) can be converted to another metric unit (e.g., gram) simply by moving the decimal place and adding the correct prefix to the base unit. Converting from a smaller unit to a larger unit requires moving up the rows shown in Table **14.1.1** shown above and moving the decimal place to the *left in the number*. Converting from a larger unit to a smaller unit means moving down the rows in Table **14.1.1** and moving the *decimal to the right*. The number of rows you moved in the table corresponds to the number of decimal places moved in the number. For example, converting 72,500.0 grams to kilograms requires moving up in the table by three rows. This means decimal three places to the left and adding the prefix "kilo" in front of the base unit, gram.

Scientific notation

There are several ways to write numbers (i.e., numerals). Most people write numbers as **Arabic numerals** (e.g., 1, 2, 3, 4). In ancient Rome, numbers were written as **Roman numerals** (e.g. 2024 = MMXXIV). Science uses Arabic numerals most of the time. However, in science, it is common to work with very large and very small numbers. For example, the diameter of a red blood cell is 0.0065 cm, the distance from the Earth to the sun is 150,000,000 km, and the number of molecules in 1 g of water is 33,400,000,000,000,000,000,000. It gets cumbersome to work with such long numbers, so numbers such as these are often written using a shorthand called **scientific notation**.

Scientific notation expresses a number as: $\boldsymbol{a \times 10^{n}}$

Where:

- a is a decimal number (called the **coefficient**) that is greater than 1 and less than 10
  - the number a is always a single digit followed by decimals
- n is an integer (positive or negative), called the **exponent**
  - the integer represents the multiples of 10 in the original number.
  - e.g., 1 x 10^2 represents 2 multiples of 10 or the number 100 (10 x 10 = 100)

Scientific notation is a simple way to represent large numbers because the exponent (e.g. 2 in the example above) tells you how many places to move the decimal of the coefficient (1 in the example above). The direction the decimal point is moved will depend on what type of conversion is being performed (Table **14.1.2**).

- When converting an Arabic number to scientific notation, moving the decimal point to the left produces a positive exponent; moving the decimal point to the right produces a negative exponent
- When converting scientific notation to an Arabic number, a positive exponent means move the decimal point to the right; a negative exponent means move the decimal point to the left

Table **14.1.2**: **Scientific Notation Conversions**

Number	coefficient	exponent (multiple of 10 to generate coefficient)	decimal place movement to convert to scientific notation	Scientific notation	decimal place movement to convert to Arabic numeral	Arabic numeral
150,000,000	1.5	8	8 places to the left	1.5×10^{8}	8 places to the right	150,000,000
33,400,000,000,000,000,000,000	3.34	22	22 places to the left	3.34×10^{22}	22 places to the right	33,400,000,000,000,000,000,000
0.0065	6.5	3	3 places to the right	6.5×10^{-3}	3 places to the left	0.0065

Attributions

Text adapted from: The Metric System: Metric and Scientific Notation by Anthony Carpi, PhD

14.1: Metric System Fundamentals is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by LibreTexts.

Index

A

accuracy
1.3: Common Biotechnology Equipment
Acid fast staining
7.2: Microbial Identification
13.15: Bacterial Identification Methods
acids
2.1: Water
adherent culture
6.1: Principles of Mammalian Cell Culture
adipose derived stem cells (ASCs)
10.4: Regenerative Medicine
adult stem cells
10.4: Regenerative Medicine
agar plate and slant preparations, lab technique
13.16: Basics of Bacterial Culture
agarose gel electrophoresis, lab technique
13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
agricultural biotechnology
1.1: Definition and a Short History of Biotechnology
9.2: Applications of "Green" Biotechnology
Agrobacterium tumefaciens
9.2: Applications of "Green" Biotechnology
9.4: Benefits of "Green" Biotechnology
air pollution control
9.3: Applications of "Gray" Biotechnology
alkaline mini prep, lab technique
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
alternative splicing
3.3: Transcription of RNA
Ames test for DNA mutations
3.5: Mutations and DNA Repair
amino acid attachment site of tRNA
3.4: Translation of the polypeptide
amino acid structure and classification
2.6: Proteins
amino functional group
2.2: Carbon
aminoacyl (A) site of the ribosome
3.4: Translation of the polypeptide
amylose and amylopectin
2.4: Carbohydrates
analytical profile index (API) strips
7.2: Microbial Identification
13.15: Bacterial Identification Methods
animal biotechnology applications
8.1: Applications of Animal Biotechnology
animals as bioreactors
8.2: Genetic Engineering in Animals
antibiotic production
7.5: Medical Applications of Microbial Biotechnology
antibiotic resistance gene
4.2: Creating Recombinant DNA
antibodies
1.4: Advanced Biotechnology Equipment
10.2: Diagnostic Biotechnology
13.12: Lab Technique - Western Blotting
anticodon loop of tRNA
3.4: Translation of the polypeptide
antigens
1.4: Advanced Biotechnology Equipment
applications for rDNA technology
4.3: Applications of Recombinant DNA Technology
archaea
7.1: Microbe classification
aseptic technique principles
6.2: Principles of Aseptic Technique
13.13: Lab Technique - Cell Culture
aseptic technique, bacteria
7.3: Microbial Cultivation and Manipulation
13.16: Basics of Bacterial Culture
autoclaves
1.3: Common Biotechnology Equipment
6.2: Principles of Aseptic Technique
automated cell counters
6.3: Cell Culture Equipment

B

Bacillus Thuringiensis
9.2: Applications of "Green" Biotechnology
bacterial artificial chromosomes (BACs)
4.1: Principles of Genetic Engineering
bacterial cell wall composition
7.1: Microbe classification
bacterial cellulose
7.3: Microbial Cultivation and Manipulation
bacterial chromosome structure
3.1: DNA Structure
bacterial cultivation
7.3: Microbial Cultivation and Manipulation
bacterial culture conditions
13.16: Basics of Bacterial Culture
bacterial culture media types
7.2: Microbial Identification
7.3: Microbial Cultivation and Manipulation
bacterial identification
7.1: Microbe classification
7.2: Microbial Identification
13.15: Bacterial Identification Methods
bacterial plates and slants
7.2: Microbial Identification
bacterial smear preparation
13.15: Bacterial Identification Methods
bacterial transformation
4.2: Creating Recombinant DNA
13.6: Lab Technique - Bacterial Transformation
balances
1.3: Common Biotechnology Equipment
basal medium for cell culture
6.1: Principles of Mammalian Cell Culture
Base Excision Repair
3.5: Mutations and DNA Repair
bases
2.1: Water
batch records
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs
batch vs continuous culture of bacteria
7.3: Microbial Cultivation and Manipulation
beakers
1.3: Common Biotechnology Equipment
bioactive molecules
10.4: Regenerative Medicine
bioaugmentation
9.3: Applications of "Gray" Biotechnology
BioBricks
11.3: Synthetic Biology
bioengineering
1.2: Applications of Biotechnology
11.2: Bioprocess Engineering
biofabrication
7.4: Industrial Applications of Microbial Biotechnology
biofertilizers
9.2: Applications of "Green" Biotechnology
biofilms and microscopy
1.4: Advanced Biotechnology Equipment
biofilters
9.3: Applications of "Gray" Biotechnology
biofortification
9.4: Benefits of "Green" Biotechnology
biofuels
4.3: Applications of Recombinant DNA Technology
7.4: Industrial Applications of Microbial Biotechnology
11.1: Applications of Industrial Biotechnology
bioinformatics
5.1: Introduction to Bioinformatics
biological safety levels (BSLs)
6.1: Principles of Mammalian Cell Culture
biological scaffolds (bioscaffolds)
10.4: Regenerative Medicine
biomanufacturing
11.2: Bioprocess Engineering
biomarker types
10.2: Diagnostic Biotechnology
biomaterials
10.4: Regenerative Medicine
biopesticides
9.2: Applications of "Green" Biotechnology
biopharming
8.2: Genetic Engineering in Animals
bioplastics
11.1: Applications of Industrial Biotechnology
bioprinting
10.4: Regenerative Medicine
bioprocess engineering
11.2: Bioprocess Engineering
bioproduction
7.4: Industrial Applications of Microbial Biotechnology
bioreactors
7.3: Microbial Cultivation and Manipulation
11.2: Bioprocess Engineering
bioremediation
1.2: Applications of Biotechnology
4.3: Applications of Recombinant DNA Technology
9.3: Applications of "Gray" Biotechnology
11.1: Applications of Industrial Biotechnology
biosafety
6.1: Principles of Mammalian Cell Culture

biosafety cabinets
1.3: Common Biotechnology Equipment
6.2: Principles of Aseptic Technique
6.3: Cell Culture Equipment
biosensors
9.3: Applications of "Gray" Biotechnology
10.2: Diagnostic Biotechnology
biosurveillance
1.2: Applications of Biotechnology
biotech lab protocols
13.1: Lab Technique - Pipetting
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
13.4: Lab Technique - Restriction Digest of DNA
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
13.6: Lab Technique - Bacterial Transformation
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
13.8: Lab Technique - Quantitation of DNA
13.9: Lab Technique - DNA Sequencing
13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
13.11: Lab Technique - SDS-PAGE
13.12: Lab Technique - Western Blotting
13.13: Lab Technique - Cell Culture
13.14: Lab Technique - Light Microscopy
13.15: Bacterial Identification Methods
13.16: Basics of Bacterial Culture
biotechnological applications
1.1: Definition and a Short History of Biotechnology
biotechnology
1.1: Definition and a Short History of Biotechnology
1.2: Applications of Biotechnology
bioterrorism
1.2: Applications of Biotechnology
black biotechnology
1.1: Definition and a Short History of Biotechnology
blue biotechnology
1.1: Definition and a Short History of Biotechnology
blunt end cloning
4.2: Creating Recombinant DNA
bottles, reagent
1.3: Common Biotechnology Equipment
BRCA genes
5.5: Functional Genomics
breeding vs genetic engineering
4.1: Principles of Genetic Engineering
brightfield microscope
1.4: Advanced Biotechnology Equipment
brown biotechnology
1.1: Definition and a Short History of Biotechnology
buffers
1.3: Common Biotechnology Equipment
2.1: Water

C

carbohydrate fermentation
13.15: Bacterial Identification Methods
carbohydrates
2.3: Macromolecules
2.4: Carbohydrates
carbon
2.2: Carbon
carbon capture
2.2: Carbon
carbon skeletons
2.2: Carbon
carbonic acid bicarbonate buffer system
2.1: Water
carbonyl functional group
2.2: Carbon
carboxyl functional group
2.2: Carbon
catalase tests for bacterial identification
7.2: Microbial Identification
catalysts
2.6: Proteins
cell cloning
6.1: Principles of Mammalian Cell Culture
8.1: Applications of Animal Biotechnology
cell counting
6.3: Cell Culture Equipment
13.13: Lab Technique - Cell Culture
cell culture conditions and reagents
6.1: Principles of Mammalian Cell Culture
13.13: Lab Technique - Cell Culture
cell culture equipment
6.3: Cell Culture Equipment
13.13: Lab Technique - Cell Culture
cell line
6.1: Principles of Mammalian Cell Culture
cell strain
6.1: Principles of Mammalian Cell Culture
cellulose
2.4: Carbohydrates
central dogma of molecular biology
3.3: Transcription of RNA
centrifuge rotors
1.3: Common Biotechnology Equipment
centrifuges
1.3: Common Biotechnology Equipment
Certificate of Analysis
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs
chain termination DNA sequencing
5.3: Genome Sequencing
chemical transformation of bacteria
13.6: Lab Technique - Bacterial Transformation
chemiluminescence, Western blotting
13.12: Lab Technique - Western Blotting
chemotaxonomy for bacterial identification
7.2: Microbial Identification
chitin
2.4: Carbohydrates
cholesterol
2.5: Lipids
chromatids
3.1: DNA Structure
chromatin structure
3.1: DNA Structure
chromatography
5.6: Proteomics
class II biosafety cabinets
6.3: Cell Culture Equipment
cloning
4.1: Principles of Genetic Engineering
4.2: Creating Recombinant DNA
4.3: Applications of Recombinant DNA Technology
8.1: Applications of Animal Biotechnology
cloning plasmids
4.1: Principles of Genetic Engineering
coding sequence
3.3: Transcription of RNA
codon table
3.4: Translation of the polypeptide
common lab equipment
1.3: Common Biotechnology Equipment
common restriction enzymes
4.1: Principles of Genetic Engineering
comparative genomics
5.1: Introduction to Bioinformatics
5.2: Genomics
5.4: Comparative Genomics
competent bacterial cells
13.6: Lab Technique - Bacterial Transformation
complementary base pairing
2.7: Nucleic Acids
condensor lens, microscopy
1.4: Advanced Biotechnology Equipment
confocal microscope
1.4: Advanced Biotechnology Equipment
conserved DNA sequences
5.4: Comparative Genomics
contamination in cell culture
6.2: Principles of Aseptic Technique
continuous cell lines
6.1: Principles of Mammalian Cell Culture
contrast, microscopy
1.4: Advanced Biotechnology Equipment
cosmids
4.1: Principles of Genetic Engineering
CRISPR
8.1: Applications of Animal Biotechnology
10.3: Therapeutic Biotechnology
cryopreservation
6.3: Cell Culture Equipment
13.13: Lab Technique - Cell Culture
culture medium, mammalian culture
6.1: Principles of Mammalian Cell Culture
13.13: Lab Technique - Cell Culture
cultured cell types
6.1: Principles of Mammalian Cell Culture
cystic fibrosis and gene therapy
10.3: Therapeutic Biotechnology

D

darkfield microscope
1.4: Advanced Biotechnology Equipment
databases
5.1: Introduction to Bioinformatics
daughter DNA strand
3.2: Replication of DNA
decellularized scaffolds
10.4: Regenerative Medicine
dehydration synthesis
2.3: Macromolecules
2.4: Carbohydrates
2.5: Lipids
2.6: Proteins
deletion mutations
3.5: Mutations and DNA Repair
Deoxyribonucleic acid (DNA)
2.7: Nucleic Acids

diagnostic biotechnology
10.2: Diagnostic Biotechnology
Dicer role in gene silencing
5.5: Functional Genomics
dideoxy chain termination method of DNA sequencing
5.3: Genome Sequencing
dideoxynucleotides (ddNTPs)
5.3: Genome Sequencing
differential bacterial media
7.2: Microbial Identification
differential staining
7.2: Microbial Identification
differentiation of stem cells
10.4: Regenerative Medicine
digesting DNA with restriction enzymes
13.4: Lab Technique - Restriction Digest of DNA
dipeptides
2.6: Proteins
directional cloning
4.2: Creating Recombinant DNA
disaccharides
2.4: Carbohydrates
DNA cloning
4.1: Principles of Genetic Engineering
4.2: Creating Recombinant DNA
4.3: Applications of Recombinant DNA Technology
DNA helicase
3.2: Replication of DNA
DNA helix
3.1: DNA Structure
DNA ligase
3.2: Replication of DNA
4.2: Creating Recombinant DNA
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
DNA ligation
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
DNA microarrays
10.2: Diagnostic Biotechnology
DNA mutations
3.5: Mutations and DNA Repair
DNA packaging
3.1: DNA Structure
DNA polymerase
3.2: Replication of DNA
DNA purification methods
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
DNA repair mechanisms
3.5: Mutations and DNA Repair
DNA replication
3.2: Replication of DNA
DNA sequencing
5.3: Genome Sequencing
13.9: Lab Technique - DNA Sequencing
DNA structure
2.7: Nucleic Acids
3.1: DNA Structure
DNA supercoiling
3.1: DNA Structure
DNA vs RNA
2.7: Nucleic Acids
double digest of DNA
4.2: Creating Recombinant DNA
13.4: Lab Technique - Restriction Digest of DNA
downstream processes in biomanufacturing
11.2: Bioprocess Engineering

E

E (exit) site of the ribosome
3.4: Translation of the polypeptide
EcoRI
4.1: Principles of Genetic Engineering
edible vaccines
9.4: Benefits of "Green" Biotechnology
electron gun
1.4: Advanced Biotechnology Equipment
electron microscope types
1.4: Advanced Biotechnology Equipment
electronic balances
1.3: Common Biotechnology Equipment
electrophoresis
1.3: Common Biotechnology Equipment
13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
13.11: Lab Technique - SDS-PAGE
electroporation
13.6: Lab Technique - Bacterial Transformation
electrospinning of scaffolds
10.4: Regenerative Medicine
embryonic stem (ES) cells
10.4: Regenerative Medicine
endonucleases
3.5: Mutations and DNA Repair
endospore staining
7.2: Microbial Identification
13.15: Bacterial Identification Methods
enhancers
3.3: Transcription of RNA
enriched bacteria media
7.2: Microbial Identification
environmental biotechnology
1.1: Definition and a Short History of Biotechnology
9.3: Applications of "Gray" Biotechnology
environmental monitoring
9.3: Applications of "Gray" Biotechnology
Enzyme Linked Immunosorbent Assays (ELISAs)
10.2: Diagnostic Biotechnology
enzymes
2.6: Proteins
equipment, cell culture
6.3: Cell Culture Equipment
equipment, laboratory
1.3: Common Biotechnology Equipment
essential fatty acids
2.5: Lipids
ester bond
2.5: Lipids
euchromatin
3.1: DNA Structure
eukaryotic chromosome structure
3.1: DNA Structure
evaporation
2.1: Water
ex situ bioremediation
9.3: Applications of "Gray" Biotechnology
exonuclease types
3.5: Mutations and DNA Repair
expression plasmids
4.1: Principles of Genetic Engineering
extremophiles
7.1: Microbe classification

F

fatty acid structure and classification
2.5: Lipids
fermentation
11.2: Bioprocess Engineering
fermentation tests for bacterial identification
7.2: Microbial Identification
filtration
6.2: Principles of Aseptic Technique
first generation biofuels
7.4: Industrial Applications of Microbial Biotechnology
flasks, types
1.3: Common Biotechnology Equipment
flow cytometry
1.4: Advanced Biotechnology Equipment
fluorescent in situ hybridization (FISH)
10.2: Diagnostic Biotechnology
fluorescent microscopes and microscopy
1.4: Advanced Biotechnology Equipment
13.14: Lab Technique - Light Microscopy
fluorochromes
1.4: Advanced Biotechnology Equipment
food biotechnology
1.1: Definition and a Short History of Biotechnology
fourth generation biofuels
7.4: Industrial Applications of Microbial Biotechnology
frameshift mutations
3.5: Mutations and DNA Repair
freezers
1.3: Common Biotechnology Equipment
fume hoods
1.3: Common Biotechnology Equipment
functional genomics
5.2: Genomics
5.5: Functional Genomics
functional groups in biology
2.2: Carbon

G

gene addition
8.1: Applications of Animal Biotechnology
gene editing
8.1: Applications of Animal Biotechnology
10.3: Therapeutic Biotechnology
gene expression
3.3: Transcription of RNA
gene expression analysis
5.5: Functional Genomics
gene gun
9.2: Applications of "Green" Biotechnology

gene silencing
5.5: Functional Genomics
8.1: Applications of Animal Biotechnology
gene structure
3.3: Transcription of RNA
gene therapy
10.3: Therapeutic Biotechnology
genetic code
3.4: Translation of the polypeptide
genetic engineering
1.1: Definition and a Short History of Biotechnology
4.1: Principles of Genetic Engineering
8.1: Applications of Animal Biotechnology
8.2: Genetic Engineering in Animals
genetic maps
5.2: Genomics
genetic recombination
5.2: Genomics
genetically modified crops
1.2: Applications of Biotechnology
4.3: Applications of Recombinant DNA Technology
9.2: Applications of "Green" Biotechnology
genetically modified organism (GMO)
1.1: Definition and a Short History of Biotechnology
1.2: Applications of Biotechnology
8.2: Genetic Engineering in Animals
9.2: Applications of "Green" Biotechnology
genome sequencing
5.3: Genome Sequencing
genomes and genomics
5.1: Introduction to Bioinformatics
5.2: Genomics
5.4: Comparative Genomics
5.5: Functional Genomics
glassware
1.3: Common Biotechnology Equipment
global pairwise sequence alignment
5.4: Comparative Genomics
GLOFish
8.2: Genetic Engineering in Animals
glucose monitors
10.2: Diagnostic Biotechnology
glycerol structure
2.5: Lipids
glycogen
2.4: Carbohydrates
glycosidic bond
2.4: Carbohydrates
GMO
1.1: Definition and a Short History of Biotechnology
golden rice
9.4: Benefits of "Green" Biotechnology
Good Laboratory Practices (GLPs)
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
Good Manufacturing Practices (GMPs)
11.2: Bioprocess Engineering
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
graduated cylinders
1.3: Common Biotechnology Equipment
Gram staining
7.1: Microbe classification
7.2: Microbial Identification
13.15: Bacterial Identification Methods
gray biotechnology
1.1: Definition and a Short History of Biotechnology
9.1: "Green" vs. "Gray" Biotechnology
9.3: Applications of "Gray" Biotechnology
green biotechnology
1.1: Definition and a Short History of Biotechnology
9.1: "Green" vs. "Gray" Biotechnology
9.2: Applications of "Green" Biotechnology
9.4: Benefits of "Green" Biotechnology

H

harvesting cultured cells
13.13: Lab Technique - Cell Culture
HeLa cells
6.1: Principles of Mammalian Cell Culture
hemacytometers
6.3: Cell Culture Equipment
hematopoietic stem cells (HSCs)
10.4: Regenerative Medicine
Henrietta Lacks
6.1: Principles of Mammalian Cell Culture
HER2 role in cancer
5.6: Proteomics
heterochromatin
3.1: DNA Structure
histones
3.1: DNA Structure
homologous genes
5.4: Comparative Genomics
Hox genes
5.4: Comparative Genomics
human genome project
5.2: Genomics
Humulin
4.1: Principles of Genetic Engineering
10.3: Therapeutic Biotechnology
hydrocarbons
2.2: Carbon
hydrogen bonding
2.1: Water
hydrolysis
2.3: Macromolecules
hydrophilic and hydrophobic
2.1: Water
hydroxyl functional group
2.2: Carbon

I

ICH guidelines
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
immersion oil
1.4: Advanced Biotechnology Equipment
immortalized cells
6.1: Principles of Mammalian Cell Culture
immunoassays
10.2: Diagnostic Biotechnology
immunoblotting (Western blotting)
13.12: Lab Technique - Western Blotting
immunodiagnostics
10.2: Diagnostic Biotechnology
immunotherapy
1.2: Applications of Biotechnology
in situ bioremediation
9.3: Applications of "Gray" Biotechnology
in vitro cell culture
6.1: Principles of Mammalian Cell Culture
13.13: Lab Technique - Cell Culture
in vitro fertilization
1.2: Applications of Biotechnology
in vitro transcription systems
3.3: Transcription of RNA
incubators
6.3: Cell Culture Equipment
indirect immunofluorescence
13.14: Lab Technique - Light Microscopy
induced pluripotent stem cells (iPSCs)
10.4: Regenerative Medicine
industrial biotechnology
1.1: Definition and a Short History of Biotechnology
11.1: Applications of Industrial Biotechnology
industrial enzymes
4.3: Applications of Recombinant DNA Technology
11.1: Applications of Industrial Biotechnology
initiation factors, translation
3.4: Translation of the polypeptide
initiator tRNA
3.4: Translation of the polypeptide
inoculating bacterial media
13.16: Basics of Bacterial Culture
insertion mutations
3.5: Mutations and DNA Repair
intron splicing
3.3: Transcription of RNA
inverted microscopes
6.3: Cell Culture Equipment
ISO 9001 certification
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
ISO standards
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
isoelectric focusing
5.6: Proteomics
isolation of DNA from bacteria
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")

K

Kozak sequence of translation
3.4: Translation of the polypeptide

L

lab equipment
1.3: Common Biotechnology Equipment
lab grown meat
4.3: Applications of Recombinant DNA Technology
lab on a chip
10.2: Diagnostic Biotechnology

lab techniques
13.1: Lab Technique - Pipetting
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
13.3: Lab Technique - Agarose Gel Electrophoresis of DNA
13.4: Lab Technique - Restriction Digest of DNA
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
13.6: Lab Technique - Bacterial Transformation
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
13.8: Lab Technique - Quantitation of DNA
13.9: Lab Technique - DNA Sequencing
13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
13.11: Lab Technique - SDS-PAGE
13.12: Lab Technique - Western Blotting
13.13: Lab Technique - Cell Culture
13.14: Lab Technique - Light Microscopy
13.15: Bacterial Identification Methods
13.16: Basics of Bacterial Culture
lagging strand synthesis
3.2: Replication of DNA
laminar flow hoods
6.3: Cell Culture Equipment
land management biotechnology
1.1: Definition and a Short History of Biotechnology
large scale mutation types
3.5: Mutations and DNA Repair
lateral flow assays (LFAs)
10.2: Diagnostic Biotechnology
leading strand synthesis
3.2: Replication of DNA
ligation into a plasmid
13.5: Lab Technique - Ligation of Insert DNA into a Plasmid
light microscopes and microscopy
1.4: Advanced Biotechnology Equipment
13.14: Lab Technique - Light Microscopy
linked genes and linkage maps
5.2: Genomics
lipids
2.3: Macromolecules
2.5: Lipids
liposomal mediated transfection of DNA
13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
liposomes
8.1: Applications of Animal Biotechnology
8.2: Genetic Engineering in Animals
10.3: Therapeutic Biotechnology
liquid chromatography
5.6: Proteomics
liquid media, bacterial
7.2: Microbial Identification
13.16: Basics of Bacterial Culture
local pairwise sequence alignment
5.4: Comparative Genomics

M

macromolecules
2.3: Macromolecules
magnification
1.4: Advanced Biotechnology Equipment
mammalian cell culture
6.1: Principles of Mammalian Cell Culture
13.13: Lab Technique - Cell Culture
manufacturing biotechnology
1.1: Definition and a Short History of Biotechnology
map units (centimorgans)
5.2: Genomics
marine biotechnology
1.1: Definition and a Short History of Biotechnology
mass spectrometry
5.6: Proteomics
medical biotechnology
1.1: Definition and a Short History of Biotechnology
10.1: Principles of Medical Biotechnology
mesodermally derived stem cells
10.4: Regenerative Medicine
messenger RNA (mRNA) structure
3.3: Transcription of RNA
metabolomics
5.5: Functional Genomics
methyl functional group
2.2: Carbon
methylated cap
3.3: Transcription of RNA
metric system
14.1: Metric System Fundamentals
metrology
1.3: Common Biotechnology Equipment
microarrays, protein
5.6: Proteomics
microbial biotechnology
7.1: Microbe classification
7.4: Industrial Applications of Microbial Biotechnology
7.5: Medical Applications of Microbial Biotechnology
microbial consortia
7.3: Microbial Cultivation and Manipulation
microbial cultivation conditions
7.3: Microbial Cultivation and Manipulation
microbial identification and classification
7.1: Microbe classification
7.2: Microbial Identification
microbial manipulation
7.3: Microbial Cultivation and Manipulation
microbiology aseptic technique
7.3: Microbial Cultivation and Manipulation
micropipettes
1.3: Common Biotechnology Equipment
13.1: Lab Technique - Pipetting
microRNA (miRNA)
5.5: Functional Genomics
8.1: Applications of Animal Biotechnology
microscope types
1.4: Advanced Biotechnology Equipment
microscope, lens types
1.4: Advanced Biotechnology Equipment
microscopy
1.4: Advanced Biotechnology Equipment
13.14: Lab Technique - Light Microscopy
mini prep isolation of DNA from bacteria, lab technique
13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep")
mismatch repair
3.5: Mutations and DNA Repair
missense mutations
3.5: Mutations and DNA Repair
molecular diagnostics
10.2: Diagnostic Biotechnology
monomers
2.3: Macromolecules
monosaccharides
2.4: Carbohydrates
monounsaturated
2.5: Lipids
mordants
7.2: Microbial Identification
mRNA processing
3.3: Transcription of RNA
mRNA vaccines
10.3: Therapeutic Biotechnology
multi cloning site (MCS)
3.1: DNA Structure
4.1: Principles of Genetic Engineering
4.2: Creating Recombinant DNA
multiple sequence alignment
5.4: Comparative Genomics
multipotent stem cells
10.4: Regenerative Medicine
mutagens
3.5: Mutations and DNA Repair
mutational analysis
5.5: Functional Genomics
mutations
3.5: Mutations and DNA Repair

N

next generation sequencing
5.1: Introduction to Bioinformatics
5.3: Genome Sequencing
5.5: Functional Genomics
10.2: Diagnostic Biotechnology
nitrogen fixation
9.2: Applications of "Green" Biotechnology
nitrogenous bases
2.7: Nucleic Acids
non polar bonds
2.1: Water
non selective bacterial media
7.2: Microbial Identification
nonsense mutations
3.5: Mutations and DNA Repair
nucleic acids
2.3: Macromolecules
2.7: Nucleic Acids
nucleosomes
3.1: DNA Structure
nucleotide excision repair
3.5: Mutations and DNA Repair
nucleotide nomenclature
2.7: Nucleic Acids
nucleotide structure
2.7: Nucleic Acids
3.1: DNA Structure

O

oil eating bacteria
11.1: Applications of Industrial Biotechnology
oil immersion
13.14: Lab Technique - Light Microscopy
Okazaki fragments
3.2: Replication of DNA

one gene one polypeptide hypothesis
3.4: Translation of the polypeptide
orbitals
2.2: Carbon
organ culture
6.1: Principles of Mammalian Cell Culture
origin of replication
3.2: Replication of DNA
4.1: Principles of Genetic Engineering
orthologous genes
5.4: Comparative Genomics
oxidase tests for bacterial identification
7.2: Microbial Identification

P

P (peptidyl) site of the ribosome
3.4: Translation of the polypeptide
paired end sequencing
5.3: Genome Sequencing
pairwise sequence alginment
5.4: Comparative Genomics
parental DNA strand
3.2: Replication of DNA
PCR machine
1.4: Advanced Biotechnology Equipment
peptide types
2.6: Proteins
personal protective equipment (PPE)
1.3: Common Biotechnology Equipment
6.1: Principles of Mammalian Cell Culture
personalized medicine
5.1: Introduction to Bioinformatics
5.3: Genome Sequencing
pH meters and indicators
1.3: Common Biotechnology Equipment
pH scale
2.1: Water
phosphate functional group
2.2: Carbon
phosphodiester bond
2.7: Nucleic Acids
3.1: DNA Structure
phospholipid bilayer
2.5: Lipids
phospholipid structure
2.5: Lipids
phylogenetic reconstruction
5.4: Comparative Genomics
phylogenetic trees
5.4: Comparative Genomics
physical maps
5.2: Genomics
phytochemicals
9.4: Benefits of "Green" Biotechnology
phytoremediation
9.3: Applications of "Gray" Biotechnology
pipet aids (pipette pumps)
1.3: Common Biotechnology Equipment
pipettes
1.3: Common Biotechnology Equipment
13.1: Lab Technique - Pipetting
plasmids
3.1: DNA Structure
4.1: Principles of Genetic Engineering
pluripotent stem cells
10.4: Regenerative Medicine
point mutations
3.5: Mutations and DNA Repair
point of care diagnostics
10.2: Diagnostic Biotechnology
polar bonds
2.1: Water
polyA tail
3.3: Transcription of RNA
polyacrylamide gels
13.11: Lab Technique - SDS-PAGE
polyadenylation site
4.1: Principles of Genetic Engineering
polylinker
4.2: Creating Recombinant DNA
polymerase chain reaction (PCR)
1.4: Advanced Biotechnology Equipment
3.2: Replication of DNA
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
polymers
2.3: Macromolecules
polypeptides
2.6: Proteins
polysaccharides
2.4: Carbohydrates
polyunsaturated
2.5: Lipids
potency of stem cells
10.4: Regenerative Medicine
PPE
1.3: Common Biotechnology Equipment
precision
1.3: Common Biotechnology Equipment
precision fermentation
8.1: Applications of Animal Biotechnology
prepared slides
13.14: Lab Technique - Light Microscopy
primary culture
6.1: Principles of Mammalian Cell Culture
primary protein structure
2.6: Proteins
primary stains
7.2: Microbial Identification
primase
3.2: Replication of DNA
primer design in PCR
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
proflavin and protein translation
3.4: Translation of the polypeptide
prokaryotes vs. eukaryotes
7.1: Microbe classification
prokaryotic DNA structure
3.1: DNA Structure
promoters
3.3: Transcription of RNA
4.1: Principles of Genetic Engineering
protein denaturation
2.6: Proteins
protein folding
2.6: Proteins
protein microarrays
5.6: Proteomics
protein structure and disease, sickle cell anemia
2.6: Proteins
protein structure levels
2.6: Proteins
3.4: Translation of the polypeptide
protein translation stages
3.4: Translation of the polypeptide
protein types
2.6: Proteins
protein, alpha helix
2.6: Proteins
protein, beta pleated sheet
2.6: Proteins
proteins
2.3: Macromolecules
2.6: Proteins
proteomes and proteomics
5.1: Introduction to Bioinformatics
5.5: Functional Genomics
5.6: Proteomics
purines
2.7: Nucleic Acids
3.1: DNA Structure
pyrimidines
2.7: Nucleic Acids
3.1: DNA Structure

Q

QA/QC documentation
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs
QMS documentation
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs
Quality Assurance (QA) and Quality Control (QC)
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
Quality Management System (QMS)
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
quality standards and specifications
12.1: Principles of Quality Assurance (QA) and Quality Control (QC)
quantitation of DNA
13.8: Lab Technique - Quantitation of DNA
quantitative PCR (real time PCR)
3.2: Replication of DNA
quartz crystal microbalances
10.2: Diagnostic Biotechnology
quaternary protein structure
2.6: Proteins

R

radioimmunoassay (RIA) measurement of proteins
2.6: Proteins
radiolabeling
2.3: Macromolecules
random error
1.3: Common Biotechnology Equipment

rDNA applications
4.3: Applications of Recombinant DNA Technology
reading frame of translation
3.4: Translation of the polypeptide
3.5: Mutations and DNA Repair
real time PCR
13.2: Lab Technique - Polymerase Chain Reaction (PCR)
recombinant DNA (rDNA) technology
4.1: Principles of Genetic Engineering
4.2: Creating Recombinant DNA
4.3: Applications of Recombinant DNA Technology
recombinant plasmids
4.2: Creating Recombinant DNA
recombination and recombination frequency
5.2: Genomics
red biotechnology
1.1: Definition and a Short History of Biotechnology
10.1: Principles of Medical Biotechnology
refrigerators
1.3: Common Biotechnology Equipment
regenerative medicine
10.4: Regenerative Medicine
release factor in translation termination
3.4: Translation of the polypeptide
replication fork
3.2: Replication of DNA
reporter proteins
9.3: Applications of "Gray" Biotechnology
reproductive cloning
8.1: Applications of Animal Biotechnology
resolution, microscopy
1.4: Advanced Biotechnology Equipment
restriction digests
13.4: Lab Technique - Restriction Digest of DNA
restriction enzymes
4.1: Principles of Genetic Engineering
4.2: Creating Recombinant DNA
13.4: Lab Technique - Restriction Digest of DNA
restriction sequences and sites
4.1: Principles of Genetic Engineering
reversion mutations
3.5: Mutations and DNA Repair
Ribonucleic Acid (RNA)
2.7: Nucleic Acids
ribosomal RNA (rRNA) structure
3.3: Transcription of RNA
ribosome structure
3.4: Translation of the polypeptide
RNA Induced Silencing Complex (RISC) in gene silencing
5.5: Functional Genomics
RNA interference (RNAi)
5.5: Functional Genomics
8.1: Applications of Animal Biotechnology
RNA polymerase
3.3: Transcription of RNA
RNA primers in DNA replication
3.2: Replication of DNA
RNA sequencing
5.5: Functional Genomics
RNA structure
2.7: Nucleic Acids
RNA transcription, stages
3.3: Transcription of RNA
RNA types
3.3: Transcription of RNA

S

Sanger sequencing of DNA
5.3: Genome Sequencing
13.9: Lab Technique - DNA Sequencing
Sanger sequencing reaction components
13.9: Lab Technique - DNA Sequencing
scaffold materials
10.4: Regenerative Medicine
scanning electron microscopes (SEM)
1.4: Advanced Biotechnology Equipment
scientific notation
14.1: Metric System Fundamentals
SDS PAGE
13.11: Lab Technique - SDS-PAGE
second generation biofuels
7.4: Industrial Applications of Microbial Biotechnology
secondary culture
6.1: Principles of Mammalian Cell Culture
secondary protein structure
2.6: Proteins
secondary stains
7.2: Microbial Identification
selection markers, plasmids
4.1: Principles of Genetic Engineering
selective bacterial media
7.2: Microbial Identification
semiconservative DNA replication
3.2: Replication of DNA
sequence alignment
5.4: Comparative Genomics
sequencing by synthesis
10.2: Diagnostic Biotechnology
sequencing plasmids
13.9: Lab Technique - DNA Sequencing
serological pipettes
1.3: Common Biotechnology Equipment
13.1: Lab Technique - Pipetting
Shine Dalgarno sequence of translation
3.4: Translation of the polypeptide
shotgun sequencing
5.3: Genome Sequencing
sickle cell anemia
2.6: Proteins
10.3: Therapeutic Biotechnology
silent mutations
3.5: Mutations and DNA Repair
simple staining
7.2: Microbial Identification
13.15: Bacterial Identification Methods
single nucleotide polymorphisms (SNPs)
5.5: Functional Genomics
single stranded binding proteins (SSBs)
3.2: Replication of DNA
sister chromatids
3.1: DNA Structure
small interfering RNA (siRNA)
5.5: Functional Genomics
8.1: Applications of Animal Biotechnology
small nuclear RNA (snRNA)
3.3: Transcription of RNA
small scale mutation types
3.5: Mutations and DNA Repair
smallpox and microbial biotechnology
7.5: Medical Applications of Microbial Biotechnology
smear preparation
13.14: Lab Technique - Light Microscopy
solid bacterial media, types
7.2: Microbial Identification
13.16: Basics of Bacterial Culture
solutes
2.1: Water
solvent casting of scaffolds
10.4: Regenerative Medicine
solvents
2.1: Water
somatic cell nuclear transfer (SCNT)
1.2: Applications of Biotechnology
8.1: Applications of Animal Biotechnology
8.2: Genetic Engineering in Animals
specific heat
2.1: Water
spectrophotometers
1.3: Common Biotechnology Equipment
spectroscopy methods for bacterial identification
7.2: Microbial Identification
splice sites
3.3: Transcription of RNA
spliceosome structure
3.3: Transcription of RNA
splicing mechanism
3.3: Transcription of RNA
spore staining
7.2: Microbial Identification
13.15: Bacterial Identification Methods
squash preparation
13.14: Lab Technique - Light Microscopy
Standard Operating Procedure (SOP)
1.3: Common Biotechnology Equipment
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs
starches
2.4: Carbohydrates
start and stop codons
3.4: Translation of the polypeptide
statins
2.5: Lipids
stem cell technology
10.4: Regenerative Medicine
sterile technique, mammalian culture
6.2: Principles of Aseptic Technique
steroid structure
2.5: Lipids
sticky end
4.2: Creating Recombinant DNA
structural genomics
5.2: Genomics
substitution mutations
3.5: Mutations and DNA Repair
sugars
2.4: Carbohydrates

sulfhydryl functional group
2.2: Carbon
surface tension
2.1: Water
suspension culture
6.1: Principles of Mammalian Cell Culture
SYBR Green PCR
3.2: Replication of DNA
synteny
5.4: Comparative Genomics
synthetic biology
11.3: Synthetic Biology
systemic error
1.3: Common Biotechnology Equipment
systems biology
5.1: Introduction to Bioinformatics

T

Taq polymerase
3.2: Replication of DNA
Taqman PCR
3.2: Replication of DNA
TATA box
3.3: Transcription of RNA
temperature
2.1: Water
tertiary protein structure
2.6: Proteins
therapeutic biotechnology
10.3: Therapeutic Biotechnology
therapeutic cloning
8.1: Applications of Animal Biotechnology
thermocycler
1.4: Advanced Biotechnology Equipment
thermocycler (PCR machine)
3.2: Replication of DNA
third generation biofuels
7.4: Industrial Applications of Microbial Biotechnology
thymine thymine dimer repair
3.5: Mutations and DNA Repair
Ti plasmid
9.2: Applications of "Green" Biotechnology
tissue culture
6.1: Principles of Mammalian Cell Culture
tissue engineering
10.4: Regenerative Medicine
topoisomerases
3.1: DNA Structure
3.2: Replication of DNA
totipotent stem cells
10.4: Regenerative Medicine
traditional vaccines
10.3: Therapeutic Biotechnology
trans fatty acids
2.5: Lipids
transcription factors
3.3: Transcription of RNA
transcription initiation complex
3.3: Transcription of RNA
transcription stages
3.3: Transcription of RNA
transcriptomes and transcriptomics
5.5: Functional Genomics
transfection
8.2: Genetic Engineering in Animals
9.2: Applications of "Green" Biotechnology
10.3: Therapeutic Biotechnology
transfection of DNA
13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells
transfer RNA (tRNA) structure
3.3: Transcription of RNA
3.4: Translation of the polypeptide
transformation
4.2: Creating Recombinant DNA
10.3: Therapeutic Biotechnology
transformation of DNA into bacteria
13.6: Lab Technique - Bacterial Transformation
transgenic animal
1.1: Definition and a Short History of Biotechnology
1.2: Applications of Biotechnology
8.2: Genetic Engineering in Animals
transition mutations
3.5: Mutations and DNA Repair
translation initiation complex
3.4: Translation of the polypeptide
translation stages
3.4: Translation of the polypeptide
transmission electron microscopes (TEM)
1.4: Advanced Biotechnology Equipment
transposons
5.2: Genomics
transversion mutations
3.5: Mutations and DNA Repair
triglyceride structure
2.5: Lipids
tRNA charging mechanism
3.4: Translation of the polypeptide

U

untranslated regions
3.3: Transcription of RNA
upstream processes in biomanufacturing
11.2: Bioprocess Engineering
UV radiation damage
3.5: Mutations and DNA Repair
UV spectroscopy
13.8: Lab Technique - Quantitation of DNA
UV/VIS spectrophotometers
1.3: Common Biotechnology Equipment

V

vaccine types
7.5: Medical Applications of Microbial Biotechnology
vaccines
10.3: Therapeutic Biotechnology
vectors
4.1: Principles of Genetic Engineering
viral features
7.1: Microbe classification
viral vectors
8.1: Applications of Animal Biotechnology
10.3: Therapeutic Biotechnology
visible light spectrophotometers
1.3: Common Biotechnology Equipment

W

waste management
9.3: Applications of "Gray" Biotechnology
11.1: Applications of Industrial Biotechnology
wastewater treatment
2.1: Water
9.3: Applications of "Gray" Biotechnology
water, properties
2.1: Water
Western blotting
13.12: Lab Technique - Western Blotting
wet mounts
13.14: Lab Technique - Light Microscopy
white biotechnology
1.1: Definition and a Short History of Biotechnology
11.1: Applications of Industrial Biotechnology
whole genome alignment
5.4: Comparative Genomics
whole genome sequencing
5.3: Genome Sequencing
Work instructions
12.2: QA/QC Documentation- SOPs, Batch Records, and COAs

X

xenotransplantation
8.2: Genetic Engineering in Animals

Y

yeast
7.1: Microbe classification
Yeast artificial chromosomes (YACs)
4.1: Principles of Genetic Engineering
yellow biotechnology
1.1: Definition and a Short History of Biotechnology

Glossary

ParseError: Maximum iterations exceeded! (click for details)

Detailed Licensing

Overview

Title: Biotechnology

Webpages: 90

All licenses found:

- CC BY 4.0: 95.6% (86 pages)
- Undeclared: 4.4% (4 pages)

By Page

- Biotechnology - *CC BY 4.0*
  - Front Matter - *CC BY 4.0*
    - TitlePage - *CC BY 4.0*
    - InfoPage - *CC BY 4.0*
    - Table of Contents - *Undeclared*
    - Licensing - *CC BY 4.0*
  - 1: Introduction to Biotechnology - *CC BY 4.0*
    - 1.1: Definition and a Short History of Biotechnology - *CC BY 4.0*
    - 1.2: Applications of Biotechnology - *CC BY 4.0*
    - 1.3: Common Biotechnology Equipment - *CC BY 4.0*
    - 1.4: Advanced Biotechnology Equipment - *CC BY 4.0*
  - 2: The Molecules of Life - *CC BY 4.0*
    - 2.1: Water - *CC BY 4.0*
    - 2.2: Carbon - *CC BY 4.0*
    - 2.3: Macromolecules - *CC BY 4.0*
    - 2.4: Carbohydrates - *CC BY 4.0*
    - 2.5: Lipids - *CC BY 4.0*
    - 2.6: Proteins - *CC BY 4.0*
    - 2.7: Nucleic Acids - *CC BY 4.0*
  - 3: Molecular Biology Fundamentals - *CC BY 4.0*
    - 3.1: DNA Structure - *CC BY 4.0*
    - 3.2: Replication of DNA - *CC BY 4.0*
    - 3.3: Transcription of RNA - *CC BY 4.0*
    - 3.4: Translation of the polypeptide - *CC BY 4.0*
    - 3.5: Mutations and DNA Repair - *CC BY 4.0*
  - 4: Genetic Engineering and Recombinant DNA Technology - *CC BY 4.0*
    - 4.1: Principles of Genetic Engineering - *CC BY 4.0*
    - 4.2: Creating Recombinant DNA - *CC BY 4.0*
    - 4.3: Applications of Recombinant DNA Technology - *CC BY 4.0*
  - 5: Bioinformatics- Genomics and Proteomics - *CC BY 4.0*
    - 5.1: Introduction to Bioinformatics - *CC BY 4.0*
    - 5.2: Genomics - *CC BY 4.0*
    - 5.3: Genome Sequencing - *CC BY 4.0*
    - 5.4: Comparative Genomics - *CC BY 4.0*
    - 5.5: Functional Genomics - *CC BY 4.0*
    - 5.6: Proteomics - *CC BY 4.0*
  - 6: Mammalian Cell Culture Fundamentals - *CC BY 4.0*
    - 6.1: Principles of Mammalian Cell Culture - *CC BY 4.0*
    - 6.2: Principles of Aseptic Technique - *CC BY 4.0*
    - 6.3: Cell Culture Equipment - *CC BY 4.0*
  - 7: Microbial Biotechnology - *CC BY 4.0*
    - 7.1: Microbe classification - *CC BY 4.0*
    - 7.2: Microbial Identification - *CC BY 4.0*
    - 7.3: Microbial Cultivation and Manipulation - *CC BY 4.0*
    - 7.4: Industrial Applications of Microbial Biotechnology - *CC BY 4.0*
    - 7.5: Medical Applications of Microbial Biotechnology - *CC BY 4.0*
  - 8: Animal Biotechnology - *CC BY 4.0*
    - 8.1: Applications of Animal Biotechnology - *CC BY 4.0*
    - 8.2: Genetic Engineering in Animals - *CC BY 4.0*
  - 9: Agricultural and Environmental Biotechnology – “Green” and "Gray" Biotechnology - *CC BY 4.0*
    - 9.1: "Green" vs. "Gray" Biotechnology - *CC BY 4.0*
    - 9.2: Applications of "Green" Biotechnology - *CC BY 4.0*
    - 9.3: Applications of "Gray" Biotechnology - *CC BY 4.0*
    - 9.4: Benefits of "Green" Biotechnology - *CC BY 4.0*
  - 10: Medical Biotechnology – “Red” Biotechnology - *CC BY 4.0*
    - 10.1: Principles of Medical Biotechnology - *CC BY 4.0*
    - 10.2: Diagnostic Biotechnology - *CC BY 4.0*

- 10.3: Therapeutic Biotechnology - *CC BY 4.0*
- 10.4: Regenerative Medicine - *CC BY 4.0*

- 11: Industrial Biotechnology – “White” Biotechnology - *CC BY 4.0*
  - 11.1: Applications of Industrial Biotechnology - *CC BY 4.0*
  - 11.2: Bioprocess Engineering - *CC BY 4.0*
  - 11.3: Synthetic Biology - *CC BY 4.0*
- 12: Quality Assurance and Quality Control in Biotechnology - *CC BY 4.0*
  - 12.1: Principles of Quality Assurance (QA) and Quality Control (QC) - *CC BY 4.0*
  - 12.2: QA/QC Documentation- SOPs, Batch Records, and COAs - *CC BY 4.0*
- 13: Biotechnology Lab Protocols - *CC BY 4.0*
  - 13.1: Lab Technique - Pipetting - *CC BY 4.0*
  - 13.2: Lab Technique - Polymerase Chain Reaction (PCR) - *CC BY 4.0*
  - 13.3: Lab Technique - Agarose Gel Electrophoresis of DNA - *CC BY 4.0*
  - 13.4: Lab Technique - Restriction Digest of DNA - *CC BY 4.0*
  - 13.5: Lab Technique - Ligation of Insert DNA into a Plasmid - *CC BY 4.0*
  - 13.6: Lab Technique - Bacterial Transformation - *CC BY 4.0*
  - 13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep") - *CC BY 4.0*
  - 13.8: Lab Technique - Quantitation of DNA - *CC BY 4.0*
  - 13.9: Lab Technique - DNA Sequencing - *CC BY 4.0*
  - 13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells - *CC BY 4.0*
  - 13.11: Lab Technique - SDS-PAGE - *CC BY 4.0*
  - 13.12: Lab Technique - Western Blotting - *CC BY 4.0*
  - 13.13: Lab Technique - Cell Culture - *CC BY 4.0*
  - 13.14: Lab Technique - Light Microscopy - *CC BY 4.0*
  - 13.15: Bacterial Identification Methods - *Undeclared*
  - 13.16: Basics of Bacterial Culture - *Undeclared*
- 14: Metric System and Measurements - *CC BY 4.0*
  - 14.1: Metric System Fundamentals - *CC BY 4.0*
- Back Matter - *CC BY 4.0*
  - Index - *CC BY 4.0*
  - Glossary - *CC BY 4.0*
  - Detailed Licensing - *CC BY 4.0*
  - Detailed Licensing - *Undeclared*

Detailed Licensing

Overview

Title: Biotechnology

Webpages: 90

All licenses found:

- CC BY 4.0: 95.6% (86 pages)
- Undeclared: 4.4% (4 pages)

By Page

- Biotechnology - *CC BY 4.0*
  - Front Matter - *CC BY 4.0*
    - TitlePage - *CC BY 4.0*
    - InfoPage - *CC BY 4.0*
    - Table of Contents - *Undeclared*
    - Licensing - *CC BY 4.0*
  - 1: Introduction to Biotechnology - *CC BY 4.0*
    - 1.1: Definition and a Short History of Biotechnology - *CC BY 4.0*
    - 1.2: Applications of Biotechnology - *CC BY 4.0*
    - 1.3: Common Biotechnology Equipment - *CC BY 4.0*
    - 1.4: Advanced Biotechnology Equipment - *CC BY 4.0*
  - 2: The Molecules of Life - *CC BY 4.0*
    - 2.1: Water - *CC BY 4.0*
    - 2.2: Carbon - *CC BY 4.0*
    - 2.3: Macromolecules - *CC BY 4.0*
    - 2.4: Carbohydrates - *CC BY 4.0*
    - 2.5: Lipids - *CC BY 4.0*
    - 2.6: Proteins - *CC BY 4.0*
    - 2.7: Nucleic Acids - *CC BY 4.0*
  - 3: Molecular Biology Fundamentals - *CC BY 4.0*
    - 3.1: DNA Structure - *CC BY 4.0*
    - 3.2: Replication of DNA - *CC BY 4.0*
    - 3.3: Transcription of RNA - *CC BY 4.0*
    - 3.4: Translation of the polypeptide - *CC BY 4.0*
    - 3.5: Mutations and DNA Repair - *CC BY 4.0*
  - 4: Genetic Engineering and Recombinant DNA Technology - *CC BY 4.0*
    - 4.1: Principles of Genetic Engineering - *CC BY 4.0*
    - 4.2: Creating Recombinant DNA - *CC BY 4.0*
    - 4.3: Applications of Recombinant DNA Technology - *CC BY 4.0*
  - 5: Bioinformatics- Genomics and Proteomics - *CC BY 4.0*
    - 5.1: Introduction to Bioinformatics - *CC BY 4.0*
    - 5.2: Genomics - *CC BY 4.0*
    - 5.3: Genome Sequencing - *CC BY 4.0*
    - 5.4: Comparative Genomics - *CC BY 4.0*
    - 5.5: Functional Genomics - *CC BY 4.0*
    - 5.6: Proteomics - *CC BY 4.0*
  - 6: Mammalian Cell Culture Fundamentals - *CC BY 4.0*
    - 6.1: Principles of Mammalian Cell Culture - *CC BY 4.0*
    - 6.2: Principles of Aseptic Technique - *CC BY 4.0*
    - 6.3: Cell Culture Equipment - *CC BY 4.0*
  - 7: Microbial Biotechnology - *CC BY 4.0*
    - 7.1: Microbe classification - *CC BY 4.0*
    - 7.2: Microbial Identification - *CC BY 4.0*
    - 7.3: Microbial Cultivation and Manipulation - *CC BY 4.0*
    - 7.4: Industrial Applications of Microbial Biotechnology - *CC BY 4.0*
    - 7.5: Medical Applications of Microbial Biotechnology - *CC BY 4.0*
  - 8: Animal Biotechnology - *CC BY 4.0*
    - 8.1: Applications of Animal Biotechnology - *CC BY 4.0*
    - 8.2: Genetic Engineering in Animals - *CC BY 4.0*
  - 9: Agricultural and Environmental Biotechnology – “Green” and "Gray" Biotechnology - *CC BY 4.0*
    - 9.1: "Green" vs. "Gray" Biotechnology - *CC BY 4.0*
    - 9.2: Applications of "Green" Biotechnology - *CC BY 4.0*
    - 9.3: Applications of "Gray" Biotechnology - *CC BY 4.0*
    - 9.4: Benefits of "Green" Biotechnology - *CC BY 4.0*
  - 10: Medical Biotechnology – “Red” Biotechnology - *CC BY 4.0*
    - 10.1: Principles of Medical Biotechnology - *CC BY 4.0*
    - 10.2: Diagnostic Biotechnology - *CC BY 4.0*

- 10.3: Therapeutic Biotechnology - *CC BY 4.0*
- 10.4: Regenerative Medicine - *CC BY 4.0*

11: Industrial Biotechnology – “White” Biotechnology - *CC BY 4.0*

- 11.1: Applications of Industrial Biotechnology - *CC BY 4.0*
- 11.2: Bioprocess Engineering - *CC BY 4.0*
- 11.3: Synthetic Biology - *CC BY 4.0*

12: Quality Assurance and Quality Control in Biotechnology - *CC BY 4.0*

- 12.1: Principles of Quality Assurance (QA) and Quality Control (QC) - *CC BY 4.0*
- 12.2: QA/QC Documentation- SOPs, Batch Records, and COAs - *CC BY 4.0*

13: Biotechnology Lab Protocols - *CC BY 4.0*

- 13.1: Lab Technique - Pipetting - *CC BY 4.0*
- 13.2: Lab Technique - Polymerase Chain Reaction (PCR) - *CC BY 4.0*
- 13.3: Lab Technique - Agarose Gel Electrophoresis of DNA - *CC BY 4.0*
- 13.4: Lab Technique - Restriction Digest of DNA - *CC BY 4.0*
- 13.5: Lab Technique - Ligation of Insert DNA into a Plasmid - *CC BY 4.0*
- 13.6: Lab Technique - Bacterial Transformation - *CC BY 4.0*
- 13.7: Lab Technique - Isolation of DNA from Bacteria (The "Mini-Prep") - *CC BY 4.0*
- 13.8: Lab Technique - Quantitation of DNA - *CC BY 4.0*
- 13.9: Lab Technique - DNA Sequencing - *CC BY 4.0*
- 13.10: Lab Technique - Liposomal transfection of DNA into mammalian cells - *CC BY 4.0*
- 13.11: Lab Technique - SDS-PAGE - *CC BY 4.0*
- 13.12: Lab Technique - Western Blotting - *CC BY 4.0*
- 13.13: Lab Technique - Cell Culture - *CC BY 4.0*
- 13.14: Lab Technique - Light Microscopy - *CC BY 4.0*
- 13.15: Bacterial Identification Methods - *Undeclared*
- 13.16: Basics of Bacterial Culture - *Undeclared*

14: Metric System and Measurements - *CC BY 4.0*

- 14.1: Metric System Fundamentals - *CC BY 4.0*

Back Matter - *CC BY 4.0*

- Index - *CC BY 4.0*
- Glossary - *CC BY 4.0*
- Detailed Licensing - *CC BY 4.0*
- Detailed Licensing - *Undeclared*