3.5.1: Blotting

Blotting provides a means of identifying specific molecules out of a mixture. It employs three main steps. First, the mixture of molecules is separated by gel electrophoresis. The mixture could be DNA (Southern Blot), RNA (Northern Blot), or protein (Western Blot) and the gel could be agarose (for DNA/RNA) or polyacrylamide (for protein). Second, after the gel run is complete, the proteins or nucleic acids in the gel are transferred out of the gel onto a membrane/paper that physically binds to the molecules. This “blot", as it is called, has an imprint of the bands of nucleic acid or protein that were in the gel (see figure at left). The transfer can be accomplished by diffusion or by using an electrical current to move the molecules from the gel onto the membrane. The membrane may be treated to covalently link the bands to the surface of the blot. Last, a visualizing agent specific for the molecule of interest in the mixture is added to the membrane. For DNA/RNA, that might be a complementary nucleic acid sequence that is labeled in some fashion (radioactivity or dye). For a protein, it would typically involve an antibody that specifically binds to the protein of interest. The bound antibody can then be targeted by another antibody specific for the first antibody. The secondary antibody is usually linked to an enzyme which, in the presence of the right reagent, catalyzes a reaction that produces a signal (color or light) indicating where the antibody is bound. If the molecule of interest is in the original mixture, it will “light" up and reveal itself.

Western Blots

In a western blot procedure, proteins are first separated on an SDS-PAGE gel and then transferred to a membrane. This membrane replica is treated with antibodies that specifically recognize a protein or epitope of interest. Additional processing steps generate a signal at the position of the bound antibody. Between the steps, various washes are done to increase the signal-to-noise ratio on the final, developed blot. The major steps involved in a typical western blot are as follows:

- Electrophoretic transfer of proteins from an SDS-PAGE gel to a membrane
- Blocking of nonspecific protein binding sites on transfer membranes
- Incubation of the membrane with a primary antibody specific for the epitope of interest
- Incubation with a secondary antibody that recognizes primary antibodies
- Visualization of bound antibodies

Electrophoretic transfer of proteins from an SDS-PAGE gel to a membrane

The first step in a western blot is to generate a replica of the SDS-PAGE gel by transferring proteins electrophoretically to a synthetic membrane with a high protein binding capacity. The membranes made of polyvinylidine fluoride (PVDF), a kind of plastic, are hydrophobic and the dry membranes do not wet properly with water. Therefore, PVDF membranes are first wet with methanol, then rinsed with deionized water, and finally rinsed with transfer buffer. They must not be allowed to dry out during the transfer and immunoblot procedures. If they do dry out, they must be re-wet with methanol and rinsed with water before proceeding.

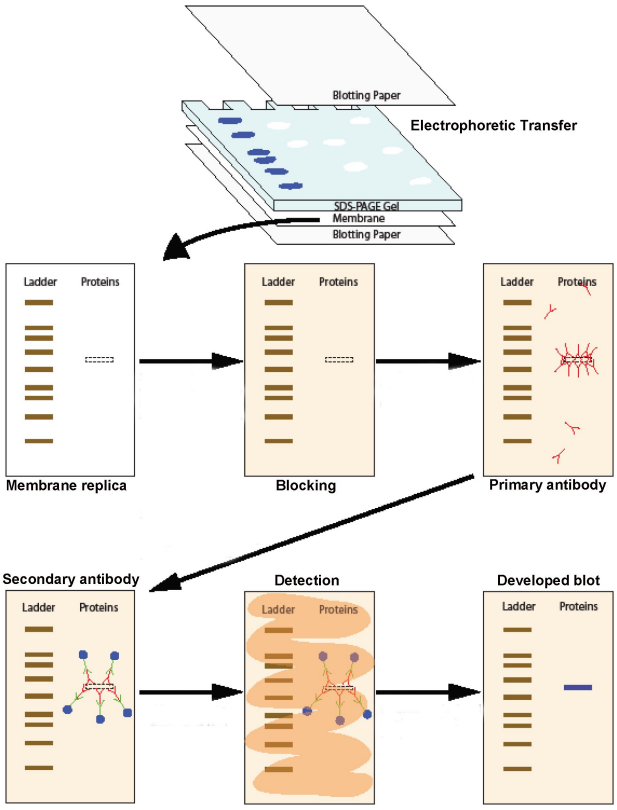

Figure 3.1.1.3: *The major steps in a typical western blot.*

During the transfer process, the gel and membrane are placed directly against each other within a “sandwich” of pre-wet filter papers and foam pads. During the electrophoretic transfer, current should flow evenly across the entire surface area of the gel. It is important, therefore, that air bubbles are not trapped between the gel and membrane. After the electrophoretic transfer, which can be done in a few hours or overnight with reduced voltage, the membrane replica with the transferred proteins can be allowed to dry out and stored for later visualization with antibodies.

Blocking of non-specific protein binding sites on membranes

The transfer membranes used in western blots bind proteins nonspecifically. Before the membranes are incubated with specific (and expensive) antibodies, they must be pretreated with blocking solutions that contain high concentrations of abundant (and cheap) proteins to saturate non-specific binding sites. If the transfer membranes are not adequately blocked before the antibody is applied, the nonspecific sites on the membranes will absorb some of the antibodies, reducing the amount of antibody available to bind the

Primary antibody binding

Either polyclonal or monoclonal antibodies can be used as the primary antibody on western blots. Antibodies can be directed toward a naturally-occurring protein or toward an epitope attached to an overexpressed protein (as we are doing). Increasingly, researchers are using epitope-tagged proteins in their experiments, because antibodies against naturally- occurring proteins are expensive and time-consuming to prepare.

Secondary antibody binding

The secondary antibodies used in western blots are designed to bind the FC fragments of primary antibodies, taking advantage of cross-species differences in antibody sequences. Secondary antisera are generally prepared by injecting an animal with FC fragments of IgGs from a second species. The first animal recognizes the FC fragments as foreign antigens and produces antibodies that bind the FC fragments.

Visualization of bound antibody

In this final step, the western blot is incubated with substrates for the enzyme that has been conjugated to the secondary antibody.

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