Chapter 15

Western blots

Western blots are one of the most widely used techniques in cell biology. In a western blot, investigators take advantage of the exquisite sensitivity of antibodies to identify proteins of interest in complex samples. In this lab, you will learn about the different kinds of antibodies used in western blots. You will use western blots to analyze Met and LacZ protein expression in your transformed yeast strains.

Objectives

At the end of this lab, students will be able to:

- explain how monoclonal and polyclonal antibodies are produced.
- identify the different functional regions of antibodies and explain how they are used in western blots
- design a strategy that uses antibodies to detect epitope-tagged proteins.
- prepare a western blot to analyze protein expression in cell extracts.
Western blots provide a method to find the proverbial “needle in a haystack.” A typical cell expresses thousands of different proteins, and it is often difficult to detect changes in expression of your favorite protein (Yfp) without a probe that is capable of discriminating the Yfp against a large background of unrelated cellular proteins. Fortunately, antibodies provide highly specific molecular probes that can be used to detect the expression of proteins on western blots. To appreciate the sensitivity of western blots, it’s helpful to have some understanding of antibody structure and antibody production during immune responses. (Disclaimer: The following paragraphs provide a highly abbreviated overview of antibodies and one segment of the complex vertebrate immune system. The Department offers an immunology course that will introduce you to the finer details of this fascinating system.)

**Antibodies are produced in response to antigens**

Antibodies are proteins produced by vertebrates with adaptive immune systems capable of responding to foreign antigens. Antigens are defined as substances that stimulate the production of antibodies. Antigens are commonly able to stimulate the production of multiple kinds of antibodies, each of which recognizes a small, distinct region on the surface of the antigen known as an epitope. Antibodies are Y-shaped proteins produced by lymphocytes that bind epitopes with high affinity.

The availability of hybridoma cells that secrete large quantities of antibodies with a single specificity has greatly facilitated structural studies on antibodies. Researchers are able to harvest antibody molecules secreted by cultured hybridoma cells and to prepare crystals for X-ray diffraction. Based on a large number of crystallographic studies, we now understand the basic architecture of antibodies, more properly known as immunoglobins. The crystal structures show that immunoglobulins (Igs) are composed of three domains that are readily apparent in the crystal structure (below). The two F\textsubscript{ab} regions (antigen-binding fragments) that form the arms of the “Y” are hypervariable regions involved in binding antigen. The F\textsubscript{c} region (crystallizable fragment) that forms the base of the “Y” is recognized by non-immune effector cells, such as mast cells and macrophages, which process antigen-antibody complexes. Each Ig class has a characteristic heavy
chain, which gives the class its name. We are using antibodies from the IgG class of immunoglobins, which have gamma heavy chains. (IgGs are also known as gamma globulins.) IgA molecules have alpha chains, IgM molecules have mu chains, etc.

Crystal structure of an IgG antibody.
This figure is derived from Protein Data Bank entry 1IGT (Harris et al., 1997).

Antibodies are produced by lymphocytes

In the initial stages of the immune response, small numbers of immature B lymphocytes are able to bind foreign antigen molecules weakly via the antibodies expressed on their surfaces. Antigen binding stimulates the lymphocytes to proliferate and to differentiate into mature lymphocytes that secrete antibodies. An amazing series of transformations occur as B lymphocytes mature in response to antigen. Antigen binding stimulates responding lymphocytes to rearrange segments of their antibody-encoding genes, producing new potential antigen-binding sites. Most rearrangements are unproductive, but some rearrangements generate antibodies with greater affinity for the antigen. Antigens act as selective agents. The lymphocytes that bind the antigen with the highest affinity receive the greatest growth signal and proliferate most rapidly, because a higher fraction of their surface antibodies are bound to antigen at any one time. In the latter stages of differentiation, a hypermutation process further increases the range of potential antibody sequences. Mature B lymphocytes that have survived the selection process are known as plasma cells. Each plasma cell secretes a single antibody with high affinity for antigen. Plasma cells are virtual antibody factories that can be identified in electron micrographs by their extensive rough endoplasmic reticulum. The scope of antibody diversity is immense - vertebrates are capable of producing billions of antibody molecules with distinct specificities.

Polyclonal vs. monoclonal antibodies

For our western blots, we will be using both monoclonal and polyclonal antibodies. As their names imply, monoclonal antibodies bind to the same epitope on an antigen. Polyclonal antibodies are actually mixtures of antibodies that bind to different epitopes on an antigen. An
animal’s response to antigen is polyclonal, because antigens stimulate the proliferation of multiple lymphocyte clones, each of which produces a different antibody to the antigen. Consequently, the serum collected from an immunized animal contains a mixture of antibodies with different specificities. The polyclonal antibodies used in the lab are purified from the sera of animals that have been inoculated with antigen.

By contrast, monoclonal antibodies are produced in the lab from cultured hybridoma cells. Hybridoma cells are generated by fusing a lymphocyte from an immunized animal, most commonly a mouse, with a cancerous myeloma cell that can divide indefinitely in culture (right). Because the lymphocytes from the spleen of an immunized mouse recognize a range of different epitopes on an antigen, the hybridomas resulting from the fusion secrete a variety of different antibodies. Standard culture techniques are then used to isolate individual hybridoma cell lines, each of which secretes a unique antibody that binds to a single epitope.

Hybridoma technology has revolutionized biomedical research since its description (Kohler & Milstein, 1975), both because monoclonal antibodies recognize well-defined epitopes and because monoclonal antibodies can be produced indefinitely by cultured hybridoma cells. Investigators often use both monoclonal and polyclonal antibodies at different steps in western blots.

Plasmid-encoded proteins have C-terminal tags

In this lab, we will be using antibodies to detect Met and LacZ fusion proteins expressed in transformed *S. cerevisiae*. These fusion proteins have been engineered to add several functional elements to the C-termini of Met and LacZ proteins. Consequently, the fusion proteins expressed from the pBG1805 (Gelperin *et al.*, 2005) and pYES2.1 plasmids have C-terminal tags that can interact with a variety of reagents. Each of the plasmids encodes epitope tag(s) that can be detected with antibodies on western blots. These epitopes correspond to highly immunogenic amino acid sequences on the surfaces of viruses that have been shown to be potent inducers of antibody production. The pBG1805 sequence encodes an 9-amino acid sequence of the human influenza virus hemagglutinin (HA) protein (Sleigh *et al.*, 1981), while the pYES2.1 plasmid encodes a 15-amino acid sequence from the P protein of simian virus V5 (Southern *et al.*, 1991). In our blots, we will use a monoclonal antibody directed against the V5 protein, hereafter referred to as anti-V5, to detect proteins expressed from pYES2.1-based plasmids.
The pYES2.1 plasmid also encodes a tag consisting of 6 histidines. This His\textsubscript{6}-tag binds tightly to metal ions, so it is commonly used to purify overexpressed proteins by passing them through a resin with immobilized zinc or cobalt ions. Unfortunately the His\textsubscript{6}-tag is not very immunogenic, so it is rarely used in western blots. Together, the V5 and His\textsubscript{6} tags add ~5 kDa to the molecular weight of the expressed protein.

Western blots involve many steps

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 in a typical western blot are diagrammed on the following page and discussed in greater detail in sections that follow:

- 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. In our experiments, we will use membranes made of polyvinylidene fluoride (PVDF), a kind of plastic. PVDF membranes 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.

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 (see the figure below). 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. Think of this step as analogous to an artist priming a canvas with a lower quality paint before the more expensive media is applied. 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 target proteins. In our experiments, we will use casein proteins from milk as blocking reagents. Because our experiments do not require high sensitivity, rehydrated non-fat dry milk (direct from the grocery store!) is an adequate source of caseins.

**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. In addition, an antibody directed against an epitope can be used to detect many different proteins carrying that same epitope. In our western blots, we will use a mouse monoclonal antibody that binds the V5 epitope on Met and LacZ proteins expressed from the pYES2.1 plasmid.

**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. The secondary antibody in our experiment is a rabbit polyclonal antibody prepared against the Fc domains of mouse IgGs. The antibody will bind the Fc domains of the mouse anti-V5 antibodies bound to the pYES2.1-encoded proteins.
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. Our secondary antibody has been conjugated to HRP, a hardy enzyme with a high turnover number. (The turnover number is the number of product molecules produced at an enzyme's active site per second.) HRP catalyzes the reaction of hydrogen peroxide and 3,3',5,5' - tetramethylbenzidine (TMB), which generates a dark blue-grey reaction product that precipitates at the reaction site on the western blot. Colored reaction product accumulates with time until the reaction is stopped by washing away unreacted substrate. The reaction should be terminated before nonspecific antibody binding becomes problematic, as evidenced by the appearance of many weakly staining bands.

Exercise 1 - Preparing the membrane replica

Separate proteins on an SDS-PAGE gel
1. Separate the proteins that will be analyzed on western blots by SDS-PAGE.
2. Remove the electrode apparatus and holder from the tank, and remove the gel from the holder. Do not remove the gel from the plates until you are ready to assemble the transfer cassette (see below).
3. Dispose of the remaining buffer down the sink. Rinse out the buffer tank with deionized water to remove residual SDS, which can interfere with the transfer process.
Prepare the transfer membrane

NOTE: DO NOT touch transfer membranes with your fingers. Wear gloves and use filter forceps when you handle transfer membranes.

1. Gather the PVDF membrane and two pieces of thick filter paper, such as Whatman 3MM™. The PVDF membrane and filter papers should be cut to a size that is slightly larger than the SDS-PAGE gel. You will also need a transfer cassette and two fiber pads.

2. Prepare the PVDF membrane. Using pencil, place an orientation mark in a corner of the PVDF membrane for later identification. Wet the membrane by placing it in a small tray containing methanol for ~30-60 seconds with gentle agitation.

3. Dispose of the methanol in the waste container and add deionized water to the tray. Gently agitate for ~1 minute.

4. Replace the deionized water with transfer buffer. Store the membrane in transfer buffer until you are ready to start the transfer.

Assemble the transfer cassette

1. Using a spatula or a green plastic wedge, remove the small glass plate from the gel. The gel will remain attached to the large glass plate. With a spatula, remove the lower right corner of the gel to serve as an orientation mark. (This corresponds to the first lane of your gel.)

2. Assemble the transfer cassette as shown below. Be sure that all parts of the transfer “sandwich” remain moist at all times.

- Place a wet fiber pad (2) on top of the black cassette face (1).
- Add two pieces of filter paper (3,4).
- Position the gel (5) on top of the filter paper while it is still attached to the glass plate. Use a spatula to carefully release the gel from the plate. You may find it easier to remove the gel by beginning at the bottom edge near the dye front.
- Place the PVDF membrane (6) on top of the gel. Orient the gel so that the pencil mark on the membrane corresponds to the clipped corner of the gel. **Be sure that there are NO air bubbles between the gel and the membrane.**
- Add the remaining filter papers (7,8) and the fiber pad (9).
- Fold the clear cassette face (10) over the gel assembly and carefully slide the clamp into place.
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**Electrophoretic protein transfer**

1. Place the transfer sandwich into the cassette holder with the black face of the transfer cassette aligned with black side of the cassette holder and the clear face aligned with red side of the cassette holder (right). **NOTE**: Each cassette holder can hold two transfer cassettes.

2. Place the cassette holder and assembled cassettes into the electrophoresis tank. Add an ice pack to the tank.

3. Fill the electrophoresis tank to the top with transfer buffer.

4. Place lid on tank by aligning black with black and red with red.

5. Transfer proteins at 100 V for 1 hour at room temperature or at 20 V overnight in the cold room. If you are transferring overnight, be sure to label the tank clearly and to coordinate the following day’s activities with others sharing the tank with you.

6. When the transfer is complete, remove the transfer cassette from the tank. Pour the transfer buffer back into its original bottle so that it can be reused.

7. Disassemble the transfer cassette. Depending on your schedule:
   - If you will be continuing with the western procedure, skip the rehydration step (step 1) below and continue with the blocking step (step 2). Be careful that the membrane remains moist!
   - If you will be processing the membrane at a later time, allow the membrane to dry out. Wrap the membrane in plastic wrap and save it for a later lab period.

**Exercise 2 - Immunodetection**

This is a multi-day procedure. Timing may vary for different classes.

**Membranes are rehydrated and treated with blocking reagents**

1. Wearing GLOVES, unwrap the dry blot from the plastic wrap. Use the prestained standards to identify the side of the membrane to which the proteins are bound. Submerge the membrane in methanol with this side facing up. Gently agitate the membrane by hand rocking for 30-60 seconds until the membrane has been uniformly wet with methanol. Decant the methanol into the appropriate container and fill the tray half way full with deionized water. Gently agitate the membrane for an additional minute.

2. Decant the water and replace it with sufficient TBS-T (Tris buffered-saline containing 0.05% Tween 20) to cover the blot. Place the blot on a rocking platform. Equilibrate the blot in TBS-T for 5 minutes with slow rocking. At the end of 5 minutes, drain off the TBS-T.

3. Pour enough blocking solution (5% nonfat milk in TBS-T) onto the blot to cover it.

4. Cover the tray with a small piece of plastic wrap. Label the tray clearly and place the tray on a rocking platform in the cold room. The blot should float freely in the tray so that both sides are washed. Incubate the blot for at least an hour or up to 24 hours at 4°C.
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Membranes are washed and incubated with primary antibody (~24 hours)
1. Locate your blot in the cold room and bring it back to the lab room.
2. Remove the plastic wrap from the container holding the blot and pour off the blocking solution. SAVE the plastic wrap! You will need it to cover the container again!
3. Add enough TBS-T to cover the blot and place the container on the rocking platform. Rock for 5 minutes.
4. Pour off the TBS-T. Add 15 mL of primary antibody diluted in blocking buffer.
5. Cover the container with the same piece of plastic wrap and place the tray on the rocking platform in the 4˚C cold room. Make sure that the blot floats freely in the tray and that the standards are on the top face of the blot. Incubate overnight at 4˚C with slow rocking. NOTE: The timing of this step is the most critical in the procedure. Shortening the incubation time with primary antibody may reduce the sensitivity of the western blot.

Secondary antibody binding and detection (1.5-2 hours)
1. Locate your blot in the cold room and bring it to your lab classroom.
2. Carefully drain the antibody from the blot into the test tube marked “Used primary antibody”. (Antibodies are expensive. Fortunately, the solutions can be re-used.)
3. Fill the tray with the blot about half-full with TBS-T. Place the tray on a rocking platform and wash the membrane for 5 minutes to remove unbound primary antibody. Drain the TBS-T when the wash is complete.
4. Repeat step 3 once more, for a total of two washes.
5. Add enough secondary antibody to cover the blot and incubate the membrane for 1 hour with gentle rocking at room temperature. The secondary antibody, which is conjugated to horseradish peroxidase (HRP), has been diluted in TBS-T.
6. Carefully drain the antibody from the blot into the test tube marked “Used secondary antibody.”
7. Wash the membrane 3 times for 5 minutes each with TBS-T, as in step 3.
8. Drain the TBS-T from the blot. Using a P1000 micropipette, cover the blot with 1 mL of 3,3’5,5’-tetramethyl benzidine (TMB), a colorigenic substrate for HRP. Let the color continue to develop until distinct bands are apparent. Bands will probably become apparent within minutes. Do not allow the blot to over-develop, when nonspecific bands become apparent.
9. Stop color development by diluting the substrate with an excess of deionized water. Drain the diluted substrate into the waste container.
10. Allow the blot to dry on a piece of filter paper. Record your data with your cell phone camera.
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References


