

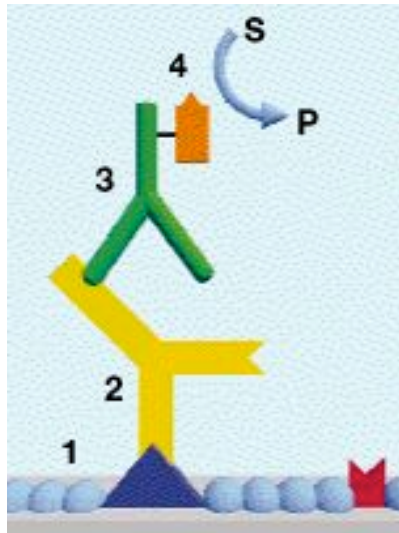
## Chem 405 Biochemistry Lab I Western Blot (immunoanalysis) Developing of MGH

### Background

Immunoblotting (western blots) uses specific interactions of antibodies to detect a protein of interest. Western blotting can be divided into two steps: transfer of the protein from the gel to the matrix (paper) and detection of the epitope with antibodies. Last week we transblotted the SDS-PAGE gel onto the nitrocellulose paper (transfer paper) and left the blot in blocking solution until this week. We will now finish the second half of the immunoblot, detection.

There are several methods to develop a western blot including radioactivity, chemiluminescence, fluorescence and colorimetric systems. Each of these methods use a series of incubations and washes to allow specific antibodies to react with the antigens or target proteins. The antibody that recognizes the protein on the paper is considered the *primary antibody*. A second antibody is then used to detect the primary protein and this second protein is called the *secondary antibody*. This secondary antibody is often linked to an enzyme that will allow for the detection of a protein. Chemiluminescent techniques use secondary antibodies conjugated (linked) to horseradish peroxidase to generate light wherever the complex of antigen, primary and secondary proteins/antibodies are located. this signal is detected by exposing the blot to x-ray film.

We will be using secondary antibodies conjugated to *alkaline phosphatase*. This enzyme will metabolize the substrate (bromochloroindoyl phosphate/nitro blue tetrazolium, or BCIP/NBT) to give a blue black color. The product of the alkaline phosphatase with the BCP/NBD is insoluble and will permanently precipitate onto the paper. This type of detection gives very sharp bands with low background staining of the membrane. This is one of the easiest methods for western blot detection.



**Figure 1: Specific enzymatic detection of membrane-bound antigens.**

1. Dry milk blocks the unoccupied sites on the membrane.
2. Primary antibody to a specific antigen is incubated with the membrane.
3. An antibody enzyme conjugate is added to bind to the primary antibody.
4. Color development reagent is then added to the blot. The HRP or AP enzyme catalyzes the formation of a colored precipitate from the substrate at the site of the antigen-antibody complex

There are two disadvantages to this method: the signal or bands fade over time when exposed to light, and second, it is not as sensitive as some of the other methods such as chemiluminescence.

The steps are listed below. Blocking with dry milk covers *un-reacted portions* of the blotted paper. Remember the only protein on the paper

is from the gel. If we were to add the antibodies directly to the paper most of the primary antibody will non-specifically bind to the paper. The blot is then washed off with salt and detergent (tween 20) and the primaries are added. tween 20 is essential in washing to eliminate overall non-specific hydrophobic interactions. At 0.5%, tween 20 will not disrupt binding of primary antibodies to antigens, but will optimize detection by eliminating the non-

specific interactions. Inclusion of the blotting solution will help reduce non-specific interactions with proteins from the gel and the antibody (nothing works perfect). The primaries are washed away the blot.

and secondary antibody added. Again the blotting buffer is included to reduce background and non-specific signals. The secondary antibodies are washed away and color substrate is added to develop

### **Molecular weight determination of MGH by SDS-PAGE.**

We still need to finish up some of the experiment from last week. SDS-PAGE is frequently used to determine the molecular weight of a protein since protein migration is generally proportional to the mass of the protein. A standard curve is generated with proteins of known molecular weight, and the molecular weight of the protein of interest can be extrapolated from this curve

#### **For the MGH Paper:**

- Following gel electrophoresis and coomassie blue staining, take a picture of the gel. This will be done following instructions posted next to Dr Provost's computer in the research lab.
- Later you will measure the distance of migration of the proteins as well as that of the tracking dye (bromophenol blue). Distance of migration is measured from the beginning of the resolving gel to the leading edge of a protein band.
- Calculate the Rf values for each of the protein standards and the GFP using the formula below

$$Rf = \text{Distance of protein migration} / \text{distance of tracking dye migration}$$

- Plot the log of the known protein molecular weights as a function of the Rf. The area in the middle of the gel should yield a straight line.
- Interpolate the molecular weight of the MGH from the graph
- HOW do you know your protein is the biggest band? THIS should be addressed in your paper? IMPORTANT POINT!
- Does this molecular weight match that which you calculated from the amino acid sequence?

### **Western Blot Procedure.**

1) Wash the blot

- pour out old solution and rinse with 5-10 ml of TTBS.
- incubate at room temp with shaking for 2 min.

2) Incubate with primary antibody

- decant TTBS
- add in 5 ml of 1:2000 diluted primary antibody in TTBS
- incubate at room temp with rocking for 45 min. (by hand!)

3) Wash the blot 3 times

- pour out old solution and rinse with 10-20 ml of TTBS.
- incubate at room temp with shaking for 3-5 min.
- repeat last two steps a total of three times

5) Incubate with secondary antibody

- pour out TTBS
- add in 10 ml of 1:5000 diluted secondary antibody in TTBS
- incubate at room temp with shaking for 30 min.

6) Wash the blot 3 times

- pour out old solution and rinse with 20 ml of TTBS.
- incubate at room temp with shaking for 5 min.
- repeat last two steps a total of three times

7) Develop Blot (do this step just before needed)

- pour out old solution
- add 100  $\mu$ l of BCIP and 100  $\mu$ l NBT to 10 ml of color developing buffer (stock color developing buffer is 25X)
- incubate with shaking at room temp until color develops

8) Wash with water and let dry on test tube rack in drawer.

9) Take picture of blot results with camera

**Points to include in your paper:** Compare the gel vs the blot. Do the bands you believe is the MGH from the SDS-PAGE gel correlate to the band identified by the western blot? Based on the gel, how pure is the preparation? Are there other protein bands that are found in the western blot? Why are they there? How do the molecular weight of the MGH determined by the SDS-PAGE gel match the results determined by the amino acid sequence? Why might they be different? Don't always assume the differences are mistakes or one is better than another. Rather think of the different conditions that each measurement was taken and relate that to differences in results.