Labs 7-9: Labeled Immunoassays

Background to Labeled Immunoassays

The need to develop rapid, specific and sensitive assays to determine the presence of important biologically active molecules ushered in a new era of testing in the clinical laboratory. Labeled immunoassays are designed for antigens and antibodies that may be small in size or present in very low concentrations. The presence of such antigens or antibodies is determined indirectly by using a labeled reactant to detect whether or not specific binding has taken place. Labeled immunoassays have made possible rapid quantitative measurement of many important entities such as virus antigens in patients infected with human immunodeficiency virus (HIV) and other infectious agents. The ability to detect antigen or antibody very early during the course of an illness has revolutionized diagnosis, monitoring, and determining treatment options for numerous diseases.

Constituents of Labeled Assays

Current techniques include the use of fluorescent, radioactive, chemiluminescent and enzyme labels. The underlying principle of all of these techniques is essentially the same. In all of these assays the following are usually a part of the assay:

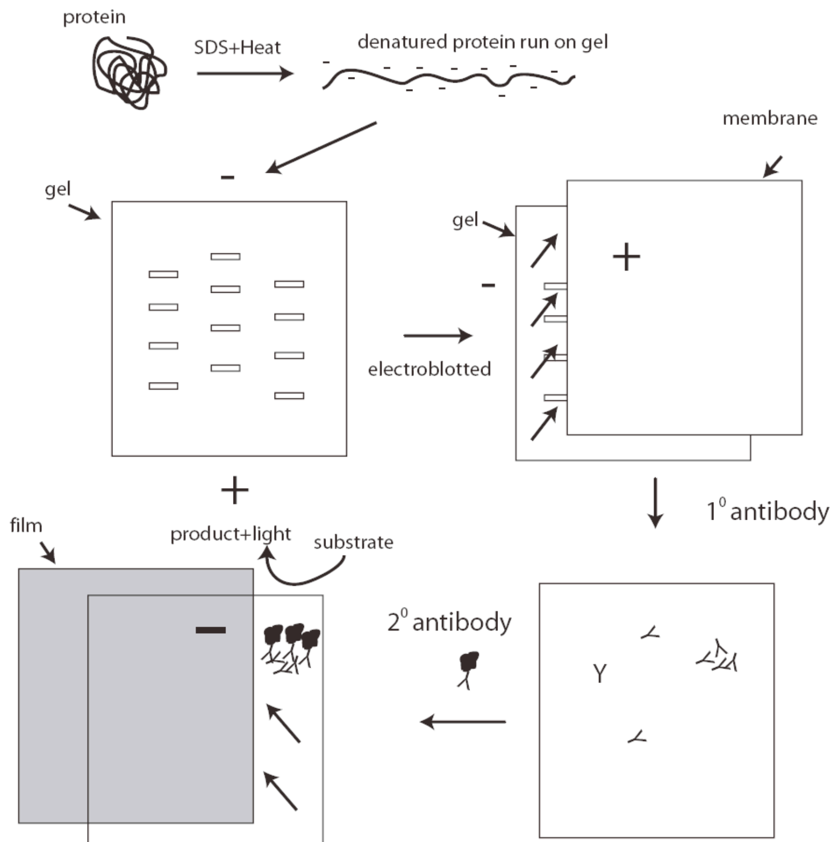
1) antibody to the antigen in question, which is specific, and of high affinity
2) standards or calibrators of known quantities
3) a means of separation of the bound vs. free components such precipitation, adsorption to particles or solid-phase adsorption
4) a means of detection of the label such as radioactivity, fluorescence, chemiluminescence or a colorimetric probes.

Fluorescent molecules are commonly used for detection in immunofluorescence assays and Flow Cytometry. **Colorimetric** probe detection is used in Enzyme-linked Immunosorbent assays (ELISA). Western Blots can be detected using **colorimetric** probes or via **chemiluminescent** detection. Immunohistochemistry, which entails antigen detection on tissue sections, usually uses colorimetric probes.

Lab 7: Western Blots

Principle of Western Blotting

A Western Blot is used to detect a specific protein from within a complex mixture of proteins. By this technique, electrophoresis of antigenic material yields separation of the antigenic components by molecular weight. Briefly in electrophoresis a sample is first lysed in a solution to release proteins. Samples are coated with sodium dodecyl sulfate (SDS) to uniformly coat the samples with a negative charge. The lysate is then separated via polyacrylamide gel electrophoresis (PAGE) where the electric current drives the proteins toward the positive pole and separates them based upon their molecular weights. The separated protein bands are then transferred from the gel to a nitrocellulose membrane by a second electrophoresis, and individual protein bands can subsequently be identified by incubating the nitrocellulose membrane with a monoclonal or polyclonal antibody that is specific for the protein of interest. The identification of antigen is due to reactivity with specific antibodies. The antibody that is specific to the antigen is called the **primary antibody**. A second antibody is then used which binds to the constant portion of the primary antibody and is the **secondary antibody**. The enzyme-linked **secondary antibody** is used to detect the antigen, with **alkaline phosphatase (AP)** and **horseradish peroxidase (HRP)** being the two most commonly used enzymes. Chemiluminescent substrates are now also commonly used which have approximately 10-fold increased sensitivity. Electrophoresis of known molecular weight standards allows for the determination of the molecular weight of each antigenic band to which antibodies may be produced. A schematic of the Western blot immunoassay is shown in **Figure 1.**



Uses of Western Blot analysis

Western blots are commonly used to identify antigens in research. It is also useful in clinical medicine as a more selective assay, where the patients' serum is the source of antibody. For example, western blots are the most widely accepted confirmatory assay for the detection of HIV antibodies (anti-HIV-2 specific) and is considered the "gold standard" for the validation of HIV infection. A positive Western blotting result not only confirms the presence of antibodies reactive with HIV in the infected individual but also permits identification of the specific viral components to which that individual has raised a detectable humoral immune response. Recognized viral antigens produce bands at p18, p24, p31, gp41, p51 p55, p66, gp 120 and gp 160 (numbers refer to apparent molecular masses, measured in kilodaltons). By performing serial dilutions of the test sample, it can also be used to grade the intensity of the individual components of that response qualitatively and, in some cases, quantitatively over time. By comparison with control sera as well as with internal reference standards, results can be scored visually in terms of the pattern and number of antibody bands present. Densitometry techniques allow quantitation of bands. Such quantitation is useful in evaluating the humoral response to candidate AIDS vaccines in noninfected seronegative recipients following primary and booster immunizations.

Today's Lab:

In this lab you will conduct a Western Blot to detect antibodies to 2 subunits of cytochrome b, a component of NADPH oxidase of neutrophils. One subunit has a molecular weight of 22 kDa and the other subunit a molecular weight of 91 kDa.

WESTERN BLOT PROTOCOL

1. Block blot (proteins run on PAGE and transferred to nitrocellulose) for 1 hr or overnight at 4° C

2. Dip blot in Blot **wash buffer (Tris Buffer + Tween 20)** once to remove excess block

3. Incubate blot in **primary antibody** for 1 hr

4. Wash 3 x 5 min in wash buffer

5. Incubate blot in **alkaline conjugated secondary antibody** for 30'

6. Wash 3x in wash buffer

7. Add Substrate (BCIP/NBT) for 1- 30 min; use 1 ml/10 ml buffer;
   put in water to stop reaction;
   NOTE: OUR reaction occurs quickly – usually within 1-5 minutes

BLOCKING BUFFER: 5% dry milk + 0.2% Tween 20 in PBS

ANTIBODIES:
Primary antibodies:
anti-p22 phox; monoclonal antibody, (mouse); diluted 1:1000

anti-gp91phox; monoclonal antibody (mouse); diluted 1: 1000

Secondary antibodies:
Goat anti-mouse-alkaline phosphatase (AP); diluted 1: 1000

Goat anti-mouse-alkaline phosphatase (AP); diluted 1: 1000

SUBSTRATE:
BCIP/NBT: Consists of BCIP (5-bromo-4-chloro-3-indoyl-phosphate), the substrate for alkaline phosphatase + nitroblue tetrazolium (NBT) which produces the colored reaction product.