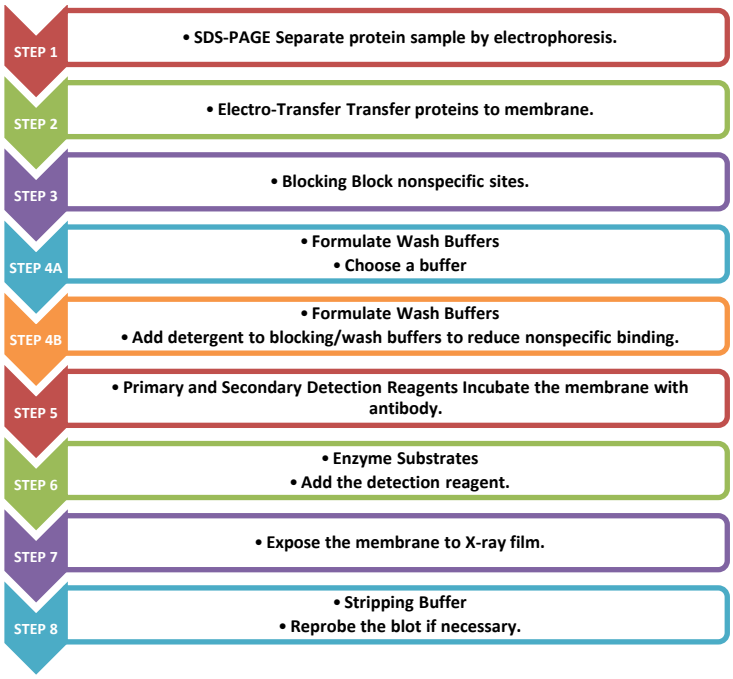
Western Blotting

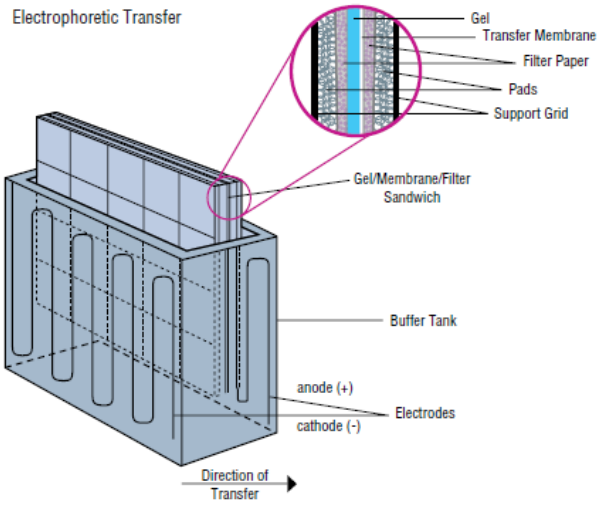
Introduction

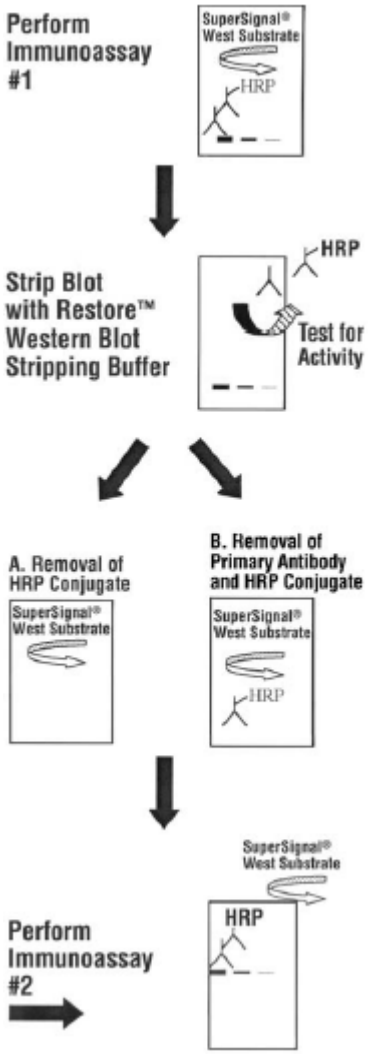
The term “blotting” refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture. Western blotting is commonly used to positively identify a specific protein in a complex mixture and to obtain qualitative and semiquantitative data about that protein.

Procedure

The first step in a Western blotting procedure is to separate the macromolecules (proteins) using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.





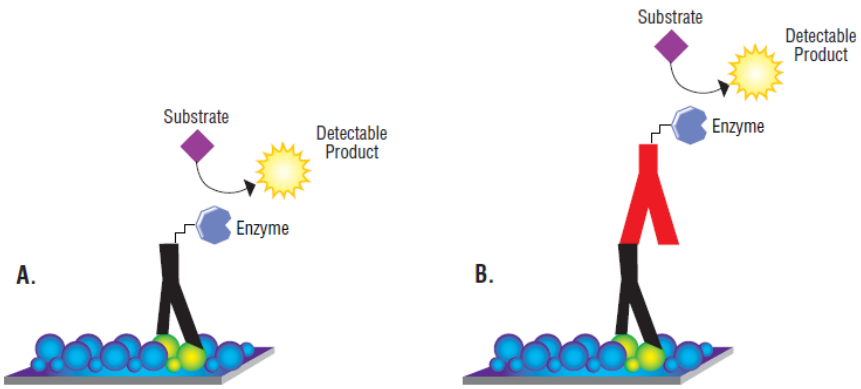


A. Electrophoretic transfer **B. Striping**

Direct vs Indirect detection

With the **direct detection** method, the primary antibody that is used to detect an antigen on the blot is also labeled with an enzyme or fluorescent dye. This detection method is not widely used as most researchers prefer the indirect detection method for a variety of reasons.

In the **indirect detection** method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase. The indirect method offers many advantages over the direct method.



1A. Direct Detection

1B. Indirect Detection

Primary and Secondary Antibodies

The choice of a primary antibody for a Western blot will depend on the antigen to be detected and what antibodies are available to that antigen. Both polyclonal and monoclonal antibodies work well for Western blotting. Polyclonal antibodies are less expensive and less time-consuming to produce and they often have a high affinity for the antigen. Monoclonal antibodies are valued for their specificity, purity and consistency that result in lower background.

Antibody Labels

Enzymatic labels are used most commonly and, although they require extra steps, they can also be extremely sensitive.

Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes that are used extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates is available for use with either enzyme.

	Horseradish Peroxidase	Alkaline Phosphatase
Size	40 kDa	140 kDa
Price	Relatively Inexpensive	Relatively Expensive
Stability (Storage)	Stable at < 0°C	Unstable at < 0°C
Number of Substrates	Many	Few
Kinetics	Rapid	Slower
pH optimum	5-7	8-10

Substrate type	Property	Examples	Enzyme
Chromogenic Substrates	After enzymatic reaction, they are converted to insoluble, colored products that precipitate onto the membrane and require no special equipment for processing or visualizing.	TMB (3,3´,5,5´-tetramethyl benzidine), 4-CN (4-chloro-1-naphthol) and DAB (3,3´-diaminobenzidine tetrahydrochloride)	HRP
		NBT (nitro-blue tetrazolium chloride), BCIP (5-bromo-4-chloro-3´-indolylphosphate p-toluidine salt) and Fast Red (naphthol AS-MX phosphate + Fast Red TR Salt)	AP
Fluorogenic Substrates	nonfluorescent material that is acted upon by an enzyme to produce a fluorescent compound	3-(p-hydroxyphenyl) propionic acid	HRP
		Rhodamine	AP
Chemiluminescent Substrates	When energy in the form of light is released from a substance because of a chemical reaction the process is called chemiluminescence.	SuperSignal® West Femto	HRP
		DynaLight Substrate with RapidGlow Enhancer	AP