Overview of Western Blotting

What is a western blot?

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. A western blot experiment, or 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 target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semi-quantitative data about the protein of interest.

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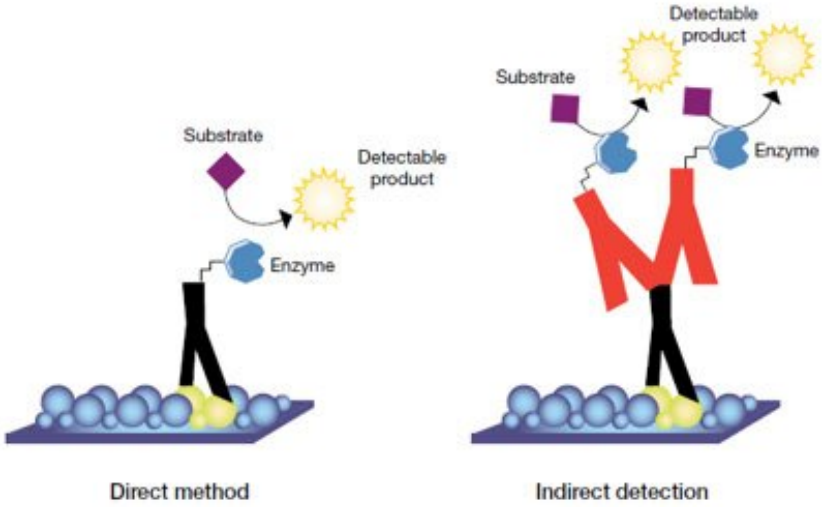
Introduction

The first step in a western blotting procedure is to separate the macromolecules in a sample using gel electrophoresis. Subsequently, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Most commonly, the transferred protein is then probed with a combination of antibodies: one antibody specific to the protein of interest (primary antibody) and another antibody specific to the host species of the primary antibody (secondary antibody). Often the secondary antibody is complexed with an enzyme, which when combined with an appropriate substrate, will produce a detectable signal. **Chromogenic substrates** produce a precipitate on the membrane resulting in colorimetric changes visible to the eye. The most sensitive detection methods use a **chemiluminescent substrate** that produces light as a byproduct of the reaction with the enzyme conjugated to the antibody. The light output can be captured using film. However, **digital imaging instruments** based on charge-coupled device (CCD) cameras are becoming popular alternatives to film for capturing chemiluminescent signal. Alternatively, **fluorescently tagged antibodies** can be used, which require detection using an instrument capable of capturing the fluorescent signal. Fluorescent blotting is a newer technique and is growing in popularity as it affords the potential to multiplex (detect multiple proteins on a single blot). Whatever system is used, the intensity of the signal should correlate with the abundance of the antigen on the membrane.

Procedures vary widely for the detection step of a western blot experiment. One common variation involves direct versus indirect detection. With the direct detection method, an enzyme- or fluorophore-conjugated primary antibody is used to detect the antigen of interest on the blot. 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, an unlabeled primary antibody is first used to bind to the antigen. Subsequently, the primary antibody is detected using an enzyme- or fluororophore-conjugated secondary

antibody. Labels (or conjugated molecules) may include biotin, fluorescent probes such as Invitrogen Alexa Flour or DyLight flourophores, and enzyme conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). The indirect method offers many advantages over the direct method, which are described below.



Direct method	Indirect method
Advantages • Requires only one antibody • Eliminates problems with secondary antibody cross-reactivity **Disadvantages** • Label may interfere with target binding • Potential for high background if antibody specificity for target is weak • Conjugated primary antibodies may be costly • Selection of conjugated primary antibodies may be limited	**Advantages** • Signal amplification by secondary antibody • Vast selection of conjugated secondary antibodies • One secondary antibody may be used with a number of different primary antibodies • Use of secondary antibody does not inhibit primary antibody target binding • Use of labeled secondary antibodies provides options for multiple detection methods **Disadvantages** • Nonspecific staining may increase background • Additional steps are required when using indirect method

View western blot analysis videos

Electrophoretic separation of proteins

Gel electrophoresis is a technique in which charged molecules, such as protein or DNA, are separated according to physical properties as they are forced through a gel by an electrical current. Proteins are commonly separated using polyacrylamide gel electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. When combined with western blotting, PAGE is a powerful analytical tool providing information on the mass, charge, purity or presence of a protein. Several forms of PAGE exist and can offer different types of information about the protein(s) of interest. For example, nondenaturing PAGE, or native PAGE, separates proteins according to their mass-charge ratios. In contrast, sodium dodecyl sulfate-PAGE, or SDS-PAGE, separates proteins according to mass due to the negative charge imparted on proteins bound to the ionic SDS detergent.

Several buffering systems or gel chemistries are available for protein gel electrophoresis. Each system provides unique advantages when resolving proteins of different molecular weights.

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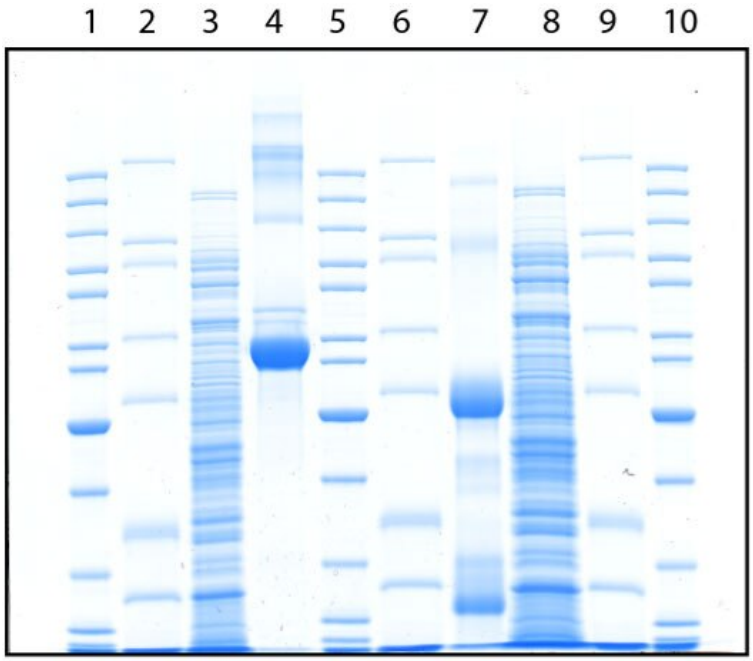
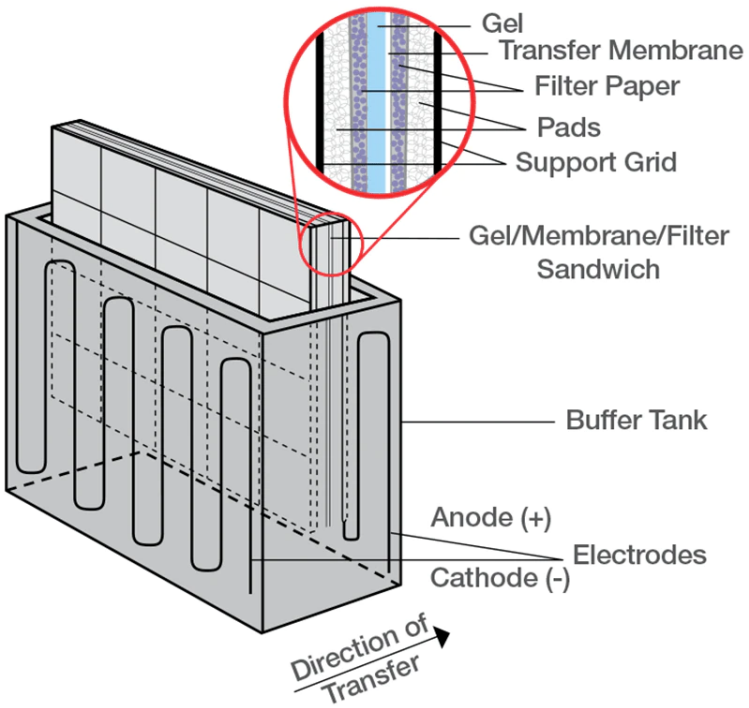
Proteins separated on a Novex Tris-Glycine protein gel and stained with Simple Blue Safe stain.



Transferring proteins to a membrane

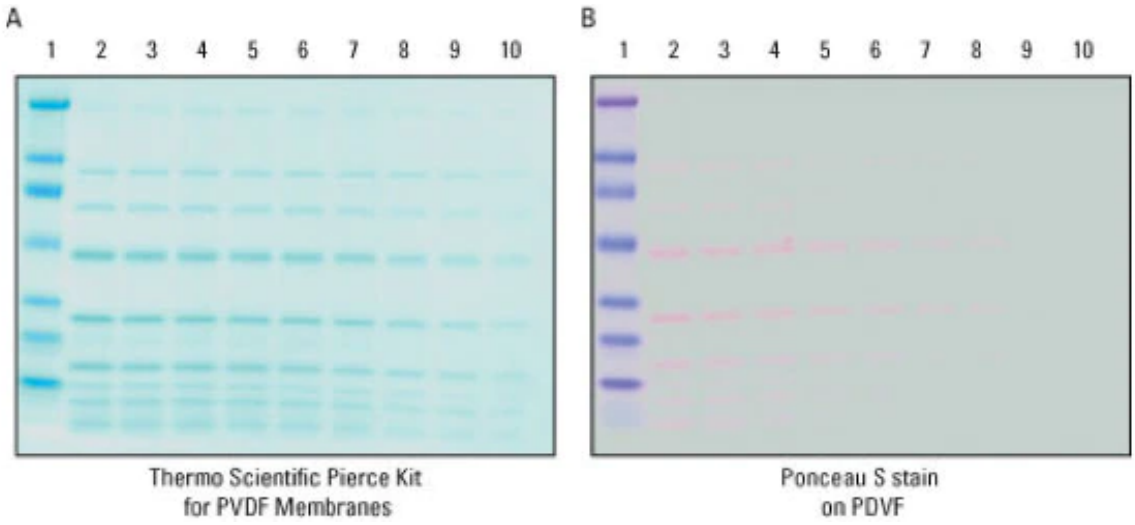
Following electrophoresis, the protein must be transferred from the gel to a **membrane**. There are a variety of methods that have been used for this process that include, but are not limited to, diffusion transfer, capillary transfer, vacuum blotting transfer, and electroelution. The transfer method that is most commonly used for proteins is electroelution or electrophoretic transfer because of its speed and transfer efficiency. This method uses the electrophoretic mobility of proteins to transfer them from the gel to the membrane. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable, protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution. The process involves the use of porous pads and filter paper to facilitate the transfer. When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel.



Western blot transfer apparatus. Schematic showing the assembly of a typical western blot apparatus with the position of the gel, transfer membrane, and direction of protein in relation to the electrode position. Although the image depicted here is representative of a vertical "wet" transfer apparatus, the orientation is applicable for horizontally positioned semi-dry transfer apparatus.

Transfer efficiency can vary dramatically among proteins, based upon the ability of a protein to migrate out of the gel and its propensity to bind to the membrane under a particular set of conditions. The efficiency of transfer depends on factors such as the composition of the gel, complete contact of the gel with the membrane, the position of the electrodes, the transfer time, size and composition of proteins, field strength and the presence of detergents and alcohol in the buffer.

After transfer and before proceeding with the western blot, total protein on the membrane can be assessed with a **protein stain** to check the transfer efficiency. The gel may also be stained to confirm that protein has moved out of the gel, but this does not ensure efficient binding of protein to the membrane. Because dyes may interfere with antibody binding and detection, a protein stain that is easily removable is ideal. Ponceau S stain is the most widely used reagent for reversibly staining proteins on a membrane, although it has limited sensitivity, does not photograph well and can fade quickly, making documentation difficult. Superior alternatives for staining protein on nitrocellulose or PVDF membranes are available, which allow the detection of low-nanogram levels of protein, are easily photographed and do not fade until removed.



Comparison of Reversible Protein Stain with Ponceau S stain. Prestained MW marker was applied to each gel (Lane 1), and unstained protein MW amrkers were serially diluted and run on each 4-20% Tris-glycine-SDS polyacrylamide gel (Lanes 2–10). Electroeluction was used to transfer proteins to PVDF membranes. Thermo Scientific Pierce Reversible Stain was applied for 1 minute according to the protocol (Panel A). Blot stained with 0.1% Ponceau S in 5% acetic acid for 5 minutes according to the protocol (Panel B).

Electrotransfer systems

Several electrotransfer strategies exist. The most common methods are wet, semi-dry and dry, each of which requires special considerations with respect to time, cost, and required reagents and apparatuses. **Wet transfer** (as referred to as tank transfer) offers high transfer efficiency, flexibility in buffer system and method choices but at a cost of time and effort. **Semi-dry** blotting provides convenience and time savings with the flexibility to use multiple types of buffer systems. However, semi-dry blotting can have lower efficiency of transfer of large molecular weight proteins (>300 kDa). **Dry transfer** offers both high quality transfers with speed as well as convenience because buffers are not required but has limited flexibility in consumables.

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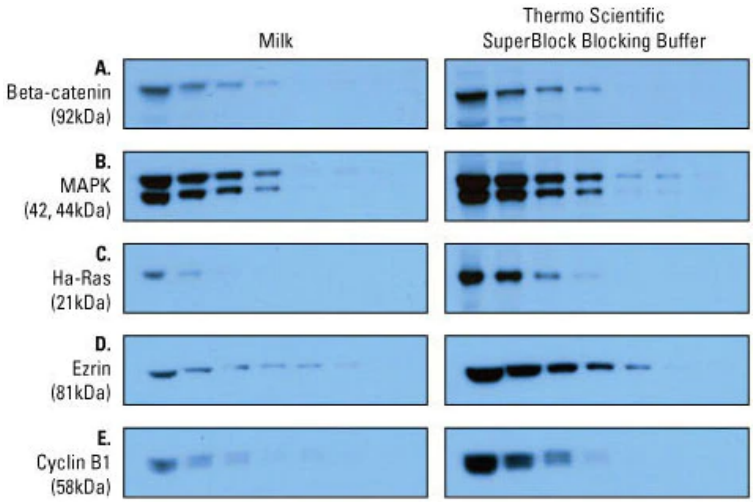
Blocking nonspecific sites

The membrane supports used in western blotting have a high affinity for proteins. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block free sites on a membrane. The blocking buffer should improve the sensitivity of the assay by reducing background interference and improving the signal-to-noise ratio. No single blocking agent is ideal for every experiment since each antibody-antigen pair has unique characteristics. Empirical testing of blocking buffers is essential in optimizing a western blot experiment. Frequently blocking buffers are made by researchers in the laboratory; however, commercially available blocking buffers offer convenience.

Comparison of SuperBlock Blocking Buffer and milk. Two-fold serial dilutions of HeLa cell lysate (20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 µg) were separated by SDS-PAGE and transferred to nitrocellulose (panels A–C) or PVDF (panels D–E) membranes. Membranes were blocked for 1 hour with 5% non-fat milk in Tris-buffered saline and 0.05% Thermo Scientific Tween 20 detergent, or Thermo Scientific SuperBlock Blocking Buffer in phosphate-buffered saline with 0.05% Tween 20 detergent. Blots were processed for 5 minutes using Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (**Cat. No. 34580**) and exposed to film. The results show that SuperBlock Blocking Buffer is superior to milk for detection of target proteins.



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Wash buffer formulations

Like other immunoassay procedures, western blotting consists of a series of incubations with different immunochemical reagents separated by wash steps. Washing steps are necessary to remove unbound reagents and reduce background, thereby increasing the signal-to-noise ratio. Insufficient washing may result in high background, while excessive washing may result in decreased sensitivity caused by elution of the antibody and/or antigen from the blot. As with other steps in western blotting blot, a variety of buffers may be used.

Tris-buffered saline (TBS) and phosphate-buffered saline (PBS) are the most commonly used wash buffers. In most cases, PBS and TBS solutions can be interchangeable. However, there are situations on when to use one over the other. For example, TBS should be used when using systems with alkaline phosphatase (AP)-conjugated secondary antibodies or when detecting phosphorylated proteins with phospo-specific antibodies.

Occasionally, wash buffer formulations consist of a detergent such as 0.05% Tween 20 to aid in the removal of nonspecifically-bound material. Depending on the specifics of the assay, the amount of detergent in the wash buffer will vary, though typical concentrations are from 0.05 to 0.5% for detergents like **Tween 20**. Another common technique is to add a 1:10 dilution of the blocking solution to the wash buffer. Including the blocking agent with the detergent may help to minimize background in the assay by preventing elution of the blocking protein from the membrane and/or allowing nonspecific interactions to occur with the protein in solution rather than those immobilized on the membrane.

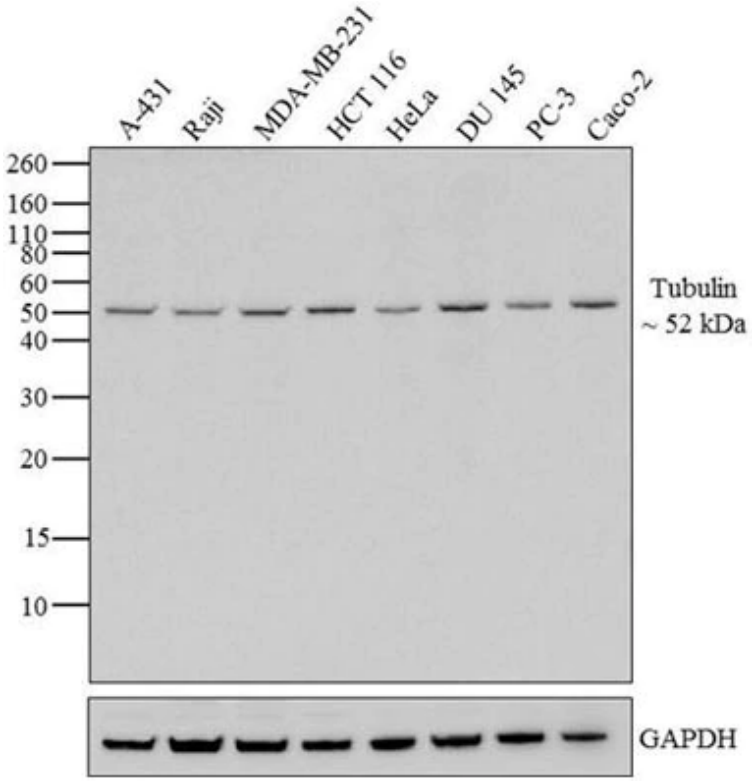
It is important to note that detergents, like the protein solutions, can promote microbial growth. While it is convenient to make pre-diluted stocks of detergents like NP-40, CHAPS, and Tween 20, fungi can grow in these solutions, which can lead to high background noise. In addition, detergents can contain significant amounts of peroxides which will cause background signal when using horseradish peroxidase substrates. Therefore, it is important to use high-purity detergents.

	TBS	**PBS**	**TBST**	**PBST**
Formulation	• 25mM Tris • 0.15M NaCl • pH 7.2	• 10mM sodium phosphate • 0.15M NaCl • pH 7.5	• 25mM Tris • 0.15M NaCl • 0.05% Tween-20 • pH 7.5	• 10mM sodium phosphate • 0.15M NaCl • 0.05% Tween-20 • pH 7.5

Primary and secondary antibodies

Western blotting is typically performed by probing the blocked membrane with a primary antibody that recognizes a specific protein or epitope on a group of proteins (e.g., SH2 domain or phosphorylated tyrosine). 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. It is also important to note that not all primary antibodies are suitable for western blotting and the application should be verified, if possible, before purchasing a new primary antibody.

Western blot using alpha (α)-tubulin antibody. Lysates from 8 cell lines were analyzed using the Invitrogen XCell Surelock Electrophoresis System and iBlot Dry Blotting System. The blot was probed for alpha (α)-tubulin protein using **alpha (α)-tubulin mouse**



monoclonal primary antibody (Cat. No. 236-10501) and **goat anti-mouse HRP conjugate secondary antibody** (Cat. No. 62-6520).

In general, the primary antibody that recognizes the target protein in a western blot is not directly detectable. Therefore, tagged secondary antibodies are used as the means of ultimately detecting the target antigen (indirect detection). A wide variety of labeled secondary antibodies can be used for western blot detection. The choice of secondary antibody depends on either the species of animal in which the primary antibody was raised (the host species) or any tag linked to the primary antibody (e.g., biotin, histidine (His), hemagglutinin (HA), etc.) For example, if the primary antibody is an unmodified mouse monoclonal antibody, then the secondary antibody must be an anti-mouse IgG secondary (or non-IgG) antibody obtained from a non-mouse host.

Antibodies for western blotting are typically used as dilute solutions, and manufacturers may recommend using ranges from a 1/100–1/500,000 dilution from a 1 mg/mL stock solution. However, the optimal dilution of a given antibody with a particular detection system must be determined experimentally. More sensitive detection systems require less antibody than lower sensitivity systems and can result in substantial savings on antibody costs and allow a limited supply of antibody to be stretched out over more experiments. Using lower amounts of antibody can also have the added benefit of reduced background because the limited amount of antibody shows increased specificity for the target with the highest affinity.

Antibody dilutions are typically made in the wash buffer. The presence of detergent and a small amount of the blocking agent in the antibody diluent often helps to minimize background, thereby increasing the signal-to-noise ratio. Conversely, adding too much blocking agent or detergent to the antibody dilution solution can prevent efficient binding of the antibody to the antigen, causing reduced signal as well as reduced background.

Continue reading: **Secondary Antibodies as Probes**
Explore: **Western Blot Antibodies**

Detection methods

While there are many different tags that can be conjugated to a secondary or primary antibody, the detection method used will limit the choice of what can be used in a western blotting assay. Radioisotopes were used extensively in the past, but they are expensive, have a short shelf-life, offer no improvement in signal-to-noise ratio and require special handling and disposal. Alternative labels are enzymes and fluorophores.

Enzymatic labels are most commonly used for western blotting and, although they require extra steps, can be extremely sensitive when optimized with an appropriate substrate. Horseradish peroxidase (HRP), and to a lesser extent, alkaline phosphatase (AP) are the two enzymes used most extensively as labels for protein detection. An array of **chromogenic**, **fluorogenic**, and **chemiluminescent substrates** are available for use with either enzyme. Alkaline phosphatase offers a distinct advantage over other enzymes in that its reaction rate remains linear, improving sensitivity by simply allowing a reaction to proceed for a longer time period. Unfortunately, the increased reaction time often leads to high background signal resulting in low signal-to-noise ratios. Horseradish peroxidase–conjugated antibodies are considered superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody due the smaller size of HRP enzyme and compatibility with conjugation reactions. In addition, the high activity rate, good stability, low cost, and wide availability of substrates make HRP the enzyme of choice for most applications.

Enzyme-conjugated antibodies offer the most flexibility in detection and documentation methods for western blotting because of the variety of substrates available. The simplest detection/documentation system is to use chromogenic substrates. While not as sensitive as other substrates, chromogenic substrates allow direct visualization of signal development. Unfortunately, chromogenic substrates tend to fade as the blot dries or during storage, making the blot itself an unreliable means of documentation. However, it is fairly straightforward to either photocopy or directly scan the blot in order to make a permanent replica of chromogenic western blot results.

Chemiluminescent blotting substrates differ from other substrates in that the signal is a transient product of the enzyme-substrate reaction and persists only as long as the reaction is occurring. If either the substrate is used up or the enzyme loses activity, then the reaction will cease and signal will be lost. However, in well-optimized assays using proper antibody dilutions and sufficient substrate, the reaction can produce stable output of light for 1 to 24 hours depending on the substrate, allowing consistent and sensitive detection that may be documented with X-ray film or digital imaging equipment. While X-ray film can be used to obtain semi-quantitative data, digital imaging is more sensitive because of the broad dynamic range of detection, allowing researchers to obtain quantitative data from western blots.

The use of fluorophore-conjugated antibodies requires fewer steps because there is no substrate development step in the assay. While the protocol is shorter, this method requires special equipment in order to detect and document the fluorescent signal due to the need for an excitation light source. Recent advances in **digital imaging** and the development of newer generation fluorophores such as infrared, near-infrared, and quantum dots has increased the sensitivity and popularity of using fluorescent probes for western blotting and other immunoassays. Although the equipment and fluorophore-conjugated antibodies can be quite expensive, this method has the added advantage of multiplex compatibility (using more than one fluorophore in the same experiment). In addition, chemical waste is further reduced compared to other blotting procedures.

Continue reading: **Chemiluminescent western blotting**
Explore: **Detection Reagents**
Explore: **Western Blot Imaging Systems**

Suggested reading

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Additional resources

Handbook: Protein Gel Electrophoresis Technical Handbook

Handbook: Protein Transfer Technical Handbook

Handbook: Protein Detection Technical Handbook

Handbook: Protein Research

Handbook: Antibody-Based Tools for Biomedical Research

Tech Tip #24: Optimize antigen and antibody concentrations for Western blots

Tech Tip #59: Choosing a secondary antibody: A guide to fragment specificity

Tech Tip #32: Guide to enzyme substrates for Western blotting

Ghana