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Advansta's Step-by-Step Guide to Western Blots

Second edition

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About Advansta, Inc.

Founded in 2005 and headquartered in San Jose, California, Advansta accelerates life science research by developing and supplying novel protein characterization tools that offer superior performance, simplicity, and convenience.

Advansta's mission is to be the leading developer and supplier of products for protein characterization.

Our expertise in chemistry and protein analysis is behind all our products, and allowed us to create the WesternBright line of substrates. With over 400 citations in 2015 alone, our flagship products have become globally recognized as the most sensitive chemiluminescent substrates on the market.

Mastering the Western Blot

The Wonderful Western

We don't think anyone will argue otherwise – the Western blot is a powerful analytical method. First described by Towbin, et. al[1] in 1979, Western blotting is a popular technique with anyone that studies proteins. Western blotting can estimate the size of a protein, confirm the presence of posttranslational modifications such as phosphorylation, and be used to quantitatively compare protein levels between samples.

Many scientists have a love-hate relationship with Western blots. When they are working well, you are graced with crisp bands on a background with no smears, spots or blotches. You see the anticipated number of bands and the data is wonderfully interpretable.

When they aren't working, Western blots are tortuous. They can produce some of the ugliest data in the lab: black rectangular-shaped boxes covering the entire blot or angry looking slashes, blotches, and spots scattered across the blots. Western blots can also give confounding, often inexplicable data; no bands or too many bands can be equally unexplainable.

One of the reasons Western blots are so difficult is because the assay is a series of almost independent steps – each fraught with its own pitfalls. Troubleshooting is a cumbersome process because each step needs to be examined. Additionally, while often the same conditions will work for multiple proteins, other proteins require different treatments and optimization must be performed on a case-by-case basis.

This guide serves to break down every step of the Western blot assay. Each section includes a description of the theory behind one step in the process, a general protocol to approaching the step, and a discussion of different methods used to successfully perform it, when appropriate. Tips, tricks, and troubleshooting guides are also given. You can use the guide in its entirety, or pick and choose the chapters that are most useful to you.

[1] Towbin, H. et. al. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 76(9):4350-4.

The Western Blot: Quick Overview

See each section for more in-depth details.

Step 1: Sample Preparation

Proteins are extracted from biological material using mechanical and/or chemical disruption. Total protein is quantified and samples are mixed with a buffer prior to loading them on a gel for electrophoresis.

Starting materials include plant or animal tissues, cultured cells, yeast, or bacteria.

Mechanical disruption with a homogenizer will break up tissues. Further steps can achieve subcellular fractionation.

Buffers containing detergent are used for cellular lysis.

Step 2: Gel Electrophoresis

Protein samples are loaded onto a gel where they are separated by size via electrophoresis. Gel electrophoresis can be performed under native or denaturing conditions.

Loading dye is added so that sample migration can be monitored.
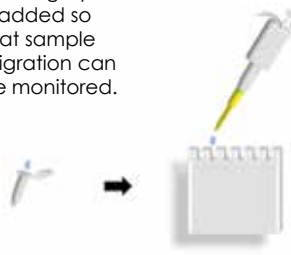
A pipette is used to load the sample into the well of a polyacrylamide gel.

A power supply provides voltage.
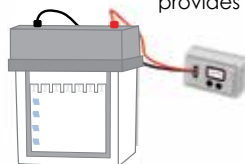
The gel is placed in an electrophoresis tank filled with buffer that will conduct current. The negatively charged proteins migrate away from the anode.

Step 3: Gel Transfer

Proteins are transferred electrophoretically to a membrane support, where they are retained for subsequent immunodetection.

The gel and membrane are placed between filter paper and sponge pads.
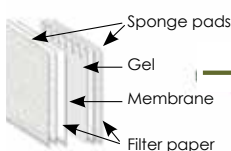
Voltage is applied in a buffer tank and the proteins move from the gel to the membrane.

Sponge pads
Gel
Membrane
Filter paper
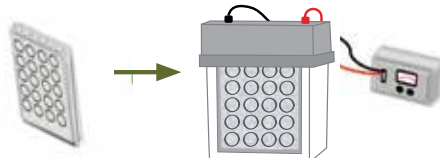
A cartridge applies pressure, maintaining close contact between the gel and membrane.

Step 4: Membrane Blocking

TThe membrane is removed from the transfer stack, placed in a container and incubated with a blocking buffer.
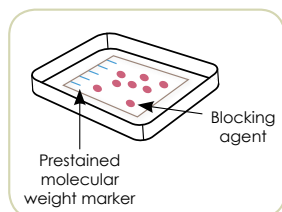
Step 5: Antibody Incubation

The blot is incubated with a primary antibody specific for an epitope present on the target protein. Following several washes, the membrane is typically then incubated with a labeled secondary antibody to provide a means of detection.
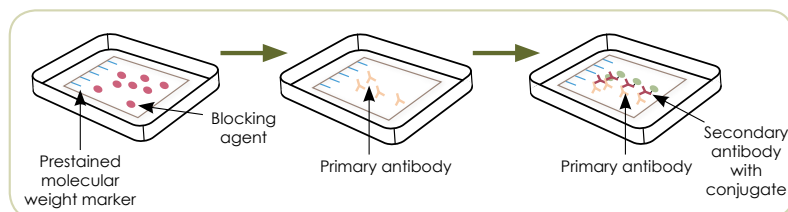
Step 6: Detection

After washing the membrane to remove unbound antibodies, the label on the secondary antibody is used to visualize the protein of interest. Antibodies can be tagged with enzymes that produce color and light, or directly labeled with fluorescent tags for visualization of the protein. Colorimetric assays can be directly visualized and documented by photography or scanning. When using light, the results are detected using film or digital imaging. Fluorescent dyes are detected with a digital imager.
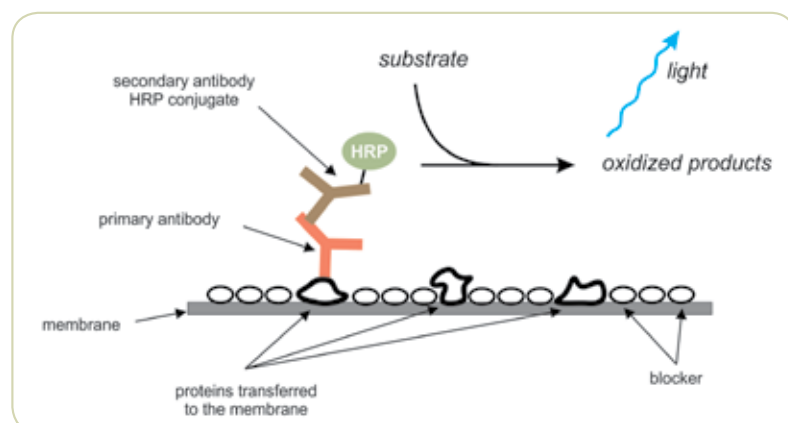
Preparing Your Samples

Step 1: Sample Preparation

The first step in a successful Western is the preparation of the protein sample. It sounds simple: you lyse your biological samples and solubilize the proteins in a buffer compatible with gel electrophoresis. You then quantitate the protein recovery and calculate how much protein to run on your gel. However, inefficient lysis or improper handling of samples can lead to poor recovery and/or degradation of proteins.

> **"Before anything else, preparation is the key to success."**
>
> *– Alexander Graham Bell*

Sample preparation involves 2 steps:

A. Lysis of samples and recovery of proteins
B. Protein quantification

A. **Lysis of Cells**. Efficient cell lysis is an important step in preparing quality samples for Western blot analysis. Inefficient lysis results in incomplete isolation and poor recovery of proteins, and decreases your ability to detect the protein of interest. Sample degradation can also occur, resulting in loss of protein integrity.

A variety of methods exist for harvesting samples, but they must all achieve the same results:

- Disruption of membranes and organelles that retain the protein of interest
- Solubilization of protein in the liquid phase
- Isolation of proteins away from cellular debris

Lysis of samples needs to be balanced with the needs of the experiment. Harsh lysis disrupts protein:protein interactions and can destroy antibody epitopes, while inefficient lysis can result in incomplete recovery of proteins, decreasing your ability to detect the protein of interest. Therefore, the type of sample, composition of the cell, and nature of the individual experiment should all be taken into consideration when choosing a lysis method.

Plants, bacteria, fungi, algae, and archaea all contain cell walls that are resistant to lysis. They will often require mechanical methods (physical disruption) to disrupt the complex cell wall matrix. Mammalian cells are bounded by plasma membranes, protein-rich lipid bilayers that are easily disrupted. Frequently, you can rupture cultured cells using detergent buffers in the absence of mechanical methods.

Table 1.1 lists some of the more common methods for lysing cells.

1. **Physical Disruption.** Grinding, blending, squeezing samples through small spaces, any way you can mash or pop open a cell physically is a method of physical disruption. Traditional physical methods of disrupting cells can be used with a wide variety of source material. Moreover, physical disruption can easily accommodate different materials by simply changing applied force or altering the duration of treatment.

Although popular, physical methods must be used with care. Physical forces create local hot spots in samples leading to denaturation and aggregation of proteins. Reproducibility between experiments can be difficult as it is challenging to define the amount of force generated by a particular experimenter. This is particularly true with homogenization and grinding. Likewise, individual differences between equipment can also contribute to a lack of reproducibility between laboratories. Depending on the method, physical disruption can also be labor intensive and/or time consuming, limiting the number of samples processed at one time.

Method	Use	Disadvantages
Mechanical Disruption • Blades • Beads	• Large amounts mammalian tissue • Large volumes yeast • Bacteria, yeast, algae, hyphae and plant and animal tissue	• Susceptible to local heating
Liquid Homogenization • Dounce • Potter-Elyebiem • French Press	• Small volumes and cultured cells • Cells, not tissues • Samples in liquid form; often used for bacterial cells	• Susceptible to local heating • Reproducibility can be difficult
Sonication	• Bacteria, fungi, single cell suspensions	• Creates local hot spots • Time consuming
Freeze/Thaw	• Bacteria • Mammalian cells	• Time consuming • Inefficient
Grinding with Liquid Nitrogen	• Plant cells • Fungi • Bacterial • Mammalian cell pellets	• Dangerous due to generation of aerosols • Loss of material • Labor intensive and time consuming

Table 1.1. Physical Methods for Lysis of Cells

a. Mechanical Disruption

i. Blades. Hershey and Chase made blenders famous in the lab when they used mechanical disruption to remove bacteriophage from cells to prove that DNA is the carrier of genetic material. Blenders can also be used to mechanically disrupt and tear samples.

You can use the traditional blender (Waring®) or a Polytron® homogenizer, both of which are useful in extracting proteins from complex mammalian tissue and in disrupting large volumes of yeast samples. While blenders are preferred for larger sample sizes, the Polytron can accommodate samples ranging from 0.1mL to 10 L, depending on the model.

ii. Beads. If you need to lyse small volumes, then glass beads are simple and inexpensive tools to use. To lyse cells, suspend glass beads in the sample and vortex the sample repeatedly. The beads disrupt cell membranes through shear forces, grinding between the beads, and collision with the beads.

BE CAREFUL: Vortexing with beads is not as powerful as other physical methods and may not completely disrupt samples.

For larger volumes and more efficient disruption using beads, you can purchase a specialized bead instrument (e.g. a bead mill) that "shakes" the mixture of sample and beads. You can adjust the density, quantity, and diameter of the beads to increase or decrease cell disruption. This method is ideally suited to lysing large sample volumes.

Beads can be used to lyse bacteria, yeast, algae, hyphae, and plant and animal tissue. To avoid heat, pre-chill samples and containers and use multiple short cycles, chilling samples in between cycles. Alternatively, you can use fewer beads and/or adjust the vigor of the shaking.

b. Liquid Homogenization. Liquid homogenization is the most popular method for lysing small volumes and cultured cells. Liquid homogenization works by creating shearing forces when samples are forced through a small space.

Similar to other physical disruption methods, liquid homogenization is susceptible to local heat production. To prevent heating of samples during homogenization, pre-chill all instruments that come in contact with the sample and, when possible, perform homogenization on ice. Chill samples in between strokes to prevent denaturation of proteins.

Three different types of homogenizers are commonly used.

i. Dounce homogenizer. Dounce homogenizers are inexpensive glass pestles that you manually push into a glass tube. They work best when mildly lysing single cell suspensions. You can purchase different size homogenizers to accommodate a variety of volumes. They are easy to use, clean and decontaminate. However, using Dounce homogenizers can be time consuming and it is difficult to lyse many samples at one time.

ii. Potter-Elvehjem homogenizer. The Potter-Elvehjem homogenizer is a polytetrafluoroethylene (PTFE) pestle that you can either manually push into a rounded or conical vessel or use a motor to push the pestle. This homogenizer is good at disrupting cells but not tissue. Similar to the Dounce homogenizer, it is easy to clean and use. Although relatively inexpensive, mechanically driven pestles can be costly.

iii. French press. A French press is a piston that applies high pressure to a sample, forcing the sample through a small hole in the press. While it is more expensive than Dounce or Potter-Elvehjem homogenizers, the French press produces more consistent homogenization as the high pressure results in efficient lysis in two passes. The French press is often the instrument of choice when lysing bacterial cells mechanically. Prior to using a French press, your samples must already be in liquid form. Sample sizes are also limited precluding high throughput.

BE CAREFUL: The number of strokes and speed with which the homogenization is performed affect cell lysis and changes can cause irreproducibility between experiments.

c. Sonication. Sonication uses high frequency sound waves to lyse cells. To sonicate a sample, you insert a probe directly into your sample and turn the machine on. Energy flowing from the tip of the probe generates microscopic bubbles that implode (a process known as cavitation) sending shock waves throughout the sample and lysing the cells. Use sonication when you need to lyse cells with less-resistant cell walls such as bacteria and fungi. It is also the most effective method for physical disruption of single cell suspensions.

Sonication can create local hot spots. To prevent heating, apply the treatment in multiple short bursts while the sample is immersed in an ice bath. Also, place your samples on ice in between treatments. Sonicating can be time-consuming and is therefore not amenable to high-throughput sample preparation. The sample volumes are also usually restricted to <100 mL.

d. **Freeze/Thaw.** The simplest method for physically disrupting cells requires a piece of equipment that all labs have: a freezer. Multiple freeze/thaw cycles can be used to lyse bacterial and mammalian cells. Simply freeze your samples quickly in a dry ice/ethanol bath or toss them in a freezer. Take them out and let them thaw. Repeat. Cycles of freezing and thawing cause cells to swell and break open as ice crystals form and contract.

While it's easy, the freeze/thaw process can be time-consuming as cells usually require multiple freeze/thaw cycles for efficient lysis.

e. **Grinding with Liquid Nitrogen.** Grinding is an inexpensive, common method for breaking down plant cells, fungi, bacteria or mammalian cell pellets. The liquid nitrogen keeps the cells cool and makes the cell structures brittle and easy to grind.

Grinding can be dangerous because it generates aerosols and it is difficult to thoroughly clean the mortar and pestle. This may limit its utility when working with biological hazards. It can also be difficult to recover material from the mortar, so it is best to use when samples are abundant. Grinding is labor intensive and the number of samples that can be processed at one time is limited.

f. **Additives to Promote Lysis**. If you find that simple physical disruption measures are not efficient in completely lysing your samples, there are several additives that you can use to promote the break down of cellular structures (shown in Table 1.2).

i. **Hypotonic buffer.** To cause cells to swell making them easier to disrupt, suspend them in a hypotonic buffer (e.g. 10mM Tris-HCl, pH 7.5) prior to mechanical treatment. The cells will be more fragile and more likely to break apart.

ii. **Lysozyme.** When working with bacteria or yeast, use lysozyme (200 µg/mL). The enzyme will break down cell wall components.

iii. **Nucleases.** The release of nucleic acids into solution can increase the viscosity of your samples gumming up the works. Add DNase (25-50 µg/mL) and/or RNase (50 µg/mL) to your samples to reduce the viscosity and make them easier to pipet.

Additive	Working Concentration
Hypotonic Buffer	10 mM Tris-HCl, pH 7.5
Lysozyme	200 µg/mL
DNase	25-50 µg/mL
RNase	50 µg/mL

Table 1.2. Additives for Promoting Lysis

2. **Detergent-Based Lysis.** Detergent-based lysis is popular and has several advantages over physical disruption. It does not require special equipment and it is:

- Efficient
- Flexible
- Easy to use

Detergents give a one-two punch. Their chemical structures enable them to disrupt membranes and solubilize proteins. Detergents have both a polar and nonpolar portion, and can be classified by the characteristics of the polar group: ionic if the polar group is positive or negatively charged, nonionic if it is uncharged, or zwitterionic if it contains positive and negative charges with a net charge of zero. The chemical structure of the detergent determines its "harshness" during lysis, or the amount of breakdown of structures the detergent facilitates.

The detergent you choose to lyse your samples will depend in part on the location of the protein within the cell, whether you need to preserve protein:protein interactions, and the effect the detergent has on the protein. In addition, as different detergents can disrupt different cellular structures, you can use various detergents to isolate organelle fractions.

Table 1.3 includes a list of common detergents, their characteristics, and their uses.

Detergent	Protein Location
NP-40; RIPA*	Whole Cell
Triton X-100	Cytoplasmic (cytoskeletal bound)
NP40; RIPA	Membrane bound
RIPA	Nuclear
RIPA	Mitochondrial

*RIPA buffer is a denaturing buffer containing several detergents: SDS, NP-40 or Triton X-100 and sodium deoxycholate.

Table 1.3. Common Detergents for Cell Lysis

a. **Ionic/Denaturing.** Ionic/denaturing detergents (anionic or cationic) are considered "harsh" detergents because they alter protein structure to a greater extent than nonionic detergents. Denaturing detergents completely disrupt cellular structures and denature proteins by breaking protein:protein interactions. The degree of denaturation depends on the particular detergent and its interaction with individual proteins. Sodium dodecyl sulfate (SDS) and sodium deoxycholate are two commonly used ionic detergents.

While they efficiently disrupt all cellular structures, denaturing detergents have disadvantages. Because they disrupt protein:protein interactions, you cannot use them for coimmunoprecipitation of proteins. Denaturing detergents cause release of nucleic acids and the lysis solution can became viscous, making it difficult to pipet. You can work around this by including nucleases in your lysis buffer (see f. Additives to promote lysis). Alternatively, you can pass lysed cells through a small-bore needle to shear genomic DNA.

b. **Nonionic/Non-denaturing.** Nonionic detergents are considered "mild" detergents; they are less efficient at denaturing proteins and allow proteins to retain their native structures. They disrupt lipid:lipid and lipid:protein interactions, but not protein:protein interactions. Nonionic detergents are great when working with membrane proteins. Common nonionic detergents are Tween®-20, Triton™ X-100 and Nonidet™ P40.

c. **Zwitterionic.** Zwitterionic detergents are the "in-between" detergents: they have characteristics of both ionic and nonionic detergents. Similar to the milder noninonic detergents, they are less denaturing, yet they are more efficient at breaking protein:protein interactions. CHAPS (3- [(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate) is a good example of a zwitterionic detergent and is especially useful for solubilizing membrane proteins.

3. **Avoiding Protein Degradation**. When you break open cell membranes (either through physical disruption or detergent lysis), you release multitudes of proteases – proteins that will indiscriminately degrade proteins in your sample, including your protein of interest.

There are several things you can do to protect the integrity of your sample:

a. **Sample Handling.** Keep your samples cool! Proteases work more efficiently at higher temperatures, so cooling will slow them down. Keep your samples as cool as possible during the lysis process by pre-cooling all glassware, plasticware, instruments, and buffers that come in contact with the sample. Use numerous short cycles when physically disrupting cells. Keep samples on ice whenever possible. Work in a cold room if samples cannot be kept on ice.

Work quickly and efficiently. If you cannot immediately process your samples, flash-freeze them. Otherwise, process and use or freeze your samples immediately.

Do not repeatedly freeze/thaw your samples once you have them. Aliquot your samples into multiple small tubes prior to freezing then store the samples at -80°C, not -20°C. Take out an aliquot to use for an experiment, then throw it away.

b. **Use Protease Inhibitors.** You should include protease inhibitors in your lysis buffers. Protease inhibitors are compounds that inhibit protein degradation by either reversibly or irreversibly binding to proteases. Unfortunately, there is no magical protease inhibitor that is effective in inhibiting all proteases. Therefore, you need to use a mixture or "cocktail" of several protease inhibitors to prevent protein degradation. You can buy protease inhibitors individually or as a pre-made cocktail.

Common protease inhibitors and their targets are shown in Table 1.4.

Inhibitor	Target	Working Concentration
Aprotinin	Trypsin, Chymotrypsin, Plasmin	1-2 µg/ml
Leupeptin	Lysosomal	1-2 µg/ml
Pepstatin A	Aspartic proteases	1 µg/ml
PMSF	Serine, Cysteine proteases	0.1-1 mM
EDTA	Mg++/Mn++Metalloproteases	1-10 mM
EGTA	Ca++ Metalloproteases	1-10 mM

Table 1.4. Common Protease Inhibitors

B. **Protein Quantification**. Whether you grind, press or use detergents to prepare your samples, you should always quantify the total amount of protein in your samples before proceeding with Western blotting. Accurate quantitation of your samples avoids bloating of your lanes and signal saturation from overloading the gel while still allowing adequate detection of your protein. Proper quantitation is also critical when performing quantitative Westerns.

There are several different methods for quantifying protein concentration in samples. Some (e.g. measuring nitrogen content or radioactively labeling cells) do not rely upon absorptive properties of the protein sample. While these are sensitive and accurate assays, they have fallen to the wayside in favor of the more popular spectrophotometric methods for measuring protein concentration.

Spectrophotometric determination of protein concentration is popular because it is easy, sensitive, and does not rely on the use of hazardous agents. The only piece of equipment you need is a spectrophotometer compatible with the range specified by the assay.

There are several different methods for using a spectrophotometer to measure your protein concentration. Most of these have been developed into easy-to-use, cost effective kits available from many suppliers.

The pro and cons for each method are summarized in Table 5.

1. **Absorbance at 280 nm.** One way to quickly measure protein concentration is to measure the absorbance of the sample at 280 nm. While nucleic acids have an absorbance maxima at 260 nm, most proteins have an absorbance maxima at 280 nm. Protein concentration can be determined using the following formula:

    Protein concentration (mg/ml) = A_{280}/path length (in cm)

    Although nucleic acids can interfere with protein measurements in the 280 nm range, you can factor out any contamination by measuring absorbance at both 260 nm and 280 nm and using the following calculation:

    Protein mg/mL = 1.55 A_{280} – 0.76 A_{260}

    Absorbance is best used to quantify and compare multiple samples containing the same, single protein. Aromatic residues (tryptophan, tyrosine, and phenylalanine) are responsible for protein absorbance at 280 nm, thus proteins lacking these residues cannot be measured using this assay. An absorbance measurement requires at least 50 µg of protein for accurate readings.

2. **Biuret Method.** The Biuret method is a test for detecting the presence of peptide bonds. The Biuret reagent contains sodium hydroxide, hydrated copper (II) sulfate, and potassium sodium tartrate (to stabilize the complexes). When these are mixed together under alkaline conditions, the copper is reduced, causing a shift in color, which can be read at 550 nm. Your protein concentration is directly proportional to the intensity of color. The linear range of the assay is typically 0.5-20 mg protein.

3. **The Lowry Method.** The development of the Lowry method (named after Oliver Lowry) introduced a more sensitive assay for determining protein concentration. In addition to being sensitive, the Lowry method is highly reproducible, inexpensive, and easy to perform.

   Lowry modified the Biuret test by adding a reagent called the Folin-Ciocalteu reagent. Reduction of the Folin-Ciocalteu reagent under alkaline conditions results in an intense blue color (heteropolymolybdenum blue) that absorbs at 750 nm. The Lowry method is best used with protein concentrations of 1.0 microgram/mL to 1.0 mg/mL. One drawback to the Lowry method is that it is not compatible with some detergents commonly used in sample preparation.

4. **Bicinchoninic Acid Assay (BCA).** The BCA is similar to the Lowry method, except bicinchoninic acid (BCA) is used instead of the Folin-Crocalteu reagent. After reduction of Cu^{2+} ions, two molecules of BCA chelate with each Cu^+ ion resulting in formation of an intense purple color that absorbs at 560 nm.

   BCA is as sensitive as the Lowry method and works well with protein concentrations from 0.5 µg/mL to 1.5 mg/mL. The use of BCA reduces interference from detergents but other contaminants can interfere with the reaction. Proteins containing higher numbers of aromatic amino acids can react more strongly with the reagent when the reaction is performed at room temperature. However, at higher temperatures (37-60°C), peptide bonds also contribute to formation of the product. Therefore, it is recommended to perform the reaction at higher temperatures to increase the sensitivity and decrease the variability due to amino acid composition.

5. **Dye-Binding Assays.** Dye-binding assays rely on an absorbance shift that occurs when a dye binds to proteins. Several different dyes can be used, such as Coomassie Brillant Blue G-250, Bromocresol green, pyrogallol red, and eosin Y.

   The most commonly used dye-binding assay is the Bradford Assay.

   a. **Bradford Assay.** The Bradford assay, named after its developer, Marion M. Bradford, is an easy, sensitive, and accurate method for protein quantification and is based on the binding of Coomassie blue dye to protein. Under acidic conditions, dye binding causes a shift of the dye from red (465 nm) to blue (595 nm). Similar to the Lowry assay, the Bradford assay can experience interference from some detergents. Solutions with a protein concentration of 20 microgram/mL to 2mg/mL can be measured using the Bradford assay.

Method	Sensitivity	Measurement	Advantages	Disadvantages
Absorbance	>50 µg	260 and 280 nm	• Good for measuring a single protein • Useful in chromotography to determine elution profile of protein • Easy, quick assay • Moderate sensitivity • Does not require a standard curve	• Need spectrophotometer capable of reading in the UV region • Need to use quartz cuvettes • Can not use with proteins that do not contain aromatic residues • Nucleic acid contamination can be a problem
Biuret Method	0.5 – 20 mg	550 nm	• Usually doesn't rely on the amino acid composition of the protein • Good for whole tissue samples and other sources of high protein concentration • Relatively few materials interfere with the assay	• Cannot measure concentration of proteins precipitated using ammonium sulfate • Not as sensitive as other methods • Nucleic acid contamination can be a problem • Proteins with abnormally high or low percentage of aromatic amino acids can give high or low readings
The Lowry Method	0.01 – 1.0 mg/mL	750 nm	• Easy to use • Highly reproducible • Inexpensive • Sensitive • Broad linear range	• Timing and mixing of reagents must be precise • Sensitivity can depend on composition of protein as reaction partly dependent on polar amino acids • Interference from some buffers, particularly detergents • Best suited for determining concentration of samples of the same protein rather than as an absolute measurement • Assay can be lengthy
Bicinchoninic Acid Assay (BCA)	0.5 µg/ml – 1.5 mg/ml	560 nm	• Easy to use • Highly reproducible • Inexpensive • Sensitive • Broad linear range	• Interference from carbohydrates, catecholamines, tryptophan, lipids, phenol red, cysteine, tyrosine, impure sucrose or glycerol, uric acid, iron and hydrogen peroxide • Color continues to develop over time, but is stable for measurement after 30 minutes at 37°C
Bradford Assay	0.02 – 2 mg/ml	595 nm	• Easy to use • Sensitive • Broad linear range • Quick • Compatible with many buffers	• Reagent stains cuvettes • Often need to dilute samples prior to analysis • Depends strongly on amino acid composition • Sensitive to detergents, although some companies offer detergent-compatible Bradford reagents

Table 5. Advantages and Disadvantages of Protein Quantification Methods

Tips for Physical Lysis

1. Optimize lysis by adjusting duration of treatment and size of instrument.

2. Prechill equipment/tubes/samples.

3. Keep samples cool by lysing on ice when possible, or by doing multiple short treatments while placing samples on ice between treatments.

4. Use a hypotonic buffer and/or lysozyme to promote lysis.

5. Reduce viscosity of samples by treating with nucleases.

6. Process hazardous material in a hood or surround with plastic.

7. Avoid aerosols.

8. Check efficiency of lysis by examining an aliquot of the sample under a microscope.

9. Try to be consistent with treatment between experiments.

Tips for Detergent Lysis

1. When making lysis buffer, make sure the detergent is completely solubilized.

2. Try different detergents to optimize lysis.

3. Avoid bubbles and frothing when pipetting/mixing samples.

4. Check efficiency of lysis by examining an aliquot of the sample under a microscope.

5. Reduce viscosity due to nucleic acids by using nucleases, or pass sample through a small-bore needle or spin through a column.

6. Make sure detergent is compatible with downstream applications, including protein quantification.

Sample Spectrophotometric Protein Quantification Assay

1. Decide which assay is compatible with samples. Look at buffer compatibility and linear range of measurement.

2. Prepare samples for standard curve.

    a. Use pure protein standard (e.g. bovine serum albumin [BSA]).

    b. Dilute standard in buffer used for protein samples.

    c. Be sure to include a buffer-only control.

3. Mix reagent per manufacturer's instructions.

    a. Make enough reagent for all standards and samples (including duplicates).

4. Mix reagent with samples and incubate per manufacturer's instructions.

5. Read samples on spectrophotometer.

6. Plot absorbance of each standard versus concentration.

7. Determine the best fit of the data to a straight line using y=mx+b (y = absorbance at 595 nm and x = protein concentration).

8. Use the equation to calculate concentration of protein samples.

9. If the absorbance of the test sample is outside the absorbance range of the control samples, then repeat assay using a different dilution of the sample.

10. Calculate amount of sample to use to load equal amounts of protein from each sample.

Running the Gel

Step 2: Running the gel

The second step in a Western blot is the "running of the gel" in which you load your samples onto a gel matrix and then use electricity to push and pull the proteins through the gel. Electrophoresis of proteins through the gel matrix separates the proteins prior to transfer of the proteins to a membrane support. After addition of a sample loading buffer to the sample and the layering of the sample into a well of the gel, electricity is applied. The proteins then migrate toward the positively charged electrode, to a position determined by the protein's molecular weight or mass:charge ratio, depending on the type of gel used. Gel electrophoresis can be performed under two conditions: denaturing or native.

> **"The true delight is in the finding out rather than the knowing."**
>
> *– Isaac Asimov*

Most Western blot samples are run on **denaturing gels** (typically called SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis). In denaturing gels, the sample loading buffer contains a detergent, sodium dodecyl sulfate (SDS), and a reducing agent (dithiothreitol or ß-mercaptoethanol) and the sample is heated prior to loading. The SDS linearizes proteins and makes them negatively charged while the reducing agent breaks disulfide bonds and disassembles the proteins' tertiary structures. The end result is linear proteins that migrate based on their molecular weight. Smaller proteins migrate faster through the gel while larger proteins migrate slower.

In **native gel electrophoresis**, proteins retain their three dimensional folded states and interactions with other proteins. Because the proteins are not denatured and linearized, separation on the gel occurs by the proteins' mass:charge ratios and cross-sectional areas. You can run a native gel by not adding SDS to the samples and by loading the samples without heating. You can also run samples under nonreducing conditions by leaving out the reducing agent from the sample loading buffer. Many researchers turn to native gels when their antibody only recognizes an epitope on the folded protein or when they are studying a protein within a protein complex.

This chapter focuses on the set-up and running of denaturing gels.

A. **Setting Up and Pouring the Gel**. While many companies sell pre-made SDS-PAGE gels, you can easily make your own in the lab. Pre-made gels have the distinct advantage of convenience with little gel-to-gel variability, but are not as cost effective as those made in-house and do expire over time.

   1. **Stacking and Resolving Gels.** The most common type of denaturing gel actually consists of two gels stacked one on top of the other.

      The top gel is called the stacking or focusing gel. The stacking gel contains large pores and a lower pH, which results in concentration of proteins several fold into a thin layer within a few minutes.

      The bottom gel is called the resolving or separating gel. This portion of the gel contains a smaller pore size and a higher pH. Proteins are separated separated based on their size, or molecular weight.

      Proteins are loaded onto the top of the stacking gel. They then run through the stacking gel and into the resolving gel.

2. **Choosing the Correct Thickness and Pore Size of the Gel.** Prior to pouring the gel, you will have to determine which thickness of gel you need and the pore size of the resolving gel appropriate for your protein(s) of interest. These factors are determined by the volume of the samples you need to load on the gel, transfer efficiency, and the molecular weight of your protein(s) of interest.

a. **Gel thickness.** You will cast your gel between two thin plates of glass and use gel spacers to determine the thickness of the gel. Spacers are available in several standard sizes: 0.75 mm, 1.0 mm, and 1.5 mm. To choose the thickness of your gel, consider several factors:

- sample volume (also factor in number of wells)
- transfer efficiency of proteins
- time
- manipulation of gel

Thinner gels are more fragile and difficult to handle when setting up the transfer and accommodate smaller sample volumes. However, proteins elute more efficiently out of thinner gels and the proteins tend to be more sharply focused in the gel.

Thicker gels are more durable and hold larger sample volumes but they require longer run times and proteins may be more diffuse. Proteins in thicker gels may also not migrate out of the gel as efficiently during transfer and therefore also require longer transfer times.

Check the individual manufacturer's instructions for the volume of sample that can be loaded into each well based on the thickness of the gel and number of wells on the comb.

TIP: In general, gels that are 0.75 mm thick are ideal for Western blots.

b. **Pore Size (Acrylamide Percentage) of Resolving Gel.** The resolving power of the gel is determined by the percentage of acrylamide in the final gel solution. Proteins with smaller molecular weights are resolved well in a higher percentage gel while larger proteins require a lower percentage gel for good resolution. Therefore, you must choose the percentage of your gel based on the molecular weight of your target protein.

Table 2.1 shows the resolving power for different percentage gels.

Protein MW Range, kDa	Recommended Gel %
~10-43	15
~12-60	12
~20-80	10
~30-95	8
~50-200	6

Table 2.1. Resolution of Proteins Based on Gel Percentage

TIP: Protein gels are composed of two polymerization components: acrylamide and bisacrylamide. Each bisacrylamide molecule cross-links with two acrylamide molecules creating a gel matrix. Although the acrylamide:bisacrylamide ratio can be varied, it is generally about 1:35.

3. **Setting Up and Pouring the Gel.** If you are pouring your own gel, you will need to use a gel-casting system, available from multiple manufacturers. While each system is different, they generally consist of the same components:

- glass plates for encasing the gel
- side spacers for determining the thickness of the gel
- combs for creating wells
- a casting stand or clamping device to hold the plates while pouring the gels and waiting for polymerization

Prior to assembling the components, clean all plates, spacers, and combs with detergent and rinse with alcohol to remove any debris that might interfere with gel polymerization. Dry all components with a cloth that does not leave lint on the equipment. Follow the individual manufacturer's instructions for setting up the gel casting form. Make sure all the edges of the plates are lined up and there are no gaps where the gel solution might leak out.

Mix the reagents for the resolving gel well (see Table 2.2) and quickly pour the mixture into the gel casting form. Overlay the gel mixture with a gel topping solution (e.g. 100% ethanol) to prevent contact between the gel edge and the air. The acrylamide will polymerize in about 30 minutes.

Acrylamide %	6	8	10	12	15
H_2O	5.2 ml	4.6 ml	3.8 ml	3.2 ml	2.2 ml
Acrylamide/Bis-acrylamide	2.0 ml	2.6 ml	3.4 ml	4.0 ml	5.0 ml
1.5M Tris (pH8.8)	2.6 ml	2.6 ml	2.6 ml	2.6 ml	2.6 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
10% ammonium persulfate	100 µl	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl

Table 2.2. Gel Components for Resolving Gel Based on Gel Percentage

TIP: Save some of the leftover gel solution in a tube to confirm polymerization.

BEWARE: Acrylamide is a neurotoxin. Wear gloves when handling the gel solution.

Once the acrylamide has polymerized, pour off the gel topping solution, rinse with water and blot to make sure all of the solution is removed. Mix the stacking gel components and pour into the gel casting form on top of the resolving gel. Carefully insert the comb into the gel solution and make sure all air bubbles are removed. Allow the resolving gel to set for approximately 30 minutes.

B. **Standards, Controls, and Experimental Design**. Before loading the gel, you should make sure you design an experiment that incorporates appropriate standards and controls to validate your results. The types of standards/controls used in protein electrophoresis include:

1. **Molecular Weight Standards**. Every gel should contain a lane of molecular weight standards. Molecular weight standards consist of a mixture of purified proteins with known molecular weights. Standards are used to approximate the molecular weight of the sample proteins run on the gel. In addition to serving as standards, you can use prestained markers to monitor the progress of the gel as it runs and also to check transfer efficiency. You can buy molecular weight standards that are either unstained or prestained with dyes. Alternatively, standard proteins can be labeled for detection by chemiluminescence or fluorescence.

   TIP: Prestained molecular weight standards purchased from the same company can migrate differently based on the amount of dye incorporated into each standard. Always note lot number and size of standards.

2. **Positive Controls**. A positive control is important to use to verify that the primary antibody is binding to the correct protein. Positive controls are especially important to use when you are working with a new antibody or to confirm the assay is working when it is unknown if the samples contain the protein of interest.

   If available, a sample of the purified protein of interest is an ideal control, however you can also use cell lines expressing the protein of interest or samples of tissues or organs known to express the protein.

3. **Negative Controls.** Negative controls are used to verify the specificity of the primary antibody and can be particularly useful when multiple bands are observed on a blot. A control cell line, organ or tissue that does not express the protein of interest is recommended.

   Alternatively, some vendors offer blocking peptides for their antibodies. You can mix the blocking peptide with the primary antibody prior to incubation with the membrane to prevent specific binding of the antibody to its target antigen. A loss of signal from the target antigen indicates that the antibody is specific for the protein.

4. **Loading Controls.** A loading control is used to normalize and to check whether the total protein loaded per lane is equal, so that you can compare levels of your target protein between samples. For true normalization, you need to compare the protein of interest to another protein(s) in the same lane on the same blot. There are different ways to accomplish this.

   a. **Housekeeping Genes**. A traditional method for normalizing is to compare the expression of your protein of interest to the expression of a "housekeeping gene". Housekeeping genes maintain the basic metabolic processes of the cell and are believed to be expressed constitutively in most cell types. However, recent research suggests that housekeeping gene expression is more varied than orignially thought. Typical housekeeping genes used for protein normalization include glyceraldehyde 3-phosphate (GAPDH), beta-actin, or tubulin.

To normalize the blot, you detect your protein of interest and the housekeeping protein individually. Then you calculate the ratio of the abundance of your protein to the housekeeping protein for each sample. Although this type of comparison is generally considered only semi-quantitative when using chemiluminescent detection, it is highly quantitative when performing fluorescent Westerns (see Chapter 6). For qualitative studies, you can present the protein of interest and the loading control in an image for visual comparison.

The use of housekeeping genes has some disadvantages. As mentioned above, studies have shown that housekeeping gene expression is not always consistent. In addition, since true normalization can only be performed using proteins on the same blot, the house keeping gene needs to be sufficiently separated from your protein of interest by gel electrophoresis when using colorimetric or chemiluminescent detection. Although some laboratories strip and reprobe blots for normalization, this is not quantitative. Finally, using this method, expression of your protein is only compared to one other protein.

TIP: Some treatments can alter the expression levels of housekeeping proteins. Always determine whether experimental conditions alter the expression of loading controls.

b. **Total Protein Normalization.** Total protein normalization (TPN) is a technique used to quantify the abundance of your target protein without relying on housekeeping genes. TPN is also more compatible with detecting proteins of lower abundance. Traditionally, TPN is performed by incubating the membrane with a total protein stain, either before or after detection with antibodies. Then the abundance of your protein of interest is normalized to the total amount of protein in each lane, removing variations associated with comparing abundance to a single protein.

There are numerous stains available for total protein normalization. While some stains can be used prior to immunodetection, some are incompatible with downstream antibody detection (See Table 2.3 for description of different protein stains). In addition, some stains fade quickly making it difficult to document the results. A newer technique, called the stain-free approach, increases the sensitivity and reduces the complexity while also saving time.

Technique	Limit of Detection	Staining Time	Advantages	Disadvantages
Anionic Stains				
Ponceau S	100-1000 ng	5-10 minutes	• Rapid • Inexpensive	• Difficult to photograph • Fades quickly
Amido Black	50 ng	10 minutes	• Rapid	• Not compatible with downstream detection
Fluorescent Stains	1-8 ng	30-60 minutes	• Highly sensitive	• Requires equipment for detection • Some stains produce hazardous waste
Colloidal Gold	1 ng	1-2 hours	• Highly sensitive • Use silver enhancement for extra sensitivity	• Not compatible with downstream detection • Expensive
Stain-Free	1 ng	None	• Rapid • Highly sensitive	• Requires equipment for detection

Table 2.3. Total Protein Stains

i. **Stain Free TPN.** Stain-free total protein detection is rapid and sensitive. The technology uses a trihalo compound that is directly incorporated into the gel. Upon UV exposure, the compound modifies tryptophan residues in proteins causing them to fluoresce. You can then detect the fluorescent signal using a CCD camera.

Some benefits to TPN using stain-free gels include:

- Visualization of proteins in the gel prior to transfer and on the membrane after transfer
- Normalization to multiple proteins for increased accuracy
- Stain-free detection does not interfere with downstream immunodetection
- You can purchase precast stain-free gels or
- You can make stain-free gels in the lab by mixing a trihalo compound with acrylamide

TIP: Stain-free technology cannot detect proteins that do not contain tryptophan and it is recommended that a protein contain at least 2 tryptophan residues to be readily detected.

5. **Placement of Samples in the Gel.** An important thing to consider before you load your gel is the order in which you load your samples. You should load lanes in an order that makes the most sense for your experiment and will require the least amount of digital manipulation when making figures. For example, do not load positive controls next to low expressing samples as they can obscure the signal. Instead, place positive controls in the first or last lanes or load molecular weight standards between the positive control and experimental samples.

AdvanStain™ Ponceau

✓ **QUICK**
✓ **REVERSIBLE**
✓ **COMPATIBLE**

AdvanStain Ponceau rapidly detects proteins on nitrocellulose and PVDF membranes, allowing you to check the quality of protein transfer before proceeding to Western blotting. With AdvanStain Ponceau you can quickly make sure that protein transfer has been even across the entire blot, and that no signs of bubbles or other transfer artifacts are present. The staining is reversible and after destaining, membranes can immediately be used for Western blotting.

AdvanStain™ Scarlet™

✓ **SENSITIVE**
✓ **CONVENIENT**
✓ **FLEXIBLE**
✓ **REVERSIBLE**
✓ **NO SPECKLING**
✓ **SAFE**
✓ **COMPATIBLE**

AdvanStain Scarlet is a fluorescent stain for gels and blots that allows sensitive and quantitative visualization of proteins. Gels and blots stained with AdvanStain Scarlet can be imaged on any fluorescent imaging system, including laser- and CCD-based systems, with a detection limit of less than 1 ng of protein per band or spot. Fully compatible with downstream Western blotting or mass spectrometry, AdvanStain Scarlet is non-toxic and biodegradable, for safe and simple disposal.

C. **Running the Gel.** To run the gel, prepare samples by mixing the samples with sample loading buffer and load the samples in to the appropriate wells prior to adding current to the apparatus.

1. **Sample Loading Buffer.** Sample loading buffer serves several purposes. Components include a buffer that is compatible with the electrophoresis system, glycerol to increase the density of the sample to aid in loading the gel, and a small amount of dye to monitor the progress of the samples. Denaturing buffers also contain SDS, which denatures proteins and applies a negative charge to each protein in proportion to its mass. Additionally, sample loading buffer can also contain a reducing agent (e.g. dithiothreitol or ß-mercaptoethanol) to reduce disulfide-linked tertiary structures. Sample loading buffers are made concentrated and are diluted to a 1x concentration by mixing with the sample. A recipe for a gel loading buffer for a denaturing SDS-PAGE gel is shown in Table 2.4.

Component	2x Stock
Tris-HCl, pH 6.8	0.125M
SDS	4%
Beta-mercaptoethanol*	5%
Dithiothreitol*	0.15M
Glycerol	20%
Bromophenol Blue	0.01%

*Use either beta-mercaptoethanol or dithiothreitol

Table 2.4. Sample Recipe for SDS-PAGE Sample Loading Buffer

2. **Loading and Running the Gel.** Set up the gel in the tank following the manufacturer's instructions. Fill the tank with running buffer and rinse wells out with running buffer. Mix your sample with the sample loading buffer by pipetting up and down. If running a denaturing gel, immediately heat samples to > 95°C for 5 minutes and spin briefly. Load samples into the wells using a pipettor. Drawn-out pipette tips can be purchased to aid in loading samples.

TIP: Load 20-40 µg of total protein per lane in a volume that is consistent with the well. It is important to know the total volume that can be loaded for the chosen well size, as overloading can result in loss of sample or spillage into adjacent wells.

Immediately after loading the samples, attach the leads to the tank and the power supply and turn the unit on. Ensure the samples are migrating in the correct direction. Monitor the progress of the gel by following the dye front and the position of the molecular weight standards (if using prestained markers). Stop the gel when the dye front runs off the gel into the buffer.

Protein Sample Loading Buffers

✓ **CONVENIENCE**
✓ **QUALITY**
✓ **FLEXIBILITY**

Pre-mixed loading buffers for polyacrylamide electrophoresis. Advansta provides ready-to-use Laemmli loading buffers for the preparation of protein samples for SDS-PAGE. Choose from reducing or non-reducing buffers; each contains bromophenol blue, to enable visualization of the progress of electrophoresis by observing migration of the dye front.

www.advansta.com

How to Pour an SDS-Page Gel

1. Choose spacers and comb for correct thickness of gel and number of samples.

2. Clean all plates, spacers, and combs with detergent and warm water. Rinse with 100% ethanol and dry.

3. Set up gel plates and spacers according to the manufacturer's instructions making sure there are no gaps or holes.

4. Measure anticipated height of resolving gel and mark with a Sharpie® on glass plate.

5. Mix resolving gel components (stacking gel components could also be mixed and saved for later).

6. Add TEMED and APS to resolving gel mixture and pour immediately between gel plates. Avoid air bubbles.

7. Overlay resolving gel with gel topping solution.

8. Allow resolving gel to polymerize (~30 minutes).

9. Pour off gel topping solution completely.

10. Mix stacking gel components, add TEMED and APS and pour between plates.

11. Carefully place comb into gel without creating air bubbles.

12. Allow stacking gel to polymerize.

Transferring the Proteins

Step 3: Transferring the Proteins

Before you can use antibodies to detect your protein(s) of interest, you must first elute the proteins out of the gel onto a solid support (the membrane). Similar to the electrophoresis of proteins through the gel, proteins can be induced to migrate out of the gel using an electric field (termed electrophoretic transfer). In most transfer systems, negatively charged proteins move out of the gel towards the positively charged electrode.

> **"No amount of experimentation can ever prove me right; a single experiment can prove me wrong."**
>
> *– Albert Einstein*

You can transfer proteins either using wet conditions in a tank apparatus or under semi-dry conditions on a solid support (see below). Regardless of the type of apparatus, you perform the transfer by surrounding the gel and membrane together with filter paper (often referred to as "the stack" or "the sandwich") and placing them between two electrodes. Proteins will migrate out of the gel and become affixed to the membrane following the current applied through a transfer buffer. The protein size, shape, and pH, gel percentage, and ionic strength of the transfer buffer all affect the elution of proteins out of the gel.

A. **Choosing the Right Membrane.** The two most commonly used membrane types for immunoblotting are nitrocellulose and polyvinylidene difluoride (PVDF). Although both work well for immunoblotting, differences in their characteristics may influence the choice of membrane.

1. **PVDF.** PVDF membranes have higher mechanical strength, are resistant to SDS, and have higher bond strength than nitrocellulose. Therefore, you must use a PVDF membrane if you are going to strip and reprobe your blot (see Step 7: Special Considerations for Western Blots). Also, if you are using fluorescent detection methods, then use a low-autofluorescence PVDF membrane that has been developed for this purpose.

   A disadvantage to PVDF is that it must be activated by pre-wetting the membrane in methanol prior to use, adding an extra step to the experiment. In addition, chicken antibodies can bind to PVDF membranes, therefore nitrocellulose should be used if using antibodies from this species.

2. **Nitrocellulose.** Nitrocellulose is favored by some researchers as it has lower background and does not require a methanol pretreatment. Nitrocellulose is also generally less expensive than PVDF. As mentioned above, you should choose nitrocellulose when using antibodies made in chickens to prevent high background.

   Nitrocellulose does have some drawbacks. It is not compatible with transfer buffers containing SDS; the use of SDS may prevent protein binding to the membrane. Nitrocellulose also autofluoresces, and therefore cannot be used in fluorescent Western blots. Nitrocellulose is fragile and can break and crack when the membrane is handled frequently.

   TIP: Nitrocellulose is available in 2 pore sizes: the 0.45 µm pore is used for proteins greater than 20 kDa while the 0.2 µm pore size is used for smaller proteins.

Which Membrane is Right For You?

Nitrocellulose

- Traditional
- Less expensive
- Binding capacity: 80–100 µg/cm^2
- Easily wetted prior to setting up the transfer
- Lower background
- Easy to block
- Use with chicken antibodies
- Comes in two pore sizes: 0.45 µm for proteins >20 kDa and 0.2 µm for smaller proteins
- Very fragile
- Autofluorescent – cannot be used with fluorescent Westerns
- Not compatible with SDS

PVDF

- Binding capacity: 150 to160 µg/cm^2
- More expensive than nitrocellulose
- Very resilient to mechanical and chemical damage
- Use when stripping and reprobing
- Compatible with fluorescent Westerns
- Compatible with SDS-containing buffers
- Requires activation step by wetting in methanol

B. **Wet Transfer.** When performing a wet transfer, you completely submerge the gel/membrane/ filter paper stack in transfer buffer in a specially designed transfer tank. To do so, you place the stack inside a plastic cassette that is inserted into a holder containing the electrodes (either wire or plate electrodes). You then fill the entire tank with transfer buffer. Many manufacturers sell different wet transfer apparatuses.

Wet transfer works well for most routine protein work and is efficient at transferring proteins of all sizes (different transfer buffers can be used to optimize transfer of proteins – see Transfer Buffers). You can also optimize the time of transfer for efficient protein transfer; with a proper cooling system, you can transfer for extended times (e.g. 24 hours) at low voltage, or rapidly (~1-2 hours) at higher voltage. With most systems you can transfer multiple gels at one time, to increase your efficiency. Wet transfer is the method of choice when you are going to quantify your results.

The biggest hindrance to wet transfer is heat. Local increases in temperature can decrease the resistance of the transfer buffer resulting in inconsistent transfer across the gel. High heat can result in breakdown of the gel itself. To compensate, most wet transfer systems are equipped with cooling mechanisms. These can be as simple as an ice block placed in the tank or as complex as a cooling coil attached to an external mechanism. You can also perform transfers with chilled transfer buffer and set up the transfer in a cold room if space allows (highly recommended for overnight transfers).

Another drawback to the wet transfer system is the volume of transfer buffer you need to use to fill the transfer tank (~1000 mL). Transfer buffer should not be reused nor diluted; thus large quantities of buffer are required in active labs.

1. **Wet Transfer Buffers.** Transfer buffers are dual purpose: they elute proteins from the gel and promote binding of the proteins to the membrane. While most labs tend to use a general all-purpose wet transfer buffer, you can improve transfer efficiency by composing a buffer based on the type of gel, the blotting application, the type of membrane, and the physical characteristics of the protein of interest.

    a. **Transfer Buffer Components.**

        i. **Conductive buffer.** The main component of a wet transfer buffer is a conductive buffer. All transfer buffers must contain a strong buffering agent that maintains the conductivity and the pH during transfer. Typical buffers include Tris, CAPS, or carbonate (see Appendix I for recipes).

        You can use 0.7% acetic acid to transfer highly basic proteins, however, the proteins will be positively charged and migrate in the opposite direction. Remember to place the membrane on the correct side of the gel.

        TIP: Never dilute your transfer buffers as dilution will decrease the buffering capacity.

        ii. **Alcohol.** Alcohol (e.g. methanol) is used in transfer buffers to foster binding of proteins to the membrane by removing SDS from SDS-protein complexes. However, alcohol can cause protein precipitation in the gel, interfering with transfer. You should optimize the percent of alcohol in the buffer for the most efficient transfer of your protein.

        Alcohol is not used when working with acidic and neutral proteins. Alcohol can also cause basic proteins to become positively charged or neutral, inhibiting their transfer.

        TIP: Only high-quality, analytical grade alcohol should be used in transfer buffers because impurities in alcohols can result in poor transfer. In many instances ethanol can be substituted for methanol without affecting transfer efficiency, although this should be determined empirically. However, Advansta's FLASHBlot transfer buffer requires methanol.

        iii. **SDS (sodium dodecyl sulfate or sodium laurel sulfate).** You can include SDS detergent in transfer buffers to promote elution of proteins out of the gel. Keep in mind that SDS inhibits binding of proteins to the membrane, particularly when using nitrocellulose membranes. Therefore, SDS should only be used with PVDF membranes and you should empirically determine the optimum SDS concentration for each protein.

SDS vs Methanol: Effects on Protein Transfer

- SDS and methanol have opposing effects on elution of proteins out of the gel and retention of proteins on the membrane.
- While SDS promotes elution of proteins from the gel, it can inhibit binding of proteins to the membrane, particularly when nitrocellulose is used.
- Methanol inhibits migration of proteins out of the gel, but increases retention of proteins on the membrane.
- Carefully titrate SDS and methanol to optimize elution and transfer of proteins.

C. Semi-Dry Transfer. In semi-dry transfer, you soak the filter papers in transfer buffer prior to setting up the transfer. The stack is then placed directly between plate electrodes. The buffer adsorbed by the filter paper is used to conduct the electricity. Because the distance between the electrodes is minimal (the thickness of the stack) high electric field strengths are achieved and transfer is rapid (< 1 h).

Semi-dry transfers are easy to perform, create less mess during set-up, and generally have fewer steps for optimization.

There are several disadvantages to semi-dry transfer that limit its utility. While semi-dry transfer works well for some proteins, the high-intensity blotting conditions can cause smaller proteins to migrate through the membrane without becoming bound.

Although transfer can be rapid, the buffering capacity is low and you can not lengthen the transfer time to accommodate higher molecular weight proteins. However, you can improve the transfer of higher molecular weight proteins by using a discontinuous buffer system (see below).

Semi-dry transfer is not recommended for quantitative Westerns.

1. **Semi-Dry Transfer Buffer.** Semi-dry transfer buffers are similar in composition to wet transfer buffers. A distinct advantage to semi-dry transfer, however, is that you can use a discontinuous transfer buffer system in which the gel is exposed to one type of buffer while the membrane is exposed to a different type of buffer. This is due to the fact that the filter papers provide all the buffer needed for transfer.

    a. **Continuous Semi-Dry Transfer Buffer.** When using a continuous buffer system, all filter paper is soaked in the same buffer. You can use a standard Tris or CAPs-based buffer for semi-dry transfer (see Appendix I).

    b. **Discontinuous Semi-Dry Transfer Buffer.** Using the discontinuous Tris-CAPS buffer system you can take advantage of a unique property of semi-dry blotting: the ability to use two different buffers during transfer.

    For discontinuous buffer system, soak the filter papers placed on either side of the membrane in two different buffers: soak the filter paper assembled on the membrane side (anode) of the blot in buffer containing methanol and soak the filter paper on the gel side (cathode) of the blot in buffer containing SDS. The SDS will promote migration of proteins out of the gel without inhibiting their ability to bind to the membrane. The alcohol will foster protein binding to the membrane without inhibiting migration of the proteins out of the gel.

D. **Assembly and Disassembly of Transfer.** Regardless of the type of transfer apparatus you are using, you should set up the transfer as soon as the gel is done running to prevent diffusion of proteins out of the gel. To do so, perform the following steps:

- Disassemble the apparatus
- Carefully remove the gel
- Cut off the stacking portion of the gel
- If the gel is symmetrical, mark one corner of the gel with a notch for orientation
- Place the gel in a clean tray and soak the gel in transfer buffer for 15 minutes

1. **Setting up a Wet Transfer**
   - Clean transfer sponges and all equipment including cassettes and buffer tank.
   - Cut membrane and 6 pieces of filter paper to the same size as the gel.
   - If using PVDF, activate the membrane with methanol by following the manufacturer's instructions.
   - Equilibrate gel, 2 transfer sponges, membrane, and filter paper in transfer buffer for 15 minutes at room temperature.
   - Set up the stack by placing the following on top of each other: a transfer sponge, three pieces of filter paper, the gel, membrane, three pieces of filter paper, and a transfer sponge. Use a roller to roll out any air bubbles and wrinkles after the addition of each layer. The stack can be built on top of one of the sides of the cassette, but make sure it is oriented such that the proteins will elute out of the gel towards the membrane.
   - Clamp the cassette tightly together and make sure uniform pressure is exerted over the entire stack.
   - Place the cassette into the cassette holder in the tank.
   - Fill tank to the indicated line with transfer buffer. Place ice blocks in tank.
   - Once the transfer is complete, remove the stack from the transfer apparatus. Carefully locate the membrane and use a pencil to mark the protein side of the membrane. Place the membrane in a clean tray for further steps.

2. **Setting up a Semi-Dry Transfer**
   - Clean electrode plates.
   - Cut membrane and 6 pieces of filter paper to the same size as the gel.
   - If using PVDF, activate the membrane with methanol by following the manufacturer's instructions.

- Equilibrate gel, membrane, and filter paper in transfer buffer for 15 minutes at room temperature. If using discontinuous transfer buffer, soak the gel and 3 pieces of filter paper in the buffer for the cathode side of the stack and the membrane and 3 pieces of filter paper in the buffer for the anode side of the stack.

- Build the stack directly on the bottom electrode plate by placing the following on top of each other: three pieces of filter paper, the membrane, the gel, and three pieces of filter paper. Use a roller to roll out any air bubbles and wrinkles after the addition of each layer.

- Place the top electrode plate firmly on top of the stack and make sure uniform pressure is exerted over the entire stack.

- Once the transfer is complete, remove the stack from the transfer apparatus. Carefully locate the membrane and use a pencil to mark the protein side of the membrane. Place the membrane in a clean tray for further steps.

E. **Checking the Efficiency of Transfer**. Once the transfer is completed, you can check the efficiency of transfer by using dyes or stains to visualize proteins. You can stain the gel itself to see what remains in the gel, or you can stain the membrane to observe the transferred proteins. Although you will not be able to correct any transfer deficiencies, staining the gel or membrane at this point can serve as a good confirmation before moving on to the next step or aid in troubleshooting when problems arise.

1. **Staining the Gel.** A standard Coomassie blue (or similar dye) staining protocol can be used to stain the gel after transfer to determine if any residual proteins are left in the gel.

2. **Staining the Membrane.** Reversible dyes, such as Ponceau S, can be used to directly visualize proteins bound to the membrane without interfering with downstream steps.

3. **Stain-Free Detection.** A newer method for observing proteins both in the gel and on the membrane after transfer uses a trihalo compound that reacts with tryptophan residues in proteins causing them to fluoresce (see Chapter 2). The compound is incorporated directly in the gel and causes fluorescence of proteins within the gel and after transfer to the membrane. You can then detect the fluorescent signal using a CCD camera.

Tips for Transfers

1. Immediately set up the transfer once the gel is done running to prevent proteins from slowly eluting out of the gel.

2. Avoid touching the membrane with bare hands. Protein and oils on the skin can adhere to the membrane and can result in spots on the blot.

3. Remove air bubbles between each layer of the stack to avoid uneven transfer. Gently roll a pipette over the gel/membrane/ filter paper "stack."

4. Do not allow excessive heat to be generated during transfer. Use cold buffer, a cooling system, or transfer in a cold room if necessary. Decrease the voltage and/or time if needed.

5. Adjust the transfer conditions for the size of the protein. Reduce the transfer time when working with smaller proteins to prevent transfer of the protein through the membrane. Increase transfer times when working with larger proteins.

6. Increase retention of smaller proteins on the membrane by reducing or removing SDS and/or by using a membrane with a smaller pore size.

7. Facilitate the transfer of larger proteins by increasing the SDS concentration and decreasing the methanol concentration.

8. Use prestained molecular weight markers for a general indication that the transfer is complete.

9. Stain the gel and/or membrane to assess the effectiveness of the transfer.

Blocking the Membrane

Step 4: Blocking the Membrane

A critical step in a successful Western is the blocking of the membrane. Membranes used for Western blotting have a high affinity for proteins, which allows them to efficiently bind and retain transferred proteins. However, before you can detect your protein of interest with an antibody, you must first incubate the membrane with a blocking buffer. The blocking buffer will block the unoccupied binding surfaces on the membrane. Efficient blocking allows antibodies to access target proteins bound to the membrane while preventing high background due to nonspecific binding of primary and secondary antibodies.

> **"Somewhere, something incredible is waiting to be known."**
>
> *– Carl Sagan*

The most important consideration when choosing a blocking solution is the signal:noise ratio. You need to optimize blocking conditions to enhance the specific signal for the antigen-antibody interaction while decreasing background noise. Inadequate blocking results in excessive background and lowers the signal:noise ratio. Excessive blocking can inhibit antibody:antigen interactions or interfere with detection reagents, lowering detection of the true signal.

Although blocking the membrane is one of the most crucial steps in Western blotting, the choice of which blocking agent to use is often overlooked as a step in optimizing Western blots. Instead, a general blocking buffer is frequently used for all applications. However, not all blocking agents are compatible with every antigen-antibody combination and optimization of blocking can greatly improve results.

Blocking buffers are composed of a salt solution, with or without detergent, and a blocking agent.

A. **Base Buffer.** The base blocking buffer is a salt solution, usually either Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.6) or phosphate-buffered saline (PBS; 140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl, pH 7.4).

   For most applications, either TBS or PBS can be used interchangeably, as long as the same buffer system is used throughout the experiment. However, PBS can interfere with alkaline phosphatase (AP) or phospho-specific antibodies, so TBS should be the buffer of choice when using these reagents.

B. **Blocking Agents.** Blocking agents are molecules used to saturate free binding sites on the membrane, preventing nonspecific binding of primary and secondary antibodies in downstream steps. Blocking agents work by covering the unoccupied areas of the membrane with a dense layer of molecules. Blocking agents can either contain proteins or be protein-free.

   1. **Detergents.** A mild, nonionic detergent used in very limiting amounts (e.g. 0.05%) can be used as a blocking agent, and is often included in blocking solutions along with other blockers. Although Tween 20 is most commonly used, Triton X-100 can also serve as a blocking agent. However, omit the detergent from the blocking solution if you are using fluorescent antibodies for detection. Some detergents can autofluoresce causing high background.

      TIP: Detergent-containing solutions can promote microbial growth and blocking buffer should be made fresh prior to use.

2. **Protein-Containing Blocking Agents.** Some of the most popular blocking agents are those that contain proteins. As membranes have a high binding capacity for protein, these blocking agents readily adsorb to all unoccupied sites on the membrane. While generally inexpensive and readily available, protein blocking agents can interfere with some antibody-antigen interactions. Additionally, some proteins may be directly recognized by primary or secondary antibodies causing high background. This issue can be overcome by using another type of protein blocking agent or by using a protein-free agent. Some of the more common protein blocking agents are described below.

   a. **Nonfat Milk.** Nonfat milk is one of the most common blocking agents used for Western blots. It is an efficient blocker because it contains all of the proteins found in milk. Nonfat milk is relatively inexpensive and is easily prepared in the lab from stocks of dry powder.

   Overall, nonfat milk is a good first choice for a blocking agent. However, milk is not recommended when detecting a phosphorylated protein. Milk contains casein, a phosphoprotein that can be recognized by anti-phospho antibodies leading to nonspecific binding and high background. Milk also cannot be used if avidin-biotin detection systems are used because milk contains biotin.

   Most protocols suggest starting with 5% nonfat milk. However, high concentrations of milk can mask some antigens, particularly if they are in low abundance. It is more advantageous to begin with lower concentrations of milk such as 1%. Milk solutions should be filtered prior to use to prevent particulates from binding to the membrane and causing a "speckled" background.

   b. **Bovine Serum Albumin (BSA).** BSA (sometimes called Fraction V) is another commonly used protein blocker derived from the serum of cows. Similar to milk, it is a good general blocking agent that is easily prepared in the lab.

   As mentioned above, BSA is usually preferred over milk when detecting phosphorylated proteins. However, some BSA preparations contain tyrosine phosphorylation and will give a high background when using anti-phosphotyrosine antibodies. Moreover, BSA is not compatible with lectin probes because it contains carbohydrates that can increase nonspecific background.

   BSA can be used in a range from 0.3-5% depending on the application. Similar to milk-containing buffers, BSA solutions should be filtered to remove particulates.

   c. **Serum (Horse or Fetal Calf).** Whole serum, which contains multiple proteins, can be used as a blocking agent at a 10% concentration. The serum is usually derived from horse or fetal calf. Serum is more expensive than milk or BSA and is less commonly used as a blocking agent. Whole serum contains immunoglobulins that can potentially cross-react with primary or secondary antibodies leading to high, nonspecific background.

   d. **Fish Gelatin.** While more commonly used in immunohistochemistry, 2% fish gelatin can be used as a blocking agent for Western blots. Gelatin is more expensive than BSA or milk, but does not cross-react with mammalian proteins. Gelatin cannot be used with avidin-biotin detection systems due to endogenous biotin. While it can decrease the background in some applications, fish gelatin can also mask some antigens.

e. **Single Purified Protein.** Purified single proteins can be used as blocking agents and many are available commercially. The use of a single protein as a blocking agent can prevent the cross-reactivity observed when milk or whole serum is used. Different proteins can be empirically tested for each type of Western blot. Whole casein protein (1%) isolated from milk is an example of a single protein used as a blocking agent. Casein protein cannot be used when detecting phosphoproteins (as mentioned above).

3. **Protein-Free Blocking Agents.** While protein-containing blocking agents are widely used in Western blots, they can have some disadvantages. As discussed above, some proteins, such as casein, can react with antibodies resulting in high background. Other blocking agents can interfere with detection substrates or mask target proteins. For this reason, several companies have developed protein-free blocking agents to provide efficient blocking without cross-reactivity or interference.

AdvanBlock™-PF

✓ **PROTEIN-FREE**
✓ **VERSATILE**
✓ **OPTIMIZED**

AdvanBlock-PF is a proprietary protein-free blocking solution compatible with both both chemiluminescent and fluorescent Western blots. AdvanBlock-PF can reduce background when used with antibodies that have a high degree of cross-reactivity with protein blocking agents. AdvanBlock-PF can also be used with protein blocking agents to increase blocking efficiency for low-quality primary antibodies.

General Blocking Protocol

1. Choose the appropriate base buffer (TBS or PBS) and blocking agent.
    • A common starting blocking buffer is 1% nonfat milk in TBS containing 0.05% Tween-20.
2. Make the blocking buffer fresh just prior to use.
3. Ensure that any detergent is fully dissolved.
4. Make sure all particulates are dissolved in the blocking buffer; filter if necessary.
5. Place the membrane, protein side up, in a clean plastic container.
6. Add blocking solution.
7. Agitate for 1 hour at room temperature or overnight at 4°C.
8. Keep blot covered to prevent particulates from falling in.
9. Pour off blocking buffer.
10. Rinse blot with antibody incubation buffer.

Antibody Hybridization

Step 5: Antibody Hybridization

After blocking the membrane to prevent nonspecific binding, it's time to incubate the membrane with antibodies. There are several things to consider for this step:

- Direct vs indirect detection
- Choice of enzyme or tag for detection
- Antibody type and quality

A. Direct vs Indirect Detection. Direct methods of detection use a primary antibody conjugated to a detectable label. Antibodies can be labeled in-house with commercially available labeling kits, or purchased prelabeled. Direct detection is quicker than indirect detection as it reduces the number of steps in the procedure. However, direct detection methods can be more costly, are less sensitive, and are also less flexible.

Indirect methods of detection are more commonly used. For this method, you first incubate the membrane with an unconjugated primary antibody and then with a conjugated secondary antibody that is specific for the primary antibody. Indirect detection is more sensitive than direct detection because each primary antibody molecule may be bound by multiple secondary antibodies, amplifying the signal. In addition, using conjugated secondary antibodies increases flexibility because a wide variety of label types are available on prelabeled secondary antibodies. Indirect detection is also more cost effective than direct detection because one secondary antibody can be used to recognize multiple primary antibodies in different experiments.
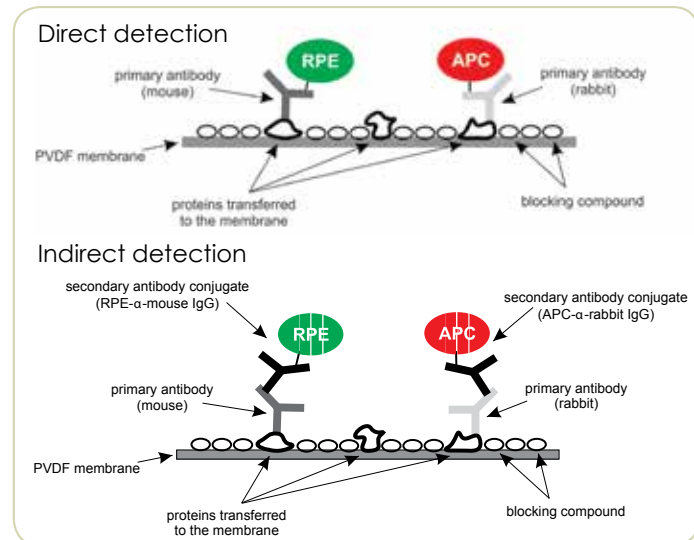
B. Choice of Enzyme/tag. Primary or secondary antibodies can be conjugated to a variety of enzymes, tags, or dyes for downstream detection. You should choose the label you will use based on the sensitivity required for detection, equipment available for visualization and documentation, and whether you need to multiplex (e.g. you need to detect proteins with similar molecular weights simultaneously).

1. **Enzyme-labeled.** Enzyme-conjugated antibodies are versatile as a variety of substrates are available for detection and documentation. Incubation of the enzyme-conjugated antibody with the appropriate substrate will produce a colored product or light.

The two most frequently used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). In general, detection using HRP is more sensitive than AP, because the enzyme has a faster catalytic rate and produces more product in a shorter time. However, the product tends to fade quickly with colorimetric detection. In addition, the activity of HRP is limited due to an oxidation reaction. While AP has a slower catalytic rate, it is not limited and the reaction rate remains linear over a longer period of time. Sensitivity can be increased by allowing the reaction to continue for a longer period of time, however, increased incubation times can lead to high background, decreasing the signal: noise ratio.

Both HRP and AP can be used for colorimetric and enhanced chemiluminescence detection methods (ECL). However, HRP is more commonly used for ECL.

2. **Fluorescently Labeled.** Fluorescently labeled antibodies conjugated to fluorophores are easy to use for detection because no substrate development steps are required. However, equipment with the appropriate excitation and emission capabilities is required for each individual fluorophore.

Fluorescently conjugated secondary antibodies are becoming increasingly popular for use in Western blotting. While the first available fluorophores were limited to a few spectral wavelengths, newly developed fluorophores have increased the options to include dyes in the infrared and near infrared ranges. Fluorescently labeled secondary antibodies are used in multiplex Western blot assays to detect multiple antigens simultaneously.

Secondary Antibodies

HRP-conjugated

✓ **PERFORMANCE**
✓ **CONVENIENCE**
✓ **PURITY**
✓ **FLEXIBILITY**

HRP-conjugated secondary antibodies for chemiluminescent immunodetection. Advansta's high-quality HRP-conjugated secondary antibodies offer unparalleled sensitivity and performance for immunoblotting and ELISA applications.

Fluorescent conjugated

✓ **PERFORMANCE**
✓ **CONVENIENCE**
✓ **FLEXIBILITY**

Fluorescently labeled antibodies for immunoblotting applications. Advansta offers a broad selection of fluorescently labeled secondary antibodies with a variety of conjugate types, excitable with both visible and near infrared (NIR) light.

Fluorescent Streptavidin conjugated

✓ **EXTREMELY BRIGHT FLUORESCENCE**
✓ **FLEXIBILITY**
✓ **COMPATIBLE**

Fluorescent streptavidin conjugates to detect biotinylated proteins. These streptavidin conjugates are prepared from phycobiliproteins. Phycobiliproteins harvest the light very efficiently; their molar extinction exceeds that of any known organic fluorescent dye by one or two orders of magnitude, and they fluoresce with extremely high quantum efficiency.

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3. **Biotin-labeled.** Biotin-labeled antibodies can be used to amplify signal intensity and increase sensitivity. After incubation with a biotin-labeled antibody, the membrane is incubated with avidin (a glycoprotein in egg white) or streptavidin (from the bacteria *Streptomyces avidinii*). The (strept)avidin is conjugated to an enzyme for colorimetric or ECL detection or a fluorophore for immediate detection. Alternatively, the target can be further incubated with additional biotin molecules forming a molecular biotin-avidin-biotin "bridge" prior to detection. Since each (strept)avidin molecule binds four biotin molecules, the signal is amplified. The biotin-avidin system is compatible with all species of antibodies.

4. **Polymer-conjugated.** A more recent antibody conjugation technology involves polymerizaton. Polymers of active enzymes (e.g. HRP) are conjugated directly to antibodies in a manner that does not inhibit antibody function. Sensitivity is enhanced because the number of active enzymes per antibody molecule is increased. Using polymer-conjugated antibodies is similar to using biotin-conjugated enzymes for increased sensitivity, however only one incubation step is required with the polymer-conjugated antibodies.

C. **Antibody Type and Quality.** Whether using a direct or an indirect detection method, it is important to choose good quality antibodies for Western blots as the specificity and sensitivity of the assay are dependent upon the antibodies.

1. **Primary Antibody.** After the membrane is blocked, the blot is incubated with the primary antibody. Generally, antibodies recognize a small sequence of amino acids (the epitope) that is exposed by removal of the higher order protein structure under reducing and denaturing conditions. The specificity of Western blotting is achieved by using a primary antibody that recognizes and binds to an unique region of the protein of interest.

a. **Monoclonal vs Polyclonal.** You can use either a monoclonal or a polyclonal primary antibody for your Western blot. The two types have distinct advantages and disadvantages and differ in how they are produced.

1. **Polyclonal.** Polyclonal antibodies are obtained directly from the serum of an immunized animal, commonly a rabbit, goat, donkey, or sheep, and are then purified and tested. Polyclonal antibodies recognize multiple epitopes within the antigen. Because of this, polyclonal antibodies can give stronger signals and recognize multiple forms of a protein simultaneously.

SpectraDye™ Antibody Labeling Kits

✓ **RAPID**
✓ **VERSATILE**
✓ **FLEXIBLE**
✓ **SAVE TIME AND MONEY**
✓ **SAVE ANTIBODY**

SpectraDye Antibody Labeling Kits contain everything you need to produce covalently labeled, fluorescent antibodies in only 30 minutes. Choose from commonly used fluorescent dyes and label your antibody in a single step. Labeled antibodies are compatible with immunofluorescence applications including flow cytometry, Western blotting, and immunofluorescence microscopy so you can validate your results across multiple platforms using the same antibody.

2. **Monoclonal.** Monoclonal antibodies recognize a single epitope on the protein. To produce a monoclonal antibody, antibody-synthesizing cells are isolated from the spleen of an immunized animal and fused with myeloma cells. The resulting hybridomas secrete antibodies into the culture media and the antibodies are then tested for their affinity for the antigen. The hybridomas with the most stable production of antibodies are selected and can be grown in culture indefinitely.

Monoclonal antibodies may not give a signal as robust as those seen with polyclonal antibodies. However, due to their specificity for a single epitope, monoclonal antibodies are useful in detecting specific forms of proteins such as phosphorylated proteins or cleavage products. Some characteristics of monoclonal and polyclonal antibodies are described in Table 5.1.

	Monoclonal Antibodies	Polyclonal Antibodies
Epitope recognition and sensitivity	• Recognize a single epitope • Less sensitive due to only one epitope for antibody binding on each protein	• Recognize multiple epitopes on a protein • More sensitive due to multiple sites for antibody binding on each protein • Good for low abundance targets
Potential for cross-reactivity	• Cross-reactions less likely • Potential to recognize other proteins having the epitope	• Cross reactions more likely • Potential for higher background due to multiple epitope recognition • More likely to crossreact with multiple animal species
Time required for production	Lengthy	Shorter
Preparation cost	Higher cost – requires specialized equipment and trained individuals	Lower cost
Variability	Batches from the same hybridoma are very stable	Prone to variability between batches
Tolerance to varying conditions	Less tolerant – may fail to recognize antigen in reducing/denaturing conditions, or if chemical modifications (glycosylation, phosphorylation) or differences in amino acid sequence due to polymorphism are present	More tolerant

Table 5.1. Monoclonal vs Polyclonal Primary Antibodies

2. **Secondary Antibody.** If you do not use the direct detection method, than you will need to incubate the membrane with a conjugated secondary antibody after incubation with the primary antibody.

Secondary antibodies are commercially available in a variety of forms, including monoclonal, polyclonal, F(ab')2 fragments, affinity purified, and preadsorbed. Several factors influence the choice of secondary antibody including the species source of the primary antibody, the class of the primary antibody, the detection method, and any special factors regarding the technique or sample that would require a higher-specificity secondary antibody.

a. **Species Specificity.** The first factor you should consider when choosing a secondary antibody is the species of animal in which the primary antibody was generated. The secondary antibody must be specific for the primary antibody. For example, if you are using a primary antibody that was made in a rabbit, then you need to use an anti-rabbit secondary antibody.

b. **Class (isotype and subclass).** Antibodies are classified into different classes (also called isotypes). Placental mammals express 5 different classes of antibody named for the type of heavy chain: IgG, IgM, IgA, IgE, and IgD. Each heavy chain can associate with one of two light chains, called kappa and lambda. Antibodies are further classified into subclasses based on minor differences in the heavy chain of each class.

The secondary antibody must be specific for the class/subclass of the primary antibody. Polyclonal primary antibodies are usually raised in rabbit, goat, donkey, or sheep and are the IgG isotype, containing a mixture of the different subclasses. For polyclonal primaries, use a general anti-IgG secondary antibody that will recognize heavy and light chains of all subclasses. This antibody may also interact with other isotypes through recognition of the light chain.

Monoclonal primary antibodies are usually raised in mouse, rabbit, or rat. Since monoclonal antibodies are clonal, each antibody molecule will be of the same subclass (for example IgG1, IgG2a, IgG2b, IgG2c, IgG3 in the mouse). While a general anti-IgG secondary antibody that will recognize all subclasses can be used, sometimes it can be advantageous to purchase a sub-class–specific antibody (e.g. anti-mouse IgG2a) to increase the specificity of the interaction or when detecting multiple primary antibodies of different classes simultaneously (multiplexing).

TIP: Birds produce a novel immunoglobulin protein called IgY and anti-IgY secondary antibodies must be used when working with primary antibodies generated in chickens.

c. **Secondary antibodies can recognize different regions of the primary antibody.** You can also use secondary antibodies that recognize specific parts of the primary antibody.

    i. **Anti-H+L.** An anti-heavy and light chain (H+L) secondary antibody recognizes both the heavy and light chains of the antibody molecule. This is a commonly used type of secondary antibody, particularly when the class of the primary antibody is unknown, because all immunoglobulin classes share the same light chains.

    ii. **Light chain–specific secondary antibodies.** Secondary antibodies that only recognize light chains can also be used. These antibodies can be very helpful when performing immunoprecipitations followed by Western blotting. Use of the anti-light chain antibody will prevent recognition of the 50 kDa heavy chain protein on the blot. However, the 25 kDa light chain protein will still be visible.

d. **Antibody Purification.** Secondary antibodies can undergo additional purification steps to increase specificity.

   i. **Affinity-purified antibodies.** Polyclonal antibodies can be affinity purified to increase sensitivity and decrease background. Undesired antibodies are removed by purifying the antibodies through a column. For example, anti-rabbit IgG antibodies can be purified through a column containing rabbit IgM and IgA. Anti-IgM and anti-IgA antibodies will remain bound to the column while anti-IgG antibodies will flow through. Affinity-purified secondary antibodies have the benefit of containing very high-affinity antibodies and therefore may be useful in detecting low-abundance proteins.

   ii. **Adsorbed secondary antibodies.** Secondary antibodies can also be purified by adsorbing the antibodies against IgG molecules from other species of animals. Adsorption removes potential nonspecific interactions among related species. It can also be used to reduce shared reactivity among heavy and light chains. For example, an anti-mouse secondary antibody might also recognize similar regions on rabbit IgG if rabbit IgG is used in the same assay. Adsorption of the anti-mouse antibody to rabbit IgG will remove this reactivity. Adsorbed antibodies are designed for particular applications, such as immunohistochemistry or multiplex assays. Care needs to be taken when using these antibodies though; adsorption can also titrate out the desired antibodies and lead to a decrease in sensitivity.

D. **General Antibody Incubation Protocol.** Perform antibody incubations and washes as follows:

- Dilute the antibody in blocking buffer, TBST, PBST, or a provided antibody dilution buffer. While the antibody vendor may recommend a starting dilution, you may need to determine the optimal antibody concentration empirically.

- Incubate the membrane either one hour at room temperature or overnight at 4°C.
  - Cover the blot completely with solution
  - Gently agitate the blot by placing on a platform shaker or rocker.

- Wash the blot with wash buffer.
  - Start with 4 washes, 5 minutes each, at room temperature. Decrease or increase the number of washes or length of washes to optimize.

- Dilute the secondary antibody in blocking buffer, TBST, or PBST.

- Incubate for one hour at room temperature.
  - Cover the blot completely with solution
  - Gently agitate the blot by placing on a platform shaker or rocker.

- Wash the blot with wash buffer.
  - Start with 4 washes, 5 minutes each, at room temperature. Decrease or increase the number of washes or length of washes to optimize.

Tips for Finding a Good Primary Antibody

1. **Dare to compare.** Take the time to comparison shop prior to buying an antibody. Use an antibody comparison site such as Biocompare, Antibodypedia, CiteAb, or Antibody Resource. Carefully compare names or antibody clones (the same antibody can be marketed by different companies), concentrations, prices, aliquot sizes, and recommended dilutions.

2. **Search the literature.** If you find an antibody using a comparison site, search PubMed for articles in which people have used the antibody. Read the methods and look at the figures to see if the antibody worked well. If you can't find an antibody using one of the comparison sites, search the literature for antibodies that may not be commercially available.

3. **Pay attention to application.** The best-case scenario is to find an antibody that has already been used in the exact manner in which you intend to use it. Search the literature, product reviews, and product data sheets to determine how the antibody has been used in the past. Be mindful that antibodies that work well in one application may not be suited for another. For example, an antibody that works well in a Western may not work well in immunoprecipitations due to conformational changes in the protein. Alternatively, posttranslational modifications, such as phosphorylation, might mask or reveal epitopes recognized by antibodies (particularly monoclonal antibodies).

   If you can't find an antibody that has been used in your application, choose one that has been used in a compatible manner. For example, antibodies used in FACS analysis on live cells might also work in native immunoprecipitations where the integrity of the protein is maintained.

   Additionally, pay attention to the species in which the antibody was generated and make sure it is compatible with your application. If you are going to be using multiple antibodies at the same time (e.g. in multiplex Westerns) make sure your antibodies won't be cross-reactive.

4. **Species, species, species.** One factor that should be taken into consideration early in your antibody search is the species of animal in which the primary antibody was generated and whether it will be compatible with your application. If you need to use a conjugated secondary antibody to detect the primary antibody (e.g. when performing immunohistochemistry) the primary antibody should be from a species as phylogenetically distinct as possible from the species of the sample. This will prevent cross-reactivity between the secondary antibody and endogenous immunoglobulins in the sample.

5. **Check how the antibody was validated.** Many antibodies are validated using recombinant proteins that are abundantly expressed. Or, they are validated using proteins that were produced in non-native hosts such as bacteria or yeast. While these validations show that the antibody can work, they do not necessarily show that the antibody will work under alternative conditions. If you need to use an antibody to detect a rare, highly glycosylated protein in mammalian cells, then an antibody that has only been shown to detect recombinant protein in bacteria might not be the best choice for you.

6. **The type of antibody matters.** Is it better to us a monoclonal or a polyclonal antibody? For some proteins, you don't have a say in the matter - you have to go with whatever antibody is available. However, if you do have a choice, choosing the right kind of antibody might increase your success. Polyclonal antibodies, which recognize multiple epitopes, are often better in Western blots. They can also be more robust and detect less-abundant proteins.

Monoclonal antibodies are useful in detecting conformation-specific epitopes and are invaluable when using multiple antibodies simultaneously (multiplexing). Due to their specificity, monoclonal antibodies often result in lower background. Because they only recognize a single epitope though, monoclonal antibodies can be more limited in application.

7. **Ask about trial samples or guarantees.** If you are not sure an antibody will work in your application, don't be afraid to ask for a trial sample. Some companies will send you a small aliquot for free or charge you a small fee for a "one-time use" aliquot. You can also suggest providing them with validation data in exchange for antibody. Some companies will guarantee that their antibody will work in a particular application. Limited risk can be very advantageous to you, but be careful of the fine print! You might only get a credit towards another antibody, or you might be asked to perform several experiments to prove it doesn't work!

Tips to Optimize Fluorescent Blocking and Washing Buffers

1. **P or T?** Similar to chemiluminescent Westerns, you can use either a phosphate- or tris-buffered system for your blocking, incubation, and washing solutions. However, phosphate can bind to some phospho-specific antibodies decreasing your ability to detect your protein of interest. This is especially important to remember for fluorescent Westerns because they lend themselves so easily to quantifying post-translational modifications.

   Whichever you choose, keep it consistent throughout the experiment.

2. **Omit Tween 20.** Tween 20 is often used in low concentrations in blocking buffers for chemiluminescent Westerns to improve blocking. But Tween 20 can be autofluorescent and actually increase non-specific background when used in fluorescent Westerns.

   To use Tween 20 in a fluorescent Western, leave it out of the blocking solution, but add it to the primary and secondary antibody incubation solutions, as well as the wash solutions. Use 0.1 - 0.2% for antibody solutions and 0.1% for washes.

3. **Add SDS.** SDS is a harsh detergent that can interfere with antibody-antigen interactions, so too much can be a bad thing. But a little SDS can go a long way in reducing background on PVDF membranes, especially when detection occurs in the 700 nm range. SDS should only be added to the secondary antibody incubation solution. A concentration of 0.01-0.02% is sufficient.

   Note: SDS should never be used with nitrocellulose membranes.

4. **Get rid of the spots.** Particulates in buffers can stick to your membrane causing fluorescent speckle artifacts. You can make particulate-free solutions by:
   • Using high-quality reagents
   • Making sure all components are completely solubilized
   • Filtering the blocking solution

5. **Watch out for lot-to-lot variations.** If you are all of a sudden encountering background where you never did before, it might be due to a new batch of membrane. Different lots of membranes are associated with different levels of background. You might have to re-optimize your blocking and washing solutions to decrease the background.

AdvanWash™

✓ **RELIABLE**
✓ **VERSATILE**
✓ **CONVENIENT**

Advansta sells two wash buffers for chemiluminescent and fluorescent Western blots. Both are provided as a 10X concentrate.

• AdvanWash is optimized for detection with Cy3- and Cy5-like labeled dyes
• AdvanWash-IR is the wash buffer to use when working with NIR antibodies

Detection

Step 6: Detection

The moment of truth has arrived. You have stuck by your blot for hours – now it is time to reveal the results of your experiment, but you have some choices to make.

There are four main methods of detection in Western blotting: radioisotopic, colorimetric, chemiluminescent, and fluorescent. Although extremely sensitive, radioisotopes have safety issues and require strict waste handling and disposal procedures. For these reasons, radioisotopes are rarely used today for standard Western blots, and will not be discussed.

However, that still leaves you with three different methods of detection: colorimetric, chemiluminescent, and fluorescent. This guide will discuss the benefits and drawbacks to all three methods. A summary is shown in Table 6.1.

Method	Advantages	Disadvantages
Colorimetric	• Simple • Cost effective • No special equipment required for detection • Can stop reaction when desired color achieved • Easy to optimize • Can use two colors for detection of multiple proteins • Ideal for abundant proteins	• Lack sensitivity • Color fades over time, can be difficult to document • Toxicity • Precipitate cannot be stripped off for reprobing • Performance of substrate can vary with manufacturer
Chemiluminescent	• Highly sensitive • Membrane can be stripped and reprobed • Results easily documented • Multiple exposures can be taken • Multiple substrates available for optimization • Can image with film or with digital imager	• Requires a darkroom or imaging equipment • Semiquantitative • Signal intensity can vary with incubation or exposure time • Cannot detect more than one protein simultaneously • Performance of substrate can vary with manufacturer
Fluorescent	• Sensitive • Can multiplex • Large dynamic range • Quantitative • No darkroom required • Can archive blots and image months later	• Less sensitive than chemiluminescence • Equipment and reagents can be costly • Need to re-optimize reagents when switching from chemiluminescence • Need to avoid reagents that autofluoresce

Table 6.1. Detection Methods for Western Blots

WesternBright™ MCF and MCF-IR

- ✓ **FLUORESCENT DETECTION**
- ✓ **ADVANBLOCK™-PF**
- ✓ **HIGH SENSITIVITY**
- ✓ **FAST RESULTS**
- ✓ **WESTERNBRIGHT MCF**
- ✓ **WESTERNBRIGHT MCF-IR**
- ✓ **ADVANWASH AND ADVANWASH-IR**

WesternBright MCF visible and near infrared (IR) fluorescent Western blotting kits allow the assay of two proteins at once, increasing the quality and quantity of information that can be gained from a single blot. Assay a control alongside a protein of interest. Assay phosphorylated and un-phosphorylated isoforms of a protein simultaneously. The fluorescent dyes provided with WesternBright MCF and WesternBright MCF-IR outperform Cy™ dyes and allow detection of low pg of protein. The WesternBright MCF protocols save time and money since there is no need for disposable film, and the blot can be imaged immediately, without drying.

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A. Colorimetric Assays. Colorimetric methods use an antibody that has been conjugated to an enzyme, usually horseradish peroxidase or alkaline phosphatase. After incubation with the conjugated antibody and washing of the membrane, the blot is incubated with a solution containing a substrate that is converted into an insoluble colored product by the enzyme. The product precipitates onto the membrane creating a band of color wherever the antibody is bound. The amount of precipitate formed is proportional to the protein level in the sample.

1. **Advantages of Colorimetric Assays.** Colorimetric assays have several advantages. They are usually very economical and products can be purchased from many companies or in bulk. After addition of the substrate, the reaction is easily monitored and stopped when the desired color is reached, aiding in optimization. Since color development occurs directly on the blot, no special equipment is needed for development and documentation. The staining intensity can then be measured spectrophotometrically or the blot can be scanned and densitometry used to measure the intensity of the color. Different proteins can be detected serially on the same blot by using substrates that produce different colors.

2. **Disadvantages of Colorimetric Assays.** In general, colorimetric assays are not as sensitive as chemiluminescent or fluorescent assays, although sensitivity can vary based on the enzyme and substrate combination. Some precipitates can fade over time so it is necessary to document the blot as soon as possible after development. Some substrates are toxic and must be handled and disposed of appropriately.

3. **Substrates for Colorimetric Assays.** Different substrates are required for each enzyme. Although individual reagents can be purchased separately, kits are also available containing all the components necessary for colorimetric detection. Some kits provide means to increase the sensitivity of the assay.

    a. **Substrates for Horseradish Peroxidase (HRP).** The two most common substrates used with HRP are 4-Chloro-1-naphthol (4CN) and 3, 3'-diaminobenzidine (DAB). In the presence of hydrogen peroxide, HRP reacts with 4CN to produce a purple precipitate and with DAB to produce a brown precipitate. Both have a limit of detection of 500 pg.

       The HRP-generated precipitates fade over time when exposed to light; the membrane needs to be photographed or scanned for permanent documentation. In addition, azide, which can be added to antibody solutions as a preservative, can inhibit HRP activity. Therefore, any antibody solutions containing azide must be thoroughly washed off the blot prior to detection.

       TIP: HRP requires the addition of hydrogen peroxide to the detection substrate. Hydrogen peroxide should be freshly added for optimum color development.

    b. **Substrates for Alkaline Phosphatase (AP).** 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) are used together with AP to produce a purple-blue precipitate that does not fade over time. AP detection systems are more sensitive than HRP detection assays (limit of detection 100 pg).

4. **Developing the color.** To perform a colorimetric assay, follow this general procedure:
   - Prepare the color substrate just prior to use by following the manufacturer's instructions.
   - After the final wash, cover the membrane with the staining solution.
   - Allow the membrane to incubate at room temperature until the desired color is achieved.
   - Rinse with stopping solution or water.
   - Document the results immediately.

B. **Chemiluminescence.** Since the advent of enhanced chemiluminescence (ECL), many substrates have been developed for light production by HRP. Chemiluminescent substrates emit light when reacted with an antibody conjugated to an enzyme. The emitted light is captured and archived on x-ray film (traditional), or through digital imaging.

Advansta sells a family of HRP products optimized for ECL with film or CCD detection…

WesternBright™ ECL & ECL Spray

✓ **STRONGER SIGNAL**
✓ **SENSITIVITY**
✓ **CONSERVE ANTIBODY**

WesternBright ECL is a sensitive substrate perfect for general film imaging. Using this substrate you can detect low pg amounts of protein while using up to 10x less antibody. The long-lasting signal lets you take your time to get the perfect exposure.

WesternBright ECL Spray gives you both components of WesternBright ECL in one convenient spray bottle. No measuring or mixing required – just spray the blot and image.

WesternBright™ Quantum™

✓ **SENSITIVE**
✓ **QUANTITATIVE**
✓ **LOW BACKGROUND**
✓ **LONG-LASTING SIGNAL**
✓ **VERSATILE**

WesternBright Quantum was specifically developed to give the broadest useful linear range when using CCD imagers. Quantum has a high sensitivity for low abundance proteins, but doesn't exhibit substrate depletion when detecting high abundance proteins. This leads to a linear range over 3 orders of magnitude. Although WesternBright Quantum was optimized for CCD imagers, it can also be used with film.

WesternBright™ Sirius™

✓ **SENSITIVE**
✓ **LOW BACKGROUND**
✓ **LONG-LASTING SIGNAL**

WesternBright Sirius was designed for the difficult-to-detect protein. With a high signal-to-noise ratio and low background, Sirius is Advansta's most sensitive substrate. It is ideal for detecting low-abundance proteins or when using a very low concentration of antibody. Sirius can be used with both film and CCD imagers.

Chemiluminescence is useful for detecting the presence or absence of a protein. For example, chemiluminescence can be used to detect the induction of exogenous protein expression, to confirm and follow purification of a known protein, or for verification of antibodies during production.

1. **Advantages of Chemiluminescent Detection.** Chemiluminescent detection is highly sensitive and can detect femtograms of protein. The results are easily documented on film or with a digital imager. Multiple exposures can be taken in succession to achieve the right image. In addition, multiple substrates with varying properties can be used to optimize results.

2. **Disadvantages of Chemiluminescent Detection.** Chemiluminescent detection has several drawbacks. The chemiluminescence is emitted at only one wavelength, therefore only proteins with significantly different molecular weights can be detected on the same blot (either by cutting the blot and incubating each section separately, by stripping and reprobing the blot, or by detecting proteins serially). Often this means that loading and normalization controls cannot be easily performed. In addition, chemiluminescence is only semi-quantitative. Many substrates have a limited dynamic range of detection and can saturate at high protein concentrations. Signal duration can be short and local differences in enzyme and substrate concentrations can affect results.

3. **Chemiluminescent Detection.** The following steps should be followed for chemiluminescent detection:
   - If stored at 4°C, prewarm substrate components to room temperature.
   - Mix substrate components according to manufacturer's instructions.
   - Carefully drain all wash solution off the membrane.
   - Completely cover membrane with substrate solution and incubate for indicated amount of time.
   - Drain off all of the substrate solution and quickly blot edge of membrane to remove excess liquid.
   - Wrap membrane in plastic to prevent drying out.
   - Document using film or digital imager.

LucentBlue™
X-ray film

✓ **PERFORMANCE**
✓ **VALUE**
✓ **FLEXIBILITY**

High sensitivity X-ray film for chemiluminescent Western blots. LucentBlue is a sensitive film ideal for imaging chemiluminescent Western blots and is compatible with horseradish peroxidase and alkaline phosphatase detection reagents as well as autoradiography.

4. **Film Versus Digital Imaging.** The chemiluminescent signal can be captured on film or with a CCD camera and digital imager.

a. **Film.** Exposing x-ray film is the traditional method for capturing chemiluminescent signals. X-ray film is coated with an emulsion that contains light-sensitive silver halide crystals or grains. Light activates the silver crystals, converting some of the silver crystals to atoms. However, you can't visualize this because the change is so small. When you develop the film, the silver atoms catalyze the reduction of the entire crystal to black metallic silver that is visible on the film.

The light emitted during development with chemiluminescent substrates is easily captured by x-ray film. To do so, the x-ray film is placed directly against the plastic-wrapped blot. The close apposition of the blot to the film allows for sensitive detection of the signal. You can make multiple exposures and adjust the exposure time to capture both high-intensity and low-intensity signals. You then develop the film using a commercial developer.

Film is ideal for documenting relative amounts of proteins, such as assessing whether a protein is expressed by a particular tissue or cell line, or when comparing large signal differences. However, a major drawback to film is that it is only semi-quantitative. Film is less sensitive to dim light and is easily saturated by moderate to high signal intensities, resulting in a narrow linear range. Although the film can be scanned and densitometry used to quantify signals relative to a loading control, this is generally not regarded as a quantitative measurement due to the limitations of film.

b. **Digital Imaging.** Digital imagers are all-in-one imaging units that contain a charged-coupled device (CCD) camera for detecting the signal and a computer unit for capturing, analyzing and storing the image.

CCD cameras convert photons into digital signals using a light-sensitive silicon chip. High-performance CCD cameras are capable of matching the speed and sensitivity of x-ray film by cooling the silicon chip to sub-zero temperatures, which reduces background noise. In addition, detection sensitivity is increased by "binning" or combining the pixels. Binning effectively increases the size of each pixel, reducing resolution but increasing the amount of light collected in the pixel area. CCD cameras detect chemiluminescent signals over a much broader range than film. Typically, a digital imager can record from 4,096-65,536 levels of intensity (approximately 2 to 4 orders of magnitude greater than x-ray film). Therefore a signal that may be saturated when captured on x-ray film is still in the linear dynamic range when using a digital imager.

To use a digital imager, place your blot in the digital imager unit and capture the signal. You can immediately manipulate the image and obtain the correct exposure without waiting for film to develop.

While digital imagers are the "gold standard" for performing quantitative Western blots, they can be costly. Depending on the performance capability of the imager, a unit can cost from $10,000 to $40,000 and higher.

c. **Choosing the Best Method for Your Experiment.** The choice of the appropriate technology for detecting and archiving Western blots will depend on the design of the experiment. If a yes/no answer is needed to determine if an abundant protein is being expressed, then film is more than adequate. However, if proteins with varying expression levels are being detected or proteins need to be accurately quantified, than digital imaging is a better choice.

Method	Advantages	Disadvantages
Film	• Traditional method • Quick • Sensitive • Ideal for detecting relative levels of single protein • Many facilities have developers	• Saturation of film • Limited dynamic range • Continuous purchase of film • Requires a dark room
Digital	• Broad dynamic range • Fluorescent Western capability • Detect more than one protein simultaneously • Quantitative • Digital documentation	• Requires purchase of digital imager

Table 6.2. Advantages and Disadvantages of Film and Digital Imaging

5. **Stripping and Reprobing the Membrane.** After imaging, antibodies can be stripped from the membrane so the blot can be reprobed for another protein. This procedure can conserve samples, but is time consuming. Stripping can also be uneven resulting in some antibodies being left on the blot or it can be too harsh and remove target proteins from the blot. For these reasons, stripping and reprobing should never be used for a quantitative Wetern blot.

C. **Fluorescent Detection**. Fluorescent Westerns are becoming increasingly popular in the lab. In fluorescent Western blotting, secondary antibodies are directly conjugated to a fluorophore, which is excited by light. The emitted light is detected by a digital imager capable of measuring fluorescence or a CCD camera with filters for the appropriate emission wavelengths. The image is digitized for data analysis.

1. **Advantages of Fluorescent Detection.** Fluorescent detection has distinct advantages over colorimetric or chemiluminescent detection methods. Unlike detecting reagents that rely on enzyme-substrate interactions and eventually saturate, the amount of dye is directly proportional to the amount of protein, making the assay truly quantitative. Fluorescent dyes are extremely stable and blots can be archived and imaged months after the experiment. Another major advantage to fluorescent Western blotting is the ability to multiplex, or detect two or more proteins simultaneously. Fluorescently labeled secondary antibodies can be purchased that have non-overlapping emission spectra. This allows detection of normalization/loading controls on the same blot as the protein of interest. In addition, post-translational modifications that do not significantly alter the protein molecular weight can be detected simultaneously with the unmodified form of the protein.

2. **Disadvantages of Fluorescent Detection.** Fluorescent Western blotting can be cost prohibitive. A digital imager is required to detect, capture, and archive the fluorescent signal. Fluorescent secondary antibodies can also be costly. Many researchers find that primary antibodies need to be re-optimized for use in fluorescent Westerns when switching from chemiluminescence; often more primary antibody is required. Care needs to be taken to avoid reagents (e.g. bromophenol blue) and supplies (e.g. nitrocellulose) that autofluoresce. Fluorescent Western blotting can also be less sensitive than chemiluminescent blotting (10-100 times less sensitive, depending on the protein). However, this can be overcome by using reagents that amplify the signal.

3. **Fluorescent Detection.** Fluorescent detection is done immediately following incubation and washing with the tagged antibody:

    • Rinse membrane with TBS or PBS to remove residual detergent.

    • Image blot using appropriate excitation and emission settings.

    • Alternatively, store the blot for up to 48 hours in TBS or PBS in the dark, or air-dry the blot and store indefinitely away from light.

Tips for Chemiluminescent Westerns

1. Don't overload the wells.

2. Try different blocking buffers.

3. Titrate primary and secondary antibodies for highest signal:noise ratio.

4. Never dilute HRP-conjugated secondary antibody in buffer with sodium azide.

5. Always handle membrane with gloved hands.

6. Use enough substrate to cover entire blot.

7. Optimize the substrate to increase sensitivity and duration.

8. Remember to equilibrate substrate to room temperature before use.

9. Use digital imaging to increase dynamic range.

Tips for Fluorescent Westerns

1. Titrate primary and secondary antibodies for highest signal:noise ratio.

   • Primary antibodies may need to be increased 2- to 5-fold compared to chemiluminescent Westerns.

2. Use a PVDF membrane with low autofluorescence.

3. Avoid dyes that fluoresce (e.g. bromophenol blue).

4. Use a pencil to mark blot as some pen ink fluoresces.

5. Always handle membrane with gloved hands.

6. If using fluorescent molecular weight markers, skip a lane before loading samples.

7. Store fluorescent antibodies in the dark.

8. When archiving blots, store away from light.

Tips for Multiplexing

1. Use primary antibodies made in different species.

2. Use secondary antibodies that are highly cross-adsorbed.

3. Use fluorophores with optically distinct spectra to avoid spectral overlap.

4. Always optimize detection of each target separately prior to simultaneous detection.

5. Detect the strongest target in the blue channel, middle in the green channel, and the weakest in the red channel.

Appendix A

Sample Recipes

A. Detergent Lysis Buffers

RIPA Buffer (denaturing lysis buffer)
- 25mM Tris-HCl, pH 7.6
- 150mM NaCl
- 1% NP-40
- 1% sodium deoxycholate
- 0.1% sodium dodecyl sulfate
- Add desired protease inhibitors just before use

NP-40 Lysis Buffer (non-denaturing lysis buffer)
- 50mM Tris-HCl, pH 7.4
- 150mM NaCl
- 1% NP-40
- Add desired protease inhibitors just before use

B. Sample Loading Buffers

SDS-PAGE Sample Loading Buffer
- 62.5mM Tris-HCl, pH 6.8
- 2% sodium dodecyl sulfate
- 2.5% beta mercaptoethanol or 75 mM dithriothreitol
- 10% glycerol
- 0.005% bromophenol blue

C. Electrophoresis Buffer

Standard Electrophoresis Buffer
- 25mM Tris base
- 192mM glycine
- 0.1% SDS

D. Transfer Buffers

Towbin Transfer Buffer (general all-purpose buffer)
- 25mM Tris-HCl, pH 8.3
- 192mM glycine
- 20% methanol
- Add SDS (0.025-0.1%) for high molecular weight proteins

Bjerrum Schafer-Nielsen Transfer Buffer (continuous buffer for semi-dry transfer)

- 48mM Tris-HCl, pH 9.2
- 39mM glycine
- 20% methanol

CAPS TransferBuffer (for basic proteins)

- 10mM 3-[cyclohexylamino]-1 propane sulfonic acid, pH 11
- 10% methanol

Dunn Carbonate Buffer (for basic proteins)

- 10mM NaHCO3, pH 9.9
- 3mM Na2CO3
- 20% methanol

Discontinuous Tris-CAPS Buffer System (for semi-dry transfer)

- Anode buffer (membrane side)
  - 60mM Tris-HCl, pH 9.6
  - 40mM 3-[cyclohexylamino]-1 propane sulfonic acid,
  - 15% methanol
- Cathode buffer (gel side)
  - 60mM Tris-HCl, pH 9.6
  - 40mM 3-[cyclohexylamino]-1 propane sulfonic acid,
  - 0.1% SDS

Acetic Acid Transfer Buffer (for basic proteins, isoelectric focusing or native-PAGE)

- 0.7% acetic acid

Note: the use of acetic acid will cause the proteins to become positively charged and migrate towards the cathode side of the gel.

E. Protein-Staining Buffers

Ponceau S Membrane Stain

- 5% acetic acid
- 0.1% Ponceau S

Amido Black Membrane Stain

- 40% methanol
- 10% acetic acid
- 0.1% Amido black

Coomassie Blue Gel Stain

- 50% methanol
- 10% acetic acid
- 0.1% Coomassie blue

F. Blocking/Washing/Incubation Buffers

PBS(T)
- 1.4mM KH2PO4
- 8mM Na2HPO4
- 140mM NaCl
- 2.7mM KCl, pH 7.3
- 0.05% Tween-20 (when desired)

TBS(T)
- 50mM Tris, pH 7.6
- 150mM NaCl
- 0.05% Tween-20 (when desired)

Traditional Milk Blocking Buffer
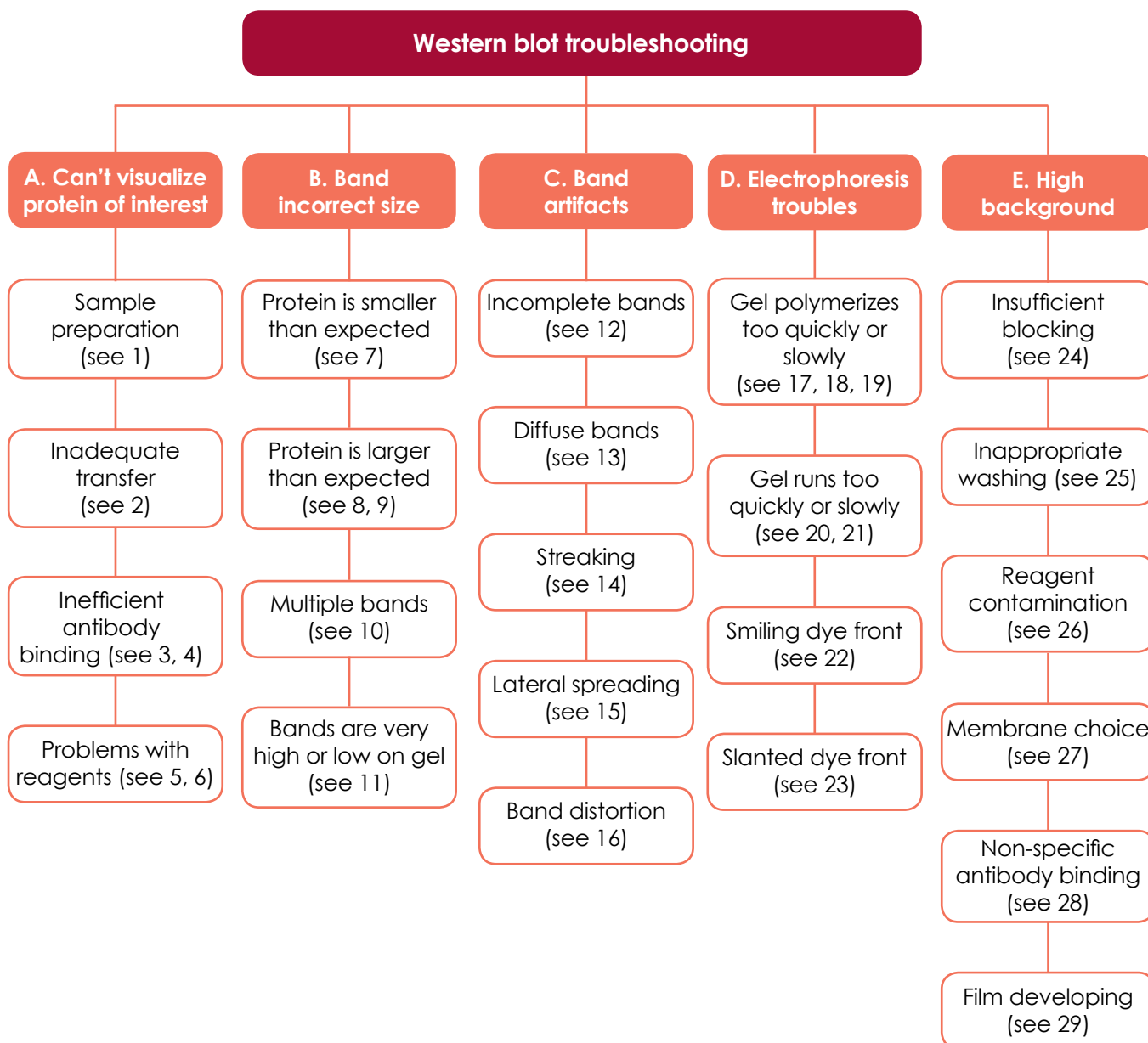- 1% lowfat milk
- PBS or TBS
- 0.05% Tween-20 (when desired, but do not use detergent in blocking buffer when using fluorescent antibodies)

Traditional Washing Buffer
- PBST or TBST

Traditional Antibody Incubation Buffer
- PBST or TBST
- For fluorescent Westerns, can add 0.01-0.02% sodium dodecyl sulfate to secondary antibody incubation buffer (never use SDS with nitrocellulose membranes)

Appendix B

Western blot troubleshooting

A. Can't visualize protein of interest

Sample preparation (see 1)

Inadequate transfer (see 2)

Inefficient antibody binding (see 3, 4)

Problems with reagents (see 5, 6)

B. Band incorrect size

Protein is smaller than expected (see 7)

Protein is larger than expected (see 8, 9)

Multiple bands (see 10)

Bands are very high or low on gel (see 11)

C. Band artifacts

Incomplete bands (see 12)

Diffuse bands (see 13)

Streaking (see 14)

Lateral spreading (see 15)

Band distortion (see 16)

D. Electrophoresis troubles

Gel polymerizes too quickly or slowly (see 17, 18, 19)

Gel runs too quickly or slowly (see 20, 21)

Smiling dye front (see 22)

Slanted dye front (see 23)

E. High background

Insufficient blocking (see 24)

Inappropriate washing (see 25)

Reagent contamination (see 26)

Membrane choice (see 27)

Non-specific antibody binding (see 28)

Film developing (see 29)

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A. Problem	Cause(s)	Solution
1. Sample preparation	Inefficient extraction	Try alternate methods; include positive control on gel
	Protein expressed at low levels in tissue or cells	Load more total protein on gel; concentrate using Afyon, or pool multiple samples
	Protein was degraded during extraction	Use protease inhibitors in lysis buffer
2. Inadequate transfer	Transfer buffer incorrectly prepared/too much methanol in buffer	Check protocol, decrease methanol
	Larger proteins may require more time/current	Repeat with longer time/higher voltage
	Insufficient contact between gel and membrane	Check fiber pad thickness; replace if too thin
3. Inefficient binding of primary antibody	Low affinity of antibody for protein or antibody is old/weak	Increase concentration of primary antibody; purchase new antibody and maintain proper storage
	Antibody has weak cross-reactivity with species of interest	Try alternate primary antibody source
	Antibody removed with washing	Use minimum number of washes; decrease salt concentration in wash buffer
	Antigen masked by blocking agent (ex., milk)	Try alternative blocker (ex., BSA)
4. Inefficient binding of secondary antibody	Incorrect species chosen	Use antibody directed against primary antibody species
	Insufficient antibody concentration or antibody is old	Increase dilution or obtain new antibody
5. Conjugate/substrate inactive	Reagents old or unstable	Mix conjugate + substrate in a tube; or luminescence in dark for ECL – obtain new reagent if no signal
	HRP inactivated by sodium azide	Avoid using solutions containing this preservative
6. Detection reagent (ECL)	Solution is old or stored improperly	Purchase new reagent

B. Problem	Cause(s)	Solution
7. Protein smaller than expected	Proteolysis; sample freeze/thaw	Use protease inhibitors/fresh samples
	Splice variant	Consult literature/use appropriate controls
8. Protein larger than expected and/or multiple bands	Naturally occurring protein modifications (glycosylation, phosphorylation, acetylation, etc.)	Consult literature to find additives to remove chemical groups
	Protein expression changing in overpassaged cell line	Use earlier passages; include positive control
9. Protein larger than expected	Protein aggregates – disulfide bonds intact	Use DTT in sample buffer; briefly spin samples prior to loading

B. Problem continued	Cause(s)	Solution
10. Extra bands	Non-specific binding of primary or secondary antibody	Decrease concentrations; try blocking peptide experiment (will remove protein of interest)
11. Band appears very high or low on the blot	Gel percentage is not optimum	Increase gel percentage for smaller protein; decrease for larger protein

C. Problem	Cause(s)	Solution
12. Incomplete bands	Bubbles between gel and membrane	Using a pipette, roll over the gel/membrane sandwich to force air bubbles out
13. Diffuse bands	Slow migration	Increase voltage; ensure proper buffer preparation
	Sample not heated correctly	Make sure sample is heated to 90°C for 2 min. prior to loading
	SDS in sample buffer is too old	Prepare new SDS for sample buffer
14. Streaking in lanes	High salt concentration in sample	Decrease salt concentration in sample buffer
	Sample too concentrated or insufficient SDS	Increase dilution/use more SDS
15. Lateral spreading of bands	Sample diffusion from wells during loading	Minimize loading time
16. Band distortion	Gel failed to polymerize completely around sample wells	Increase TEMED/AP
	Too much pressure applied to gel when pouring	Screws on the gel assembly apparatus should not be more than "thumb tight"
	Particulate matter in gel	Filter and mix gel reagents prior to preparing gel
	Excessive/uneven heating of gel	Decrease running voltage/provide cooling
	Bubbles in gel due to dirty plates	Wear gloves when handling plates
		Clean plates with ethanol and deionized H_2O
	Bubbles in gel from air introduced from pouring device (syringe or pipette)	Do not expel entire volume of gel mix
	Bubbles under comb from trapped air	Insert comb at an angle and reposition before gel solidifies

D. Problem	Cause(s)	Solution
17. Gel fails to polymerize	Failure to add TEMED and AP	Repeat with TEMED and AP
	AP solution is stable only a few days at 4°C	Prepare fresh AP
	Oxygen inhibits polymerization	Layer gel with isopropanol before pouring stacker
18. Gel polymerizes too slowly	Insufficient TEMED/AP	Increase amount of TEMED/AP
	AP solution losing activity	Prepare fresh AP

D. Problem continued	Cause(s)	Solution
19. Gel polymerizes too quickly	Excessive amount of TEMED/AP	Reduce amount of TEMED and AP, keeping the ratio the same
20. Run time unusually long	Running buffer too concentrated	Check protocol; dilute buffer if necessary
	Insufficient current	Increase voltage
21. Run time unusually short	Buffer too dilute	Check protocol; replace buffer if necessary
22. Dye front "smiling"	Migration too fast	Decrease voltage
	Heat generated	Decrease voltage; provide cooling
23. Dye front slanted	Bubble trapped between glass plates at the bottom of gel	Hold gel at an angle; place corner into lower buffer chamber; slowly move to horizontal position

E. Problem	Cause(s)	Solution
24. Insufficient blocking	Biotin in milk incompatible with streptavidin system, or milk contains antigen of interest	Use BSA
	If using AdvanBlock-PF with WesternBright MCF, some primary antibodies may require a protein blocker	Include BSA or milk in AdvanBlock-PF solution used to dilute the primary antibody
	Milk solution diluted too much	Increase to 5% milk solution
	Blocking time too short	Increase incubation time
	Some detergents not as effective in cold temperatures	Use 1 hr RT incubation instead of overnight at 4°C
25. Inappropriate wash conditions	Insufficient number of washes	Increase number of washes or duration of each wash step
	Insufficient detergent concentration	Increase detergent concentration or use stronger detergents (SDS, NP-40)
26. Reagent contamination	Bacterial or fungal growth in buffers	Check all buffers for turbidity; prepare new
27. Membrane choice	PVDF membranes may have higher background than nitrocellulose	Try nitrocellulose membranes
	Some membranes have high autofluorescence	Use only low-autofluorescence PVDF membranes with fluorescent Western blots
	Membrane dried out	Ensure membrane is hydrated during all steps
28. Non-specific binding of primary or secondary antibody	Concentration of antibody too high or antibody not affinity purified	Decrease antibody concentration; try monoclonal antibody or affinity purified
	Too much protein on gel	Decrease amount of protein loaded
29. Image overexposed	Time of exposure to film or CCD camera is too long	Reduce exposure time; if not possible, increase antibody dilutions or load less sample

Advansta provides innovative products for Western blotting,
from sample preparation to sensitive chemiluminescent detection.

Advansta. *Solutions for protein characterization.*

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D-16140-011