Protein Analysis

Electrophoresis, Blotting, and Immunodetection

advansta

Protein Analysis:

Electrophoresis, Blotting, and Immunodetection

An Advansta Corporation laboratory manual

Western blotting is a widely-used analytical technique for the study of proteins. This method, first described by Towbin, et. al[1], allows for the detection of a single protein within a mixture of proteins derived from a biological sample. The specificity of Western blotting is achieved by using an antibody that recognizes and binds to an epitope unique to the protein of interest. Western blotting can estimate the size of a protein, confirm the presence of post-translational modifications such as phosphorylation, and be used to quantitatively compare protein levels between samples. This laboratory guide describes the steps involved in performing a Western blot, including sample preparation, protein separation, blotting, and detection.

Table of Contents

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.

1. Western Blotting Overview

See following chapters for more detail about each step.

a. Sample Preparation

Proteins are extracted from biological material using mechanical and/or chemical disruption.

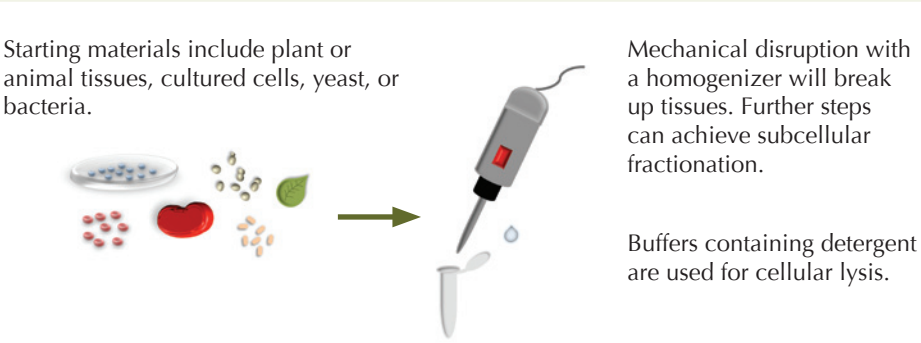


Figure 1. Sample Preparation

b. Polyacrylamide Electrophoresis

The proteins in the extract are separated according to size via electrophoresis. A gel is prepared by the polymerization of bisacrylamide and acrylamide. Sodium dodecyl sulfate (SDS) added to the gel binds to proteins, giving each a negative charge proportional to its mass.

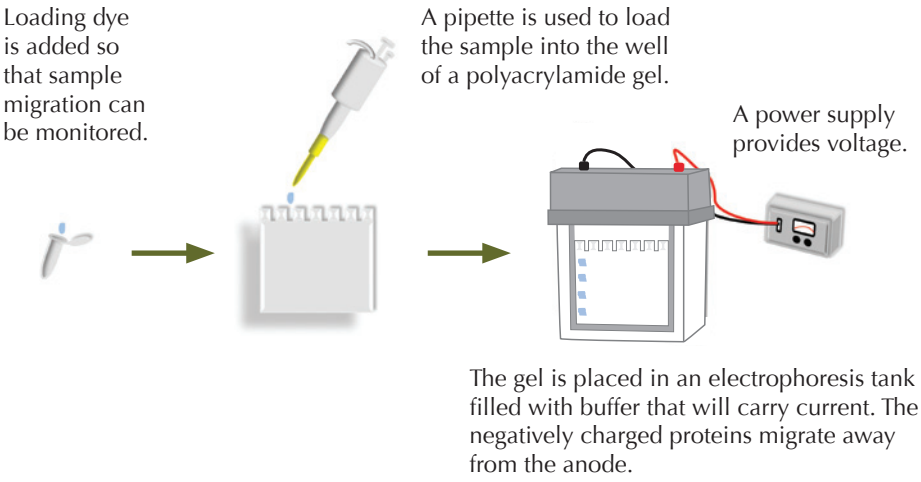


Figure 2. Polyacrylamide Electrophoresis

c. Electrophoretic transfer to membrane support

The proteins are transferred electrophoretically to a membrane, where they become immobilized.

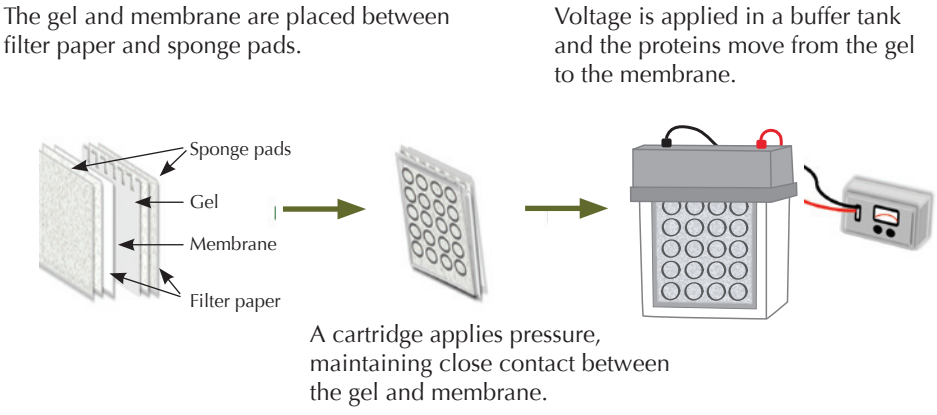


Figure 3. Transfer of Proteins to Membrane Support

d. Antibody hybridization

The membrane is blocked to prevent nonspecific binding of antibodies, and then incubated with a primary antibody directed against a specific epitope present on the target protein. Following several washes, a labeled secondary antibody binds to the primary and provides a means of detection.

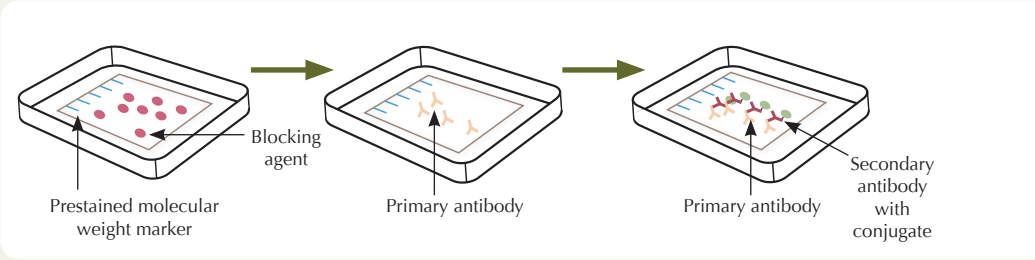


Figure 4. Antibody Hybridization

e. Detection of Bands

After washing the membrane to remove unbound antibody, the location of the protein on the blot is detected. For chemiluminescent detection, the secondary antibody is conjugated to horseradish peroxidase (HRP); addition of an HRP substrate leads to an enzymatic reaction with light as a product (Figure 5). The light can be detected using film or digital imaging. Fluorescent detection relies on light emitted by fluorophores conjugated to the antibody.

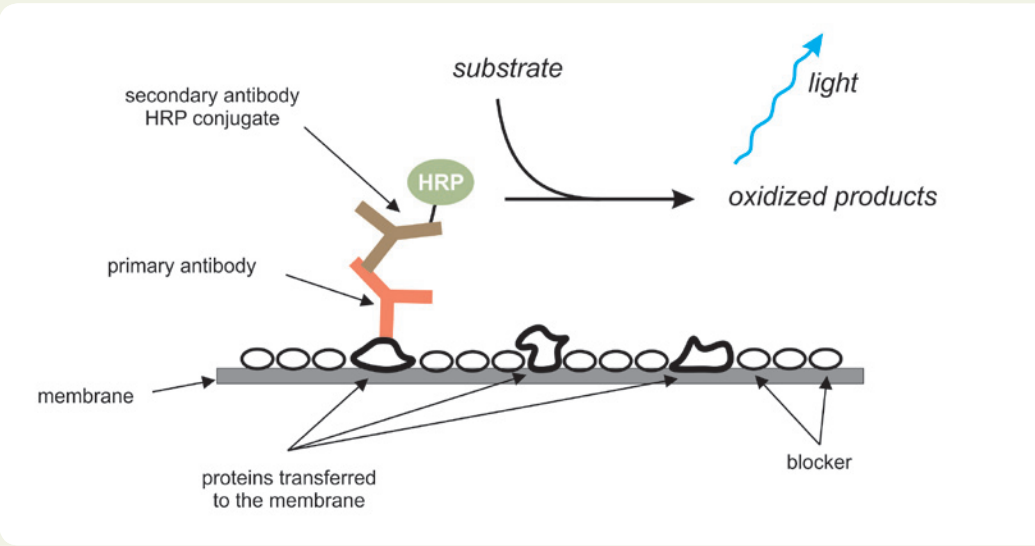


Figure 5. Chemiluminescent Detection of Protein Bands

2. Sample Preparation

Proper sample preparation is essential to a successful Western blot. For most starting materials, proteins are first extracted using chemical and/or mechanical means. The method chosen will depend on the type of sample, the subcellular location of the protein, and the conditions required by the antibody to recognize its epitope. Table 1 summarizes common physical methods used to prepare protein extracts for immunoblotting.

The use of mechanical methods is often required to disrupt the complex matrix of plant and animal samples. Mechanical disruption can be followed by cellular lysis using a buffer containing detergents to further enrich the protein of interest within the extract. Frequently, cultured cells can be ruptured using a detergent buffer without mechanical methods.

The chemical structure of detergents enables them to disrupt membranes and solublize 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.

Detergent choice depends in part on the location of the protein of interest within the cell, whether cytoplasmic, membrane-bound, or located within subcellular organelles such as the nucleus or mitochondria. Cytoplasmic proteins may be bound to cytoskeletal proteins, affecting how they partition into subcellular fractions.

Table 2 lists common buffers and detergents used to extract proteins based on their protein type and subcellular localization.

Table 1. Physical Methods of Sample Disruption

Method	Description	Type of Sample
Blender	Sample is minced by rotating blades	Large quantities of tissue
Dounce homegenizer	Glass tube with tight-fitting pestle ruptures cells by shearing action	Cells or tissue; useful for mitrochondrial or nuclear enrichment protocols
Ultrasonic homogenizer	Sound waves are emitted from a probe to disrupt cell membranes	Cells, ground tissue, bacteria
Pulverizing in liquid nitrogen	Sample is crushed into a powder using a chilled mortar and pestle	Plant or animal tissue
Glass beads	Glass beads and vortexing rupture cells	Yeast

Table 2. Buffers and detergents for protein extraction based on protein type and subcellular location

Type/Location of Target Protein	Detergent or Buffer	Comments
Native (non-denatured)	Mild, nonionic detergent	Avoid denaturing detergents (SDS, deoxycholate)
Cytoplasmic (soluble)	Tris-HCl	Can be combined with mechanical method such as a Dounce homogenizer
Cytoplasmic (cytoskeleton-bound)	Tris-Triton	The Triton series are nonionic, mild detergents
Nuclear, mitochondrial, membrane	NP-40, RIPA buffer (contains multiple detergents), Triton X-100	Enrichment procedures may be required for antigens with low expression levels
Whole cell lysate	NP-40, RIPA, Triton X-100	

When cells rupture, protease enzymes are released that can degrade the protein of interest. Therefore, it is important to prevent protease activity during sample preparation.

The following strategy will help prevent protein degradation:

- Avoid excessive freeze/thaw cycles of samples.
- Work quickly and keep samples cold during protein extraction procedures.
- Add protease inhibitors (available individually or in mixtures) to the lysis buffer.

In addition to protease activity, it may also be desirable to reduce phosphatase activity, particularly when studying protein phosphorylation. Table 3 lists common protease and phosphatase inhibitors used in protein extraction.

Table 3. Protease and Phosphatase Inhibitors Used in Protein Extraction

Protease Inhibitors	Concentration in Lysis Buffer	Target Enzymes
Aprotinin	1-2 µg/ml	Serine Proteases
EDTA	1-10 mM	Mg^{++}/Mn^{++} Metalloproteases
EGTA	1-10 mM	Ca^{++} Metalloproteases
Leupeptin	1-2 µg/ml	Serine, Cysteine Proteases
Pepstatin A	1 µg/ml	Aspartic Proteases
PMSF	(17-170 µg/ml) 0.1 - 1 mM	Serine Proteases
Phosphatase Inhibitors	**Concentration in Lysis Buffer**	**Target Enzymes**
ß-Glycero-phosphate	1 mM	Serine/Threonine Phosphatases
Sodium Fluoride	10 mM	Acidic Phosphatases
Sodium Orthovanadate	1 mM	Tyrosine phosphatases, Alkaline Phosphatases
Sodium Pyrophosphate	10 mM	Serine/Threonine Phosphatases

3. Polyacrylamide Gel Electrophoresis

In preparation for Western blotting, the proteins in the extract are separated using polyacrylamide gel electrophoresis (PAGE). Most frequently, the proteins are first coated with the negatively charged detergent sodium dodecyl-sulfate (SDS), so that they separate within the gel based on size (SDS-PAGE).

Measuring Total Protein

Prior to electrophoresis, it is important to determine the concentration of the protein sample for the following reasons:

1. Loading the correct amount of protein onto the gel.

   The optimum amount of total protein to load will depend on the prevalence of the protein of interest in the mixture, and on the sensitivity of the primary antibody. Loading too much protein on the gel can result in high background and nonspecific antibody binding. Preliminary experiments are often required to determine the optimal protein load by loading a range of protein amounts.

Note: with the Afyon™ SDS-PAGE sample preparation kit, the amount of protein loaded on the gel can be controlled by the amount of resin used, bypassing the need to assay protein concentration. For example, 5 µg of protein will be eluted from 20 µl of Afyon resin. If more or less protein is desired, adjust the amount of resin accordingly.

Ordering information

K-02101-025 Afyon™ SDS-PAGE sample preparation kit

2. Performing semi-quantitative or quantitative Western blotting.

   If the amount of protein loaded per lane is the same for all samples on a given gel, comparison of relative levels of the target protein can be made. Quantitative Western blotting is useful for studying changes in protein expression, or in protein modifications such as phosphorylation.

Protein Assay Overview

There are several methods used to measure total protein in a sample. The most commonly used protein detection methods for immunoblotting are the Lowry, Bradford, and bicinchoninic acid (BCA) assays (Table 4). These colorimetric assays are based on reactions that occur between the proteins in the sample and the detection reagents.

In order to determine the protein concentration in a sample, a standard curve must be run with each assay. This is accomplished by conducting the assay with a set of pure protein standards having increasing concentrations. The standard most often used for immunoblotting is bovine serum albumin (BSA), though the standard can be any protein made in the laboratory or purchased commercially.

A sample standard curve is shown in Figure 6. Absorbance values are plotted against the known protein content of the standards. The protein content of an unknown sample (green line in Figure 6) can be determined from its absorbance, using the linear portion of the curve. The content then can be expressed as a concentration, by correcting for the volume.

Choosing a Protein Assay

There are many commercially available protein assay kits. The choice of assay depends on many factors:

- The equipment available to read the assay (spectrophotometer, plate reader, etc.)
- The reagents in the lysis buffer—refer to the manufacturer's protocol to identify interfering substances.
- The amount of sample available
- Ease/speed of assay

Table 4. Commonly Used Total Protein Assay Methods.

Assay	Description	Advantages	Disadvantages
Bradford	Coomassie dye binds to protein and undergoes a shift in absorbance	Assays are generally simple/quick to perform	• Interference from SDS or other detergents at high concentrations • Small linear range
BCA	Cu^{2+} ions are reduced by proteins, BCA binds to Cu^{1+} ions and forms a colored product that is measured spectrophotometrically (Biuret reaction)	• Compatible with most detergents • Commercially available in format compatible with reducing agents • Less protein–to–protein variation than Bradford	Interference from copper chelators, copper reducers
Lowry	Similar to BCA (Biuret reaction)	Very well cited in literature	• Assay time may be longer than others • May not be practical for large sample groups • Precipitates may form

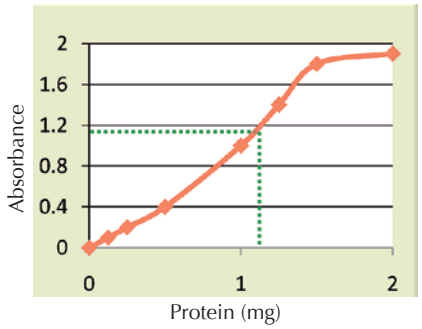


Figure 6. Example Standard Curve From a Protein Assay. The absorbance of standard solutions containing known protein amounts are plotted (red line). When the absorbance of a protein sample of unknown concentration is measured, the concentration can be determined based on the standard curve (green dotted lines).

Sample Loading Buffer

Once protein samples are obtained, they can be frozen for future use, being careful to avoid multiple freeze/thaw cycles. Alternatively, samples can be immediately combined with sample loading buffer and loaded onto a gel for electrophoresis. The components of the loading buffer will vary, depending on the desired sample conditions. The ingredients of a typical loading buffer for samples that will be detected under denaturing/ reducing conditions are described in Table 5. If non-denaturing and/or non-reducing conditions are needed, SDS and beta mercaptoethanol or dithiothreitol (DTT) are omitted.

Prior to loading on the gel, samples are boiled (95 °C for 5–10 minutes), to ensure that denaturation/ reduction is complete. Under non-reducing/denaturing conditions, the samples are not boiled. Figure 7 illustrates how SDS denatures proteins.

Note: Pre-mixed loading buffers save time and ensure that samples run in a reproducible way, lane-to-lane and gel-to-gel. Advansta provides both reducing and non-reducing loading buffers.

Ordering information

R-03018-B10 Non-reducing protein sample loading buffer (2x)

R-03019-B10 Reducing protein sample loading buffer (2x)

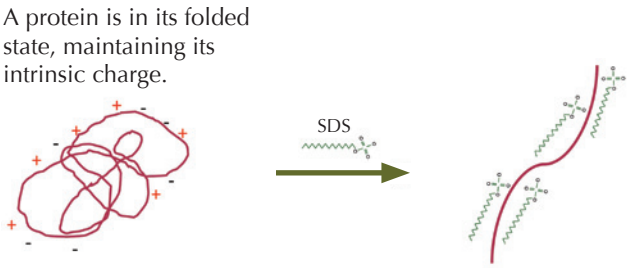


Figure 7. Mechanism of SDS Denaturation of Proteins

Table 5. Components of Loading Buffer

Reagent	Purpose
Glycerol	Viscosity/density to pull the sample to the bottom of the well and prevent it from spilling over into adjacent wells
Bromophenol blue	Small dye molecule • Adds color to the sample to aid in loading • Will migrate ahead of proteins on the gel, so that the progress of the gel can be monitored
SDS	Denaturing detergent • Hydrophobic tail surrounds polypeptide backbone • Binds protein with a constant ratio, imparting a negative charge proportional to the length of the protein • Prevents hydrophobic interactions and disrupts hydrogen bonds • Destroys secondary/tertiary structure and unfolds protein
Beta mercaptoethanol or DTT	Reducing agents • Prevents oxidation of cysteines • Will complete protein unfolding by breaking disulfide bonds between cysteine residues
Tris buffer	Maintains proper pH

Gel Electrophoresis

If native conditions are required for epitope recognition by the antibody, electrophoresis is performed without adding SDS to the gel and the running buffer. In this situation, also known as native PAGE, proteins maintain their folded state and migration in the gel is due to the mass:charge ratio of the protein, rather than the protein's size. However, most often Western blot samples are separated on polyacrylamide gels containing SDS (SDS-PAGE) and run under denaturing conditions. Under these reducing and denaturing conditions, the negatively charged proteins migrate toward the positively charged electrode when current is applied to the gel. Since SDS has equalized the negative charge on all proteins, the rate of migration is determined by their molecular weight. Smaller molecules will migrate faster than those with higher molecular weights.

SDS-PAGE gels are available commercially or can be made in the laboratory. Premade gels have the distinct advantage of convenience, but are not as cost effective as those made in-house. The chemical components of a gel for SDS-PAGE are listed in Table 6.

Table 6. Components of Acrylamide Gels Used in Protein Electrophoresis

Reagent	Purpose
Acrylamide	Molecules polymerize to form a chain
Bisacrylamide	Crosslinks acrylamide chains to form a gel
SDS	Maintains linearized state and uniform negative charge of proteins during electrophoresis
Tris buffer	Maintains proper pH
Ammonium Persulfate	Generates free radicals that catalyze polymerization reaction
TEMED	Increases free radical generation by ammonium persulfate

Selection of Gel Thickness and Pore Size

The molecular weight of the protein and the sample type dictate the choice of gel thickness and pore size.

- Gel spacers (see Figure 8) control gel thickness. They are available in several standard sizes (0.5 mm; 0.75 mm; 1.0 mm; 1.5 mm).
- Thicker gels will accommodate larger sample volumes. They will also run more slowly and require longer transfer times than thinner gels.
- Gel percentage, based on the amount of acrylamide and bis, determines pore size. The higher the gel percentage, the smaller the pores.
- Pore size determines the size range of proteins best separated by that gel (Table 7).

Table 7. Protein Sizes Best Separated by Common Gel Percentages

Gel Percentage	MW Range (kDa)
7.5	25-500
10	15-300
12	10-200
15	10-45
20	5-40

Pouring the Gel

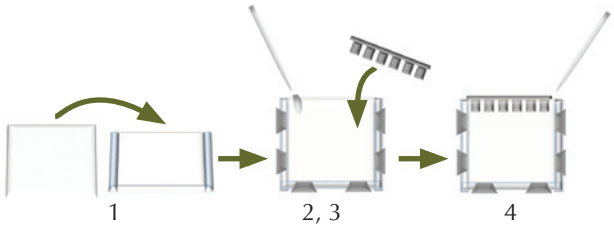
If gels are to be made in-house, the reagents are mixed and quickly poured into a gel casting form, where the acrylamide will polymerize within about 30 minutes. For improved band sharpness, the running gel is topped with a low-percentage stacking gel. There are several types of systems available for pouring a gel. Generally, they consist of glass plates, bottom and side spacers, combs, and a clamping device (Figure 8).

Experimental design; Controls and Standards

Before loading the gel, it is important to design an experiment incorporating appropriate standards and controls to validate the results. The types of standards/controls used in protein electrophoresis include:

1. Molecular weight standards
   - Consist of a mixture of purified proteins with known molecular weights
   - Used to verify that the protein detected by subsequent Western blotting is in the predicted molecular weight range
   - Available unstained or prestained and in various molecular weight ranges
   - Prestained versions can be used to monitor gel progress and verify transfer efficacy
   - Standard proteins can be labeled for detection by fluorescence or chemiluminescence.
2. Positive controls
   - Verify that the primary antibody is binding to the correct protein
   - Ideally, are a sample of the purified protein of interest, if available
   - Can be a call line known to overexpress the protein of interest
   - Can be a sample of tissue or organ known to have high levels of the protein of interest. Other species can be used for sources of control tissue if the antibody has the appropriate crossreactivity.

1. Place the bottom and side spacers between two glass plates.
2. After clamping the sides, pour the resolving gel and allow it to polymerize.
3. Place the comb between the glass plates at the top.



4. Pour the stacking gel and remove the comb after polymerization. Rinse the wells with ddH_20. The gel is ready for loading and electrophoresis.

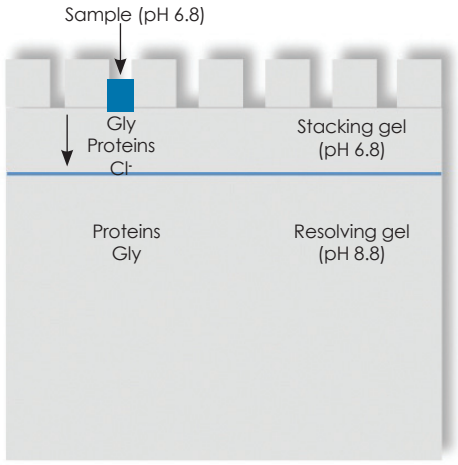
Figure 8. Preparation of Polyacrylamide Gel for Protein Separation

3. Loading controls
   - Typically, a protein that is expressed at approximately equal levels in all samples (similar to a "housekeeping gene" for molecular biology studies) is chosen as the loading control (ex: GAPDH, beta actin, etc.).
   - Keep in mind that some treatments can alter the expression levels of housekeeping proteins.
   - The blot is probed for both the loading control and the protein of interest.
   - Used to show that the total protein loaded per lane is equal, so that protein levels can be compared to one another quantitatively.
   - The quantitative expression of the protein of interest can be normalized to that of the loading control for each sample.
4. Peptide blocking experiments
   - Some vendors offer blocking peptides for their antibodies.
   - The blocking peptide is mixed with the primary antibody prior to incubation with the membrane and prevents specific binding of the antibody to its target antigen. There should then be no band where the target protein would be.
   - These studies demonstrate that the primary antibody is binding specifically to the correct target protein.

Running the Gel

The samples are loaded into the wells of the prepared gel using a pipettor. Typically, 20-40 µg of total protein is loaded per lane. It is important to know the total volume that can be loaded for the chosen well size, as overloading can result in loss of the sample or spillage into adjacent wells. Immediately after loading the samples, voltage is applied to the gel via a power supply. The buffer used during electrophoresis (running buffer) contains Tris (maintains pH), glycine (conducts electricity), and SDS (keeps proteins negatively charged). The progress of the gel is monitored by observing the dye front of the dye contained in the loading buffer and the position of the molecular weight standards if they have intrinsic color that allows them to be visualized.

The most common system for pouring and running gels is the Laemmli (discontinuous) system. In this method, the pH values for the stacking gel, the resolving gel and the electrophoresis (running) buffer differ. Figure 9 illustrates the flow of ions during electrophoresis.



- Glycine in the running buffer is negatively charged at pH 8.3.
- Voltage is applied and glycine moves away from the negatively charged electrode into the stacker gel.
- Glycine begins to lose its charge at pH 6.8 and slows down. Chloride anions in the sample and stacker move ahead of glycine. The increased resistance forces the proteins to move away from the negative electrode in a stacked fashion.
- Glycine becomes more negatively charged at the higher pH and moves ahead of the proteins.
- The proteins are separated according to their molecular weight by the sieving effect of the pores in the gel.

Figure 9. Focusing of Protein Bands Due to Ion Flow in the Discontinuous Laemmli System

4. Electrophoretic Transfer to Membrane Support

After gel electrophoresis is complete, the proteins are transferred to a membrane. The membrane is a solid support that binds and immobilizes the proteins, allowing them to be detected by antibody hybridization. Most systems for transfer use electric current to drive the negatively charged proteins toward the positive electrode. Transfer systems are available in semi-dry or wet formats. Semi-dry methods have faster transfer times and require a smaller buffer volume than wet transfers, but they may not be suitable for larger (>70 kDa) proteins and may cause increased background with certain detection systems. Both methods require that close contact be maintained between the gel and membrane for effective transfer of the proteins to the membrane.

The two most commonly used membranes types for immunoblotting are nitrocellulose and polyvinylidene difluoride (PVDF). Although both work well for immunoblotting, differences in their characteristics may influence the choice of the investigator. PVDF membranes offer the advantages of mechanical strength, resistance to SDS and higher bond strength than nitrocellulose. These properties make PVDF the membrane of choice if stripping and reprobing are desired. Nitrocellulose membranes have the advantages of lower background and not requiring a methanol pretreatment as PVDF membranes do. Low-autofluroescence PVDF membranes have been developed to reduce background when using fluorescent detection methods.

Prior to the transfer, the gel and membrane are allowed to equilibrate in pre-chilled buffer. Typically, transfer buffer includes Tris, glycine, SDS (optional), and methanol (for nitrocellulose membranes). Figure 10 illustrates how the gel and membrane assembly for a semi dry or wet transfer.

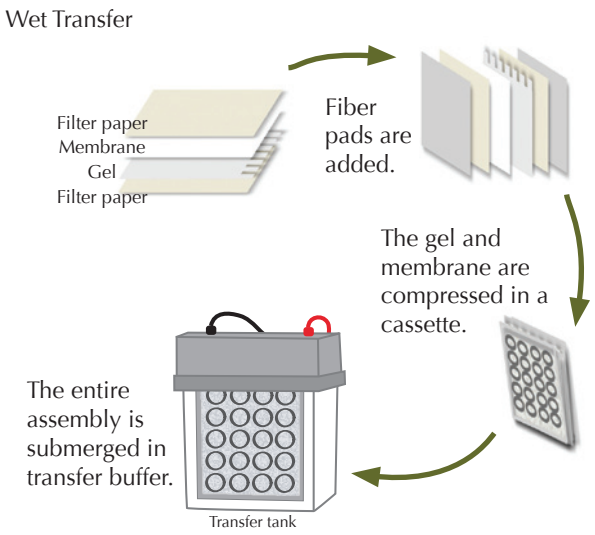


Figure 10. Protein Transfer to Membrane Support

Note: Advansta provides pre-cut nitrocellulose and low-fluorescence PVDF transfer membranes, sized for minigels. Using pre-cut membranes saves time, and avoids accidental contamination of membranes with dirty scissors or gloves.

Ordering information

L-08001-010	Low-Fluorescence PVDF Transfer Membrane 7x9 cm
L-08002-010	Nitrocellulose Transfer Membrane 0.45 µm 8x10 cm
L-08003-010	Nitrocellulose Transfer Membrane 0.22 µm 8x10 cm

The following list contains tips for a successful transfer:

- 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.
- Air bubbles between the gel and the membrane can cause an uneven transfer. To remove them, gently roll a pipette over the gel/membrane/filter paper "sandwich."
- 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.
- Adjust the transfer conditions for the size of the protein. Smaller proteins will transfer out of the gel faster, and may go through the membrane. Reducing or removing SDS or using a membrane with a smaller pore size will help in retaining smaller proteins. Increasing the SDS concentration and decreasing the methanol concentration may facilitate the transfer of larger proteins.
- The appearance of the prestained molecular weight markers on the membrane provides a general indication that the transfer is complete.
- Ponceau S stain can be used on the membrane to assess the effectiveness of the transfer. The membrane must then be destained prior to blocking. Coomassie stain can be used on the gel after transfer, to check for residual protein.

Blocking

Blocking the membrane is accomplished by incubating with a solution designed to reduce nonspecific binding by the antibody. Often, protein-based blocking solutions are used, containing nonfat dry milk or bovine serum albumin (BSA). Nonfat dry milk is often used as the first choice when starting a new protocol, because it is more cost effective than BSA. However, due to the presence of phosphoproteins and biotin in milk, it may not be the best choice when using phospho-specific antibodies or biotin detection methods. Non-protein-based blocking agents are preferable in these and other situations.

The blocking agent is diluted in TBS (Tris-buffered saline) or PBS (phosphate-buffered saline); Tween-20 detergent may also be added. Incubation of the blot for one hour (room temperature or 4 °C) is usually sufficient for blocking nonspecific antibody binding. If background levels are high when the blot is developed, alternative blocking solutions can be tried.

Note: Advansta offers blocking and washing solutions optimized for the WesternBright line of detection reagents, as well as pre-weighed buffer powders including PBS and TBS for fast, easy buffer preparation.

Ordering information

R-03024-D50	AdvanWash™, 500 ml
R-03023-D20	AdvanBlock™-PF, 200 ml
R-01038-020	Avant™ Buffer Pouches - PBS
R-01039-020	Avant™ Buffer Pouches - TBS

5. Antibody Hybridization

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. Native gels can be conducted for antibodies that require a folded protein for antigen recognition.

Primary Antibodies: Monoclonal vs Polyclonal

Both monoclonal and polyclonal antibodies can be used as primary antibodies in Western blotting. The two types have distinct advantages and disadvantages and differ in how they are produced. The first step in production of both types of antibodies is injection of an animal with an antigen to elicit an immune response. Polyclonal antibodies are obtained directly from the serum of the immunized animal, commonly a rabbit, goat, donkey or sheep, and are then purified and tested. Polyclonal antibodies recognize multiple epitopes within the antigen, in contrast to monoclonal antibodies, which recognize a single epitope on the protein. To produce a monoclonal antibody, antibody synthesizing cells are isolated from the spleen of the immunized animal and fused with myeloma cells. The resulting hybridomas secrete antibodies into their culture media, which are then tested for their affinity for the antigen. The hybridomas having the most stable production of antibodies are selected and can be grown in culture indefinitely. Some characteristics of monoclonal and polyclonal antibodies are described in Table 8.

Table 8. Monoclonal vs Polyclonal Primary Antibodies

	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 binder 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 inreducing/ denaturing conditions, or if chemical modifications (glycosylation, phosphorylation) or differences in amino acid sequence due to polymorphism are present	More tolerant

Antibody Incubation

The primary antibody is diluted in blocking buffer, TBST, or purchased antibody dilution buffer and applied to the blot. The antibody vendor may recommend a starting dilution, but often the optimal antibody concentration must be determined empirically. The affinity of the antibody for the protein of interest, the abundance of the target protein in the sample, and the sensitivity of the detection system used all will impact the amount of antibody used.

- Generally, a one-hour incubation at room temperature is sufficient. Alternatively, overnight incubation at 4 °C can be used.
- The blot should be fully covered with solution and gently agitated during the incubation. The blot can be placed in a tray and placed on a platform shaker. A smaller volume of antibody solution can be used by sealing the blot into a bag and incubating on a rotary platform.
- In some cases, the diluted antibody can be reused. If sodium azide is used as a preservative, it must be fully washed from the blot or it can quench horseradish peroxidase activity and interfere with detection methods.

Following incubation with the primary antibody, the blot is washed with wash buffer (TBS, PBS, TBST or PBST).For indirect detection protocols (see section 6), the appropriate secondary antibody is needed. The following is a list of considerations for choosing a secondary antibody.

- The species in which the primary antibody was made will dictate the choice of secondary antibody. If the primary antibody was made in a rabbit, the correct secondary antibody will be anti-rabbit. Therefore, the secondary will be raised in a species other than rabbit.
- For monoclonal antibodies, the immunoglobulin class (IgG, IgM, IgA, IgD, IgE) and possibly the subclass (ex, IgG1, IgG2a, etc.) must be considered. An antibody with broad immunoglobulin specificity should suffice, as subclass-specific secondary antibodies are used mainly in double labeling experiments in other applications.

Note: Advansta offers a complete line of secondary antibodies labeled for chemiluminescent or fluorescent detection.

Ordering information

R-05051-050	APC-goat-anti-rabbit IgG conjugate, 50 µl
R-05051-250	APC-goat-anti-rabbit IgG conjugate, 250 µl
R-05052-050	RPE-goat-anti-mouse IgG conjugate, 50 µl
R-05052-250	RPE-goat-anti-mouse IgG conjugate, 250 µl
R-05071-500	Goat-anti-mouse HRP-conjugated secondary antibody, 500 µl
R-05072-500	Goat-anti-rabbit HRP-conjugated secondary antibody, 500 µl

6. Detection

Protein detection can be achieved using direct or indirect methods, each having advantages and disadvantages. Direct methods use a primary antibody conjugated to a detectable label, such as an enzyme, biotin, or a fluorescent molecule. Antibodies can be labeled in-house with commercially available labeling kits, or obtained prelabelled. Indirect methods use two antibodies; a primary antibody and a secondary antibody directed against the species in which the primary was raised. In indirect detection, the secondary antibody is conjugated to a detectable label. Figure 11 illustrates indirect and direct antibody methods and Table 9 summarizes their advantages and disadvantages.

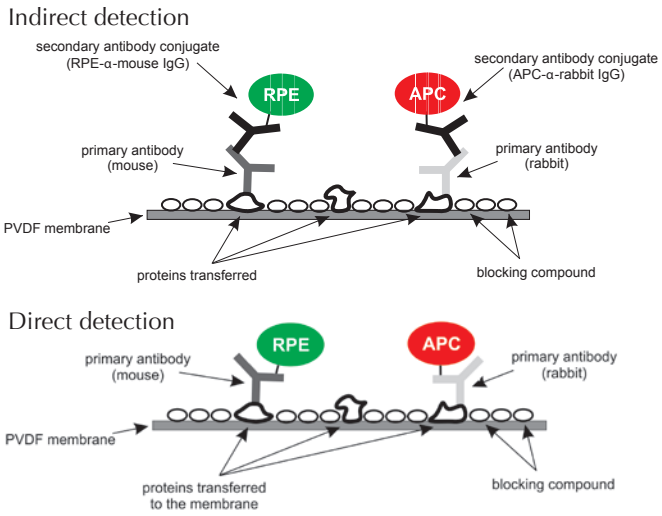


Figure 11. Indirect and Direct Detection of Protein Bands

Detection methods

The most commonly used methods for detecting proteins are: **1. Chemiluminescence**, **2. Fluorescence** and **3. Colorimetric** They are discussed in detail below.

1. Chemiluminescence

Chemiluminescent detection methods commonly use HRP-conjugated secondary antibodies. The reaction between the enzyme and substrate produces light, which can be detected by exposing the blot to x-ray film or by digital imaging using a CCD camera.

Advantages: highly sensitive, membrane can be stripped and reprobed, multiple exposures can be done with x-ray film, results easily documented.

Disadvantages: requires a darkroom (for x-ray film) or expensive imaging equipment, semiquantitative – because it relies on an enzymatic reaction, film has a limited dynamic range, signal intensity can vary with incubation or exposure time, not possible to multiplex (detecting more than one protein since only one color light is produced.

Table 9. Advantages and Disadvantages of Indirect and Direct Detection of Western Blots

	Indirect	Direct
Advantages	• Signal amplification due to multiple secondary antibodies binding to each primary antibody • Versatile – the same secondary antibody can be used for many primary antibodies made in the appropriate species	• Fewer steps • Less potential for nonspecific binding
Disadvantages	• Greater chance of nonspecific binding • More steps	• Can be more costly • Immuno-reactivity of antibody may be affected by the addition of a label

Note: LucentBlue™ film is optimized to detect the chemiluminescent signal of WesternBright HRP substrates, including WesternBright™ ECL, WesternBright Quantum and WesternBright Sirius.

Ordering information

L-07014-100	LucentBlue™ X-ray film, 8x10" sheets
L-07013-100	LucentBlue™ X-ray film, 5x7" sheets

2. Fluorescence

The secondary antibody is bound to a fluorophore, which undergoes excitation by light. The emitted light is then detected by an imager capable of measuring fluorescence or a CCD camera with filters having the appropriate wavelength. The image is digitalized for data analysis.

Advantages: sensitive (sensitivity can be increased by using biotin/streptavidin system), can be used in multiplex format with multiple dyes, large dynamic range, no darkroom required, gives quantitative data, easy to document results, can use labeled primary antibodies (for high abundance proteins) and eliminate steps.

Disadvantages: equipment and reagents can be costly, not as sensitive as chemiluminescence (may not be appropriate for low abundance proteins).

3. Colorimetric

Colorimetric methods use a secondary antibody that has been conjugated to an enzyme (peroxidase or alkaline phosphatase). The enzyme converts a dye into an insoluble product that is visible on the membrane. The amount of dye converted is proportional to the protein level in the sample. The staining intensity can then be measured spectrophotometrically or using a densitometer.

Advantages: simple to perform, does not require a darkroom, blots can be documented easily by photographing.

Disadvantages: not as sensitive as other methods, color will fade eventually.

Stripping and Reprobing the Membrane

After imaging, antibodies can be stripped from the membrane so that the blot can be reprobed for another protein. This procedure can conserve samples, but is time consuming. With multiplex fluorescent detection, multiple proteins can be assayed simultaneously, ideal for comparing a protein of interest to a loading control, or different phosphorylated isoforms of a protein. PVDF membranes are preferred over nitrocellulose for stripping protocols.

Note: WesternBright™ MCF allows you to detect two proteins on one blot using multicolor fluorescent detection. Perfect for experiments where a protein of interest will be compared to a loading control.

Ordering information

K-12021-010 WesternBright™ MCF fluorescent Western blotting kit

Note: Advansta provides a complete line of chemiluminescent detection reagents optimized for different detection methods, sample concentrations, and experiment types.

Ordering information

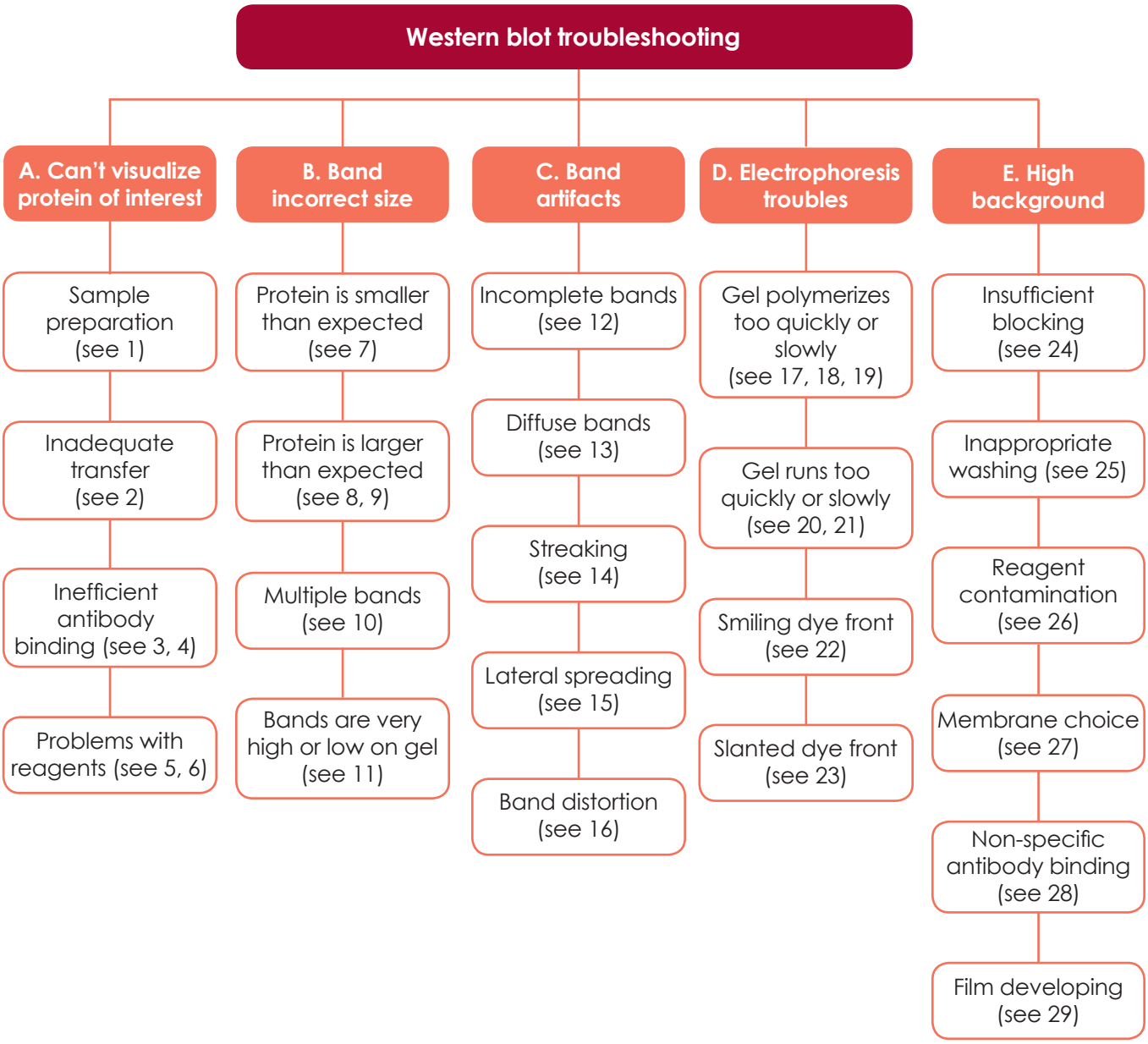
K-12042-D10 WesternBright™ Quantum Western Blotting HRP Substrate (for 1000 cm^2 membrane)

K-12043-D10 WesternBright™ Sirius Western Blotting HRP Substrate (for 1000 cm^2 membrane)

K-12045-D20 WesternBright™ ECL Western Blotting HRP Substrate (for 2000 cm^2 membrane)

K-12049-D50 WesternBright™ ECL Spray

7. Troubleshooting



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_20
	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

8. Protein Tools and Technical References

$$\text{Molarity of a solution} = \frac{\text{g of solute}}{[\text{molecular mass of solute (g/mol)}] \times (\text{L of solution})}$$

Table 10. Genetic Code and Amino Acid Abbreviations

First Position	Second Position: U	Second Position: C	Second Position: A	Second Position: G
U	UUU, UUC: Phenylalanine (Phe, F) UUA, UUG: Leucine (Leu, L)	UCU, UCC, UCA, UCG: Serine (Ser, S)	UAU, UAC: Tyrosine (Tyr, Y) UAA, UAG: STOP	UGU, UGC: Cysteine (Cys, C) UGA: STOP UGG: Tryptophan (Trp, W)
C	CUU, CUC, CUA, CUG: Leucine (Leu, L)	CCU, CCC, CCA, CCG: Proline (Pro, P)	CAU, CAC: Histidine (His, H) CAA, CAG: Glutamine (Gln, Q)	CGU, CGC, CGA, CGG: Arginine (Arg, R)
A A	AUU, AUC, AUA: Isoleucine (Ile, I) AUG: Methionine (Met, M)	ACU, ACC, ACA, ACG: Threonine (Thr, T)	AAU, AAC: Asparagine (Asn, N) AAA, AAG: Lysine (Lys, K)	AGU, AGC: Serine (Ser, S) AGA, AGG: Arginine (Arg, R)
G	GUU, GUC, GUA, GUG: Valine (Val, V)	GCU, GCC, GCA, GCG: Alanine (Ala, A)	GAU, GAC: Asparic acid (Asp, D) GAA, GAG: Glutamic acid (Glu, E)	GGU, GGC, GGA, GGG: Glycine (Gly, G)

Table 11. Converting Between Protein Mass and Moles

Molecular weight (Da)	1 µg	1 nmol
10,000	100 pmol or 6×10^{13} molecules	10 µg
50,000	20 pmol or 1.2×10^{13} molecules	50 µg
100,000	10 pmol or 6×10^{12} molecules	100 µg
150,000	6.7 pmol or 4×10^{12} molecules	150 µg

Table 12. Absorbance at 280 nm of Common Proteins

Protein	A_{280} units for 1 mg/ml
IgG	1.35
IgM	1.2
IgA	1.3
protein A	0.17
avidin	1.5
streptavidin	3.4
bovine serum albumin	0.7

Table 13. Metric Prefixes

M	mega	10^{6}	n	nano	10^{-9}
k	kilo	10^{3}	k	pico	10^{-12}
m	mili	10^{-3}	f	femto	10^{-15}
µ	micro	10^{-6}	a	atto	10^{-18}

Table 14. Abbreviations

ds	double-stranded (as in ds DNA)
ss	single-stranded (as in ss DNA)
bp	basepair
kb	kilobase: 1,000 bases or basepairs, as appropriate
Da	Dalton, the unit of molecular mass
mol	mole
M	molarity, moles of solute per liter of solution

Table 15. Half-life of Common Radioisotopes

Radioisotope	Half-life
Carbon-14 (^{14}C)	5,730 years
Iodine-125 (^{125}I)	60 days
Phosphorus-32 (^{32}P)	14.3 days
Sulfur-35 (^{35}S)	87.4 days
Tritium (^{3}H)	12.4 years

Table 16. Amino Acid Average Molecular Mass

Amino Acid Codes		Average Molecular Mass
A	Ala	71.08
C	Cys	103.1
D	Asp	115.1
E	Glu	129.1
F	Phe	147.2
G	Gly	57.05
H	His	137.1
I	Ile	113.2
K	Lys	128.2
L	Leu	113.2
M	Met	131.2
N	Asn	114.1
P	Pro	91.12
Q	Gln	128.1
R	Arg	156.2
S	Ser	87.08
T	Thr	101.1
V	Val	99.07
W	Trp	186.2
Y	Tyr	163.2

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