Protocol

Analysis of Proteins by Immunoblotting

Clara L. Kielkopf, William Bauer, and Ina L. Urbatsch

In immunoblotting (western blotting), proteins are first separated by SDS-PAGE and then transferred electrophoretically from the gel onto a support membrane that binds proteins tightly. After the unreacted binding sites of the membrane are blocked to suppress nonspecific adsorption of antibodies, the immobilized proteins are reacted with a specific polyclonal or monoclonal antibody. Antigen–antibody complexes are visualized using chromogenic, fluorescent, or chemiluminescent reactions. Immunoblotting protocols are reagent specific and, owing to the wide assortment of equipment, reagents, and antibodies available, highly diverse. Presented here is an example of a workable protocol for developing a blot using horseradish peroxidase (HRP)–conjugated secondary antibody and enhanced chemiluminescence (ECL). ECL is based on the emission of light during the HRP-catalyzed oxidation of luminal or other substrates. Emitted light is captured on film or by a CCD camera, for qualitative or semiquantitative analysis. Because ECL is so sensitive, it has become a popular detection method. This protocol can be modified for different membranes, antibodies, and detection systems. Optimal dilutions of the primary and secondary antibodies need to be determined empirically, but recommendations provided by the manufacturer are usually a good starting point.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Blocking buffer (1% [w/v] fat-free milk powder, e.g., Carnation) in TBST wash buffer

No single blocking agent is ideal for every occasion because each antibody–antigen pair has unique properties. If the background is high with 1% milk, try higher concentrations (up to 5% [w/v]) or switch to BSA (1%–5% [w/v] in TBST wash buffer), serum, casein, gelatin, or BLOTTO (Johnson and Elder 1983). Commercial blocking agents are also available (e.g., GE Healthcare, Thermo Fisher, and many others).

Deionized H_2O

Developing solution and fixative

ECL kit

Peroxide and luminol/enhancer solutions are available as a two-component kit from several suppliers (Amersham/GE Healthcare, Thermo Scientific Pierce, Life Technologies, Bio-Rad). Kits are offered in different sensitivity ranges (e.g., low sensitivity or picomolar range, high sensitivity or femtomolar range).

HRP-conjugated secondary antibody

From the Molecular Cloning collection, edited by Michael R. Green and Joseph Sambrook.

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Choose an HRP conjugate that specifically binds to the primary antibody. For example, if a monoclonal IgG antibody is used as a primary antibody, the secondary should be anti-mouse IgG antibody (almost all monoclonal IgG antibodies are generated in mice). Some monoclonals are of other isotypes, such as IgM, and require an isotype-matched secondary. If the primary antibody is polyclonal, check in which animal species it was raised (most often rabbit, sometimes sheep or another large animal), and select the appropriate HRP-conjugated secondary antibody. Following the manufacturer's recommendation to prepare a working dilution of the secondary antibody in Blocking buffer, usually between 1:5000 and 1:50,000, or 20–200 ng/mL. The optimal dilution varies depending on the particular HRP conjugate, the amount of antigen on the membrane, and the detection system.

Ponceau S stain (0.4% [w/v] Ponceau S in 1% [v/v] acetic acid)

Primary antibody

Choose an antibody that is specific to the target protein. Stocks of primary antibody can be stored short term at 4°C. For long-term storage, freeze small aliquots below −70°C. Prepare a working dilution in Blocking buffer between 1:1000 and 1:10,000, or 0.2–1 µg/mL, following the manufacturer's recommendation.

Optimal dilutions of the primary and secondary antibodies need to be determined empirically, but recommendations provided by the manufacturer are usually a good starting point.

SDS gel (see Protocol: **Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of Proteins** [Kielkopf et al. 2021])

Stripping solution (optional) <R>

TBST wash buffer <R>

Equipment

Desktop scanner

Film cassette and darkroom

Gel transfer apparatus

Plastic sheet protector (available at office supply stores)

PVDF or nitrocellulose membrane

Rocking platform

Tupperware container

METHOD

Do not let the blot dry out during the processing or development steps. Use only clean gloves and instruments when handling the membrane because contamination may lead to high background or dark spots.

1. Electrotransfer the SDS gel in an appropriate gel transfer apparatus to a nitrocellulose or PVDF membrane following the manufacturer's instructions (see also Table 1 on transfer buffers and the section Types of Membranes, below).

TABLE 1. Buffers for transfer of proteins from polyacrylamide gels to membranes

Type of transfer	Buffer	Reference
Immersion	Tris base (24 mM) Glycine (192 mM) Methanol (20%)	Towbin et al. 1979
Semidry	Tris base (48 mM) Glycine (39 mM) Methanol (20%) SDS (0.0375%)	Bjerrum and Schafer-Nielsen 1986

Methanol minimizes swelling of the gel and increases the efficiency of binding of proteins to nitrocellulose membranes. The efficiency of transfer may be affected by the presence of SDS in the electrophoresis buffer, the pH of the transfer buffer, and whether the proteins were stained in the gel before transfer. To maximize transfer of protein to membranes, the concentration of SDS should be ≤0.1% and the pH should be ≥8.0. CAPS buffer should be used for transfer if the protein is to be sequenced on the membrane (Moss 2001). Glycine interferes with this procedure.

Prestained markers are useful not only to monitor transfer efficiency of proteins out of the gel and onto the membrane, but also to identify the orientation of the gel (load the marker in Slot 1) and location of sample lanes as well as molecular weights of bands that will be detected.

2. Remove the membrane from the transfer apparatus, and rinse it briefly with deionized H_2O.
3. Stain the membrane with 0.4% Ponceau S in 1% acetic acid on a rocking platform for 5 min, decant the stain, and wash the membrane briefly in 300 mL of deionized H_2O (~1 min) until the background is destained and the bands are readily seen. Document the efficiency of transfer by scanning the wet membrane. Then rinse off the stain with deionized H_2O followed by TBST wash buffer.
4. Incubate the membrane in 20 mL of Blocking buffer for at least 20–60 min at room temperature on a rocking platform. Alternatively, the blot may be left in blocking agent overnight at 4°C at this step.
5. Rinse the membrane with 30 mL of TBST wash buffer, and drain well.
6. Immerse the membrane in primary antibody diluted in Blocking buffer. Incubate for at least 1 h on a rocking platform at room temperature or, alternatively, overnight at 4°C.

   Primary antibody dilutions can be reused several times if supplemented with 3 mM (0.02%) sodium azide and stored at 4°C.
7. Rinse six times with 30 mL of TBST wash buffer for 5 min per change, draining well after the last wash.
8. Incubate the membrane with a HRP-conjugated secondary antibody at the manufacturer's suggested dilution in Blocking buffer (i.e., 1:5000) for 30–60 min on a rocking platform at room temperature. Make a fresh dilution of secondary antibody each time.

   Sodium azide must not be added to HRP-conjugated secondary antibody because it inactivates this enzyme.
9. Rinse six times with 30 mL of TBST wash buffer for at least 5 min; longer washes and more buffer changes may help reduce background, especially when using more sensitive detection methods such as ECL.
10. Immediately before use, prepare a fresh ECL working solution by mixing peroxide solution (Solution A) and the luminol/enhancer solution (Solution B) from the ECL kit according to the supplier's directions.

    1–2 mL of working solution is usually enough to cover the surface of a 9 × 15-cm membrane.
11. Cut off the sealed edges of a plastic sheet protector to separate the two sheets. Drain the membrane, place it on one sheet, and pipette the ECL working solution directly onto the surface of the membrane. Carefully place the second sheet onto the membrane, starting at one corner and lowering the sheet slowly to avoid trapping air bubbles. Incubate the membrane with ECL working solution for 1–5 min, and protect it from unnecessary exposure to light during this incubation. Use an absorbent tissue to remove excess liquid from the corners of the sandwich.
12. Place the sheet protector/membrane sandwich protein side up into a film cassette, and tape it in place. Expose the sheet protector/membrane to film in a darkroom with an appropriate light source. A recommended first exposure time is 60 sec. Exposure time may be varied to achieve optimal results.

    Light emission is intense, and any movement between the film and membrane can cause artifacts on the film. Light emission is most intense during the first 5–30 min.
13. Develop film using appropriate developing solution and fixative. Before disassembling the film cassette, note the position of bands on the developed film relative to markers or other reference points on the blot.
14. The blot may be stripped and reprobed with a different antibody, if necessary. Incubate the membrane in Stripping solution for 5–10 min at 50°C in a sealed container (e.g., Tupperware),

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then wash extensively (~45 min) with several changes of deionized H_2O followed by TBST wash buffer. Block and reprobe the membrane beginning with Step 4.

TROUBLESHOOTING

Problem: There is no signal.

Solution: No or low signal can have many reasons including low amounts of antigen on the membrane, loss of antigenic sites (epitopes) during protein preparations, low sensitivity of the primary antibody (some are conformation dependent), and use of inappropriate secondary antibody. Loading increasing amounts of protein on the SDS gel and use of a control sample (if available) or luminescent marker proteins may help to determine the minimum antigen amount needed and to optimize the transfer efficiency to the membrane. Most often, increasing the concentrations of primary and secondary antibodies (see the Reagents list and Table 2) and their incubation times (overnight at 4°C for the primary antibody and 2 h for the secondary antibody) or switching to a higher-sensitivity chemiluminescent kit (e.g., femto versus pico ECL) will remedy the problem. Wash off the substrate with TBST as in Step 5 and resume incubation with the primary antibody (Step 6). In addition, ensure that the secondary antibody is matched to the species and isotype of the primary antibody and that sodium azide is absent in HRP-conjugated secondary antibody solutions because it inactivates this enzyme.

Problem: There is too much signal and high background.

Solutions:

- If the signal is too high and the blot glows in the darkroom, simply waiting for the glow to subside (20 min to 2 h) before taking another film exposure is an unsatisfactory solution because the relative signal intensities between saturated and faint protein bands will be obscured, making a

TABLE 2. Chromogenic and chemiluminescent methods of detection of immobilized antigens

Enzyme	Reagent	Sensitivity	Comments	Reference(s)
Chromogenic				
Horseradish peroxidase	4-Chloro-1 naphthol/H_2O_2	1 ng	The purple color of oxidized products fades rapidly upon exposure to light.	Hawkes et al. 1982; Dresel and Schettler 1984
	Diaminobenzidine/H_2O_2	250 pg	Potentially carcinogenic. The diamino-benzidine reaction generates a brown precipitate, which is enhanced by the addition of cobalt, silver, and nickel salts.	de Blas and Cherwinski 1983; Gershoni 1988
	3,3′,5,5′-Tetramethyl-benzidine	100 pg	Deep purple precipitate	McKimm-Breschkin 1990
Alkaline phosphatase	Nitro blue tetrazolium/5-bromo-4-chloroindolyl phosphate	100 pg	Steel-blue precipitate	Leary et al. 1983; Blake et al. 1984
Chemiluminescent				
Horseradish peroxidase	Luminol/4-iodo-phenol/H_2O_2	300 pg	Oxidized luminol emits blue light that is captured on X-ray film. Luminescence generated by intense bands appears within a few seconds, whereas faint bands need at least 30 min to develop.	Schneppenheim and Rautenberg 1987; Harper and Murphy 1991; Schneppenheim et al. 1991
Alkaline phosphatase	AMPPF 3-(4-methoxyspiro[1,2-dioxetane-3′2′-tricyclo-[3.3.1^{3,7}] decan]-4-yl)- phenylphosphate	1 pg	The enzymatically dephosphorylated product emits light. Because of its high turnover number, alkaline phosphatase rapidly generates a strong signal that provides an exquisitely sensitive method of immunodetection.	Gillespie and Hudspeth 1991

quantitative comparison between protein samples impossible. Instead, strip the membrane as in Step 14 and reprobe with diluted (at least fivefold) primary and secondary antibody solutions.

- If background is high or speckled, use larger volumes of Blocking and Wash buffers and increase the washing time, especially after incubation with the secondary antibody (Step 9). Increasing the milk concentration in the Blocking buffer or primary antibody solution or switching to another blocking agent (see the Reagents list) may sometimes reduce nonspecific signal.

DISCUSSION

Immunoblotting (western blotting) is used to identify and confirm the size of macromolecular antigens (usually proteins) that react with a specific antibody (Towbin et al. 1979; Burnette 1981; for reviews, see Towbin and Gordon 1984; Gershoni 1988; Stott 1989; Nelson et al. 1990). The results are reagent specific because of the wide assortment of equipment, reagents, and antibodies, and their variable affinity for a given antigen. For best results, it is important to optimize all components, including the sample amount resolved by SDS-PAGE, time of electrotransfer, choice of membrane and blocking agent, and the primary and secondary antibody concentrations used for a particular substrate detection system. For specific details, see below.

Problems that commonly arise in immunoblotting include inefficient transfer of proteins, loss of antigenic sites (epitopes), low sensitivity, high background, and nonquantitative detection. No magic formula can eliminate all of these difficulties for every antigen, but a small amount of experimentation is usually sufficient to cure all but the most obdurate technical problems. Comprehensive reviews (Bjerrum and Schaffer-Nielsen 1986; Bjerrum et al. 1988; Stott 1989; Alegria-Schaffer et al. 2009) provide catalogs of potential difficulties in immunoblotting and detailed suggestions for solving them. Useful troubleshooting information can also be found in the manuals of most western blot suppliers. For the highly sensitive chemiluminescent detection systems (ECL, SuperSignal, LumiGLO, Western Lightning), recommendations given by the manufacturers are very helpful.

Transfer of Proteins from Gel to Filter

Electrophoretic transfer of proteins from polyacrylamide gels to membranes is far more efficient and much quicker than capillary transfer. The direction of transfer is perpendicular to the plane of the separating gel, using electrodes and membranes that cover its entire surface. For transfer from SDS gels, the membrane is placed on the (red) anodic side of the gel. Most commercial electrophoretic transfer devices use large wire or plate electrodes made of graphite, platinum, or stainless steel. In older devices, the gel/membrane sandwich is submerged in a tank of transfer buffer between vertical electrodes attached to a plastic cradle. These electrophoretic transfer cells are still available for purchase, but the more efficient "semidry" format has become the more popular choice. In semidry transfer, Whatman 3MM paper saturated with transfer buffer serves as the reservoir instead of a tank (see Table 1). Optimal conditions for protein transfer depend on the type of capture membrane in use as well as the design of the apparatus. It is therefore best to consult the manufacturer's instructions.

Types of Membranes

Three types of capture membranes are used for immunoblotting: nitrocellulose, nylon, and polyvinylidene fluoride (PVDF). Individual proteins bind with different efficiencies to these membranes, and a particular epitope may be better preserved in one case over another. It is therefore worthwhile wherever possible to test detection of the protein of interest on various membranes, using several antibodies.

Nitrocellulose Membranes

Nitrocellulose (pore size, 0.45 μm) remains a standard immunoblotting membrane, although membranes with a smaller pore size (0.22 or 0.1 μm) are recommended for immunoblotting of small proteins of <14 kDa (Burnette 1981; Lin and Kasamatsu 1983). Nitrocellulose has a binding and retention capacity of 80–250 μg/cm^2, depending on the protein. Proteins are bound chiefly by hydrophobic interactions (Van Oss et al. 1987), although hydrogen bonding between amino acid side chains and the nitro group of the membrane may also contribute. In any event, partial dehydration of the proteins by methanol or salt in the transfer buffer ensures longer-lasting binding interactions. Even so, some protein may be lost from the membrane during processing, particularly if nonionic detergents are present in the buffers. Many investigators therefore fix the proteins to nitrocellulose membranes to minimize loss during washing and incubation with antibody (e.g., see Gershoni and Palade 1982). However, it is important to check that the treatment used for fixation (glutaraldehyde, cross-linking, or UV irradiation) does not destroy the epitope of interest. Fixation may also exacerbate brittleness when nitrocellulose filters are allowed to dry after transfer.

Nylon Membranes

Nylon and positively charged nylon membranes are tougher than nitrocellulose and bind proteins tightly through electrostatic interaction. Their capacity varies with membrane type and from one protein to another but is usually in the range of 150–200 μg/cm^2. These membranes have the advantage of allowing multiple rounds of probing with different antibodies. One potential disadvantage of nylon membranes is that no simple and sensitive procedure is available to stain immobilized proteins, as discussed below. Another disadvantage is the difficulty of blocking all of the unoccupied binding sites on these membranes, resulting in high nonspecific background binding of antibodies. In many cases, extended blocking in solutions containing 6% heat-treated casein and 1% polyvinylpyrrolidone (Gillespie and Hudspeth 1991) is required to achieve satisfactory results.

Polyvinylidene Fluoride Membranes

Polyvinylidene fluoride (PVDF) (Pluskal et al. 1986) is a mechanically strong, durable membrane with a binding capacity (~170 μg/cm^2) similar to that of nylon. Before transfer, it is necessary to prewet the hydrophobic surface of the membrane with methanol and then hydrate it in transfer buffer. Because proteins are bound to PVDF through strong hydrophobic interactions approximately sixfold tighter than to nitrocellulose (Van Oss et al. 1987), they are more completely retained during subsequent detection steps. Proteins immobilized on PVDF can be visualized with standard stains such as Amido Black, India Ink, Ponceau S, and Coomassie Brilliant Blue.

Staining of Proteins during Immunoblotting

Transfer of proteins from Coomassie-stained gels has sometimes been performed after complete destaining and soaking in 0.1% SDS buffer (Thompson and Larson 1992). However, the efficiency of transfer of stained (or fixed) proteins out of the gel is severely reduced in most cases, and epitopes may be masked for antibody binding. Instead, it is greatly preferable to include a lane of prestained protein markers for quality control of the electrophoresis and electrotransfer steps. Efficiency and uniformity of transfer of proteins in the sample lanes can be confirmed by staining the membrane. This is simple to perform but requires careful choice of a sufficiently sensitive stain that is compatible with the type of membrane.

Proteins bound to nitrocellulose or PVDF membranes can be stained with the removable stain Ponceau S (Muilerman et al. 1982; Salinovich and Montelaro 1986). Ponceau staining is relatively insensitive, but it provides a valuable "loading control" to verify equivalent amounts of cellular protein in each lane, as is needed for expression screening and other comparative Western blot analyses. When more permanent stains are used, such as India Ink (Hancock and Tsang 1983), Amido Black (Towbin

et al. 1979), colloidal gold (Moeremans et al. 1985; Rohringer and Holden 1985), or silver (Yuen et al. 1982), it is usually necessary to cut and separately stain a reference lane from the membrane. Brief exposure to alkali enhances staining with either India Ink or colloidal gold, perhaps by reducing loss of protein from the membrane during washing (Sutherland and Skerritt 1986). Under these conditions, it is possible to detect bands containing as little as a few nanograms of protein.

There is no satisfactory method to stain proteins immobilized on nylon or cationic nylon membranes because the high charge of these membranes produces high backgrounds that obscure all but the strongest protein bands.

Blocking Agents

Traditional blocking agents such as 1% nonfat dry milk or 5% bovine serum albumin (Johnson et al. 1984; DenHollander and Befus 1989) are often suitable for use with HRP-based detection systems. However, these agents usually harbor residual alkaline phosphatase activity and should not be used with detection systems that use antibodies conjugated with this enzyme. This is particularly true with chemiluminescent systems, in which the exquisite sensitivity of detection requires the background signal to be very low. In general, the best blocking solution for alkaline phosphatase–based systems contains 6% casein, 1% polyvinylpyrrolidone, and 10 mM EDTA in phosphate-buffered saline (Gillespie and Hudspeth 1991). The blocking solution should be heated for 1 h to 65°C to inactivate residual alkaline phosphatase and then stored at 4°C in the presence of 3 mM sodium azide. The specificity of the primary antibody should also be taken into consideration. For example, casein and milk would not be a suitable blocking agent for anti-phosphoamino acid antibodies, and bovine serum albumin (BSA) is incompatible with antibodies directed against serum proteins and many infectious agents. Consult the literature from the antibody supplier for recommended blocking agents.

Probing and Detection

The antibody that reacts with the epitope of interest can be polyclonal or monoclonal. It is seldom labeled directly but is merely diluted into an appropriate buffer for formation of antibody–antigen complexes. In general, backgrounds in immunoblotting are unacceptably high unless the primary antibody can be diluted at least 1:1000 with enzymatic methods of detection or at least 1:5000 with chemiluminescent methods. After washing, the bound antibody is detected with a specific secondary antibody, which recognizes common features of the primary antibody and is conjugated with a reporter enzyme or group. The use of two stages of antibody binding is standard practice, even though it may seem cumbersome compared with using a conjugated primary antibody. However, the ability of multiple secondary conjugated antibody molecules to bind a single primary antibody results in significant amplification of signal over a single antibody approach. In addition, a conjugated secondary antibody can be used to detect any primary antibody of the same immunoglobulin isotype, keeping reasonable the inventory of conjugates that must be kept on hand.

Secondary reagents include:

- radioiodinated antibodies or Staphylococcal Protein A, which were used in the early years of immunoblotting (e.g., Burnette 1981). Radiolabeled secondary antibodies have largely been replaced by safer and/or more sensitive detection systems such as enhanced chemiluminescence.
- antibodies conjugated to enzymes such as horseradish peroxidase or alkaline phosphatase, for which a variety of chromogenic, fluorescent, and chemiluminescent substrates are commonly used.
- biotinylated antibodies, which can be detected with labeled or conjugated streptavidin.

The results of chromogenic and chemoluminescent reactions are best recorded by conventional photography, film, or digital imaging. Table 2 shows the approximate sensitivity with which the best of these methods can detect a standard antigen using antibodies of high titer and specificity.

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RECIPES

Stripping Solution

Reagent	Quantity (for 100 mL)	Final concentration
Tris-Cl (0.5 M, pH 6.7)	12.4 mL	62 mM
SDS	2 g	2% (w/v)
β-mercaptoethanol (14.2 M)	0.7 mL	100 mM

Store at room temperature in a sealed bottle.

TBST Wash Buffer

Reagent	Quantity (for 1 L)	Final concentration
Tris-Cl (1 M, pH 7.4)	10 mL	10 mM
NaCl	9 g	0.9% (w/v)
Tween 20 (10%, v/v)	2 mL	0.02% (v/v)

Store at room temperature.

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