Western Blotting

The antigenic mixture of interest is solubilized, usually with sodium dodecyl sulfate (SDS), urea, and/or reducing agents such as 2-mercaptoethanol. Following solubilization the material is separated by SDS-PAGE (polyacrylamide gel electrophoresis), although thin-layer chromatography can also be used. The antigens are then electrophoretically transferred to nitrocellulose paper, where they are bound irreversibly. The paper is blocked to prevent nonspecific binding of antibody and probed with the antibody of interest. The antibody is detected by a horseradish peroxidase (HRPO)–anti-immunoglobulin (Ig) conjugate and visualized by incubating the filter paper in the presence of a precipitable substrate.

Materials

0.1% SDS in PBS
Electroblotting buffer
Ponceau S solution
Blocking buffer
Primary antibody
Horseradish peroxidase (HRPO)–anti-Ig conjugate
Phosphate-buffered saline (PBS; *APPENDIX 2*)
Diaminobenzidine (DAB) substrate solution

Whatman 3MM filter paper
Scotch-Brite pads (3M)
0.45-μm nitrocellulose membrane filter (Millipore #HAHY-000-10 or Schleicher & Schuell #BA85)
Electroblotting apparatus (E.C. Apparatus) or Transblot apparatus (Bio-Rad)
Heat-sealable plastic bag
Photographic equipment
Plastic box

NOTE: *Deionized, distilled water should be used throughout this protocol.*

Electrophoresing the protein sample

1. Prepare samples and separate proteins by SDS-PAGE (see *UNIT 10.2*). Bacterial colonies or lysates can be placed directly into 0.1% SDS (i.e., solubilizing buffer), loaded onto gels, and proteins separated by SDS-PAGE. Protein standards should be present in one or more gel lanes.

   A variety of gel sizes and percentages of acrylamide can be used. Most routinely used are either 14 cm × 14 cm × 1.5 mm gels or minigels (8 cm × 10 cm × 0.5 mm). Acrylamide concentrations vary from 5 to 20%, but concentrations are usually in the 10 to 15% range.

Assembling the Western blot sandwich

2. Place a piece of Whatman 3MM filter paper, cut to the same size as the gel and prewetted with electroblotting buffer, on a Scotch-Brite pad (Fig. 10.8.1).
3. Place what is arbitrarily chosen to be the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank) onto this filter paper and moisten the surface of the gel with electroblotting buffer. Remove any air bubbles between gel and filter paper by gently pushing with powder-free, gloved fingers and lifting the gel as needed.

   Use gloves when manipulating filter papers, gels, and nitrocellulose. Oil from hands blocks the transfer to the nitrocellulose paper.

4. Place a piece of cut, marked, and wetted nitrocellulose membrane filter directly

onto the anode (i.e., positive electrode) side of the gel and remove all air bubbles, as in step 3 (Fig. 10.8.1).

Many ballpoint inks come off, but Paper-Mate stays on.

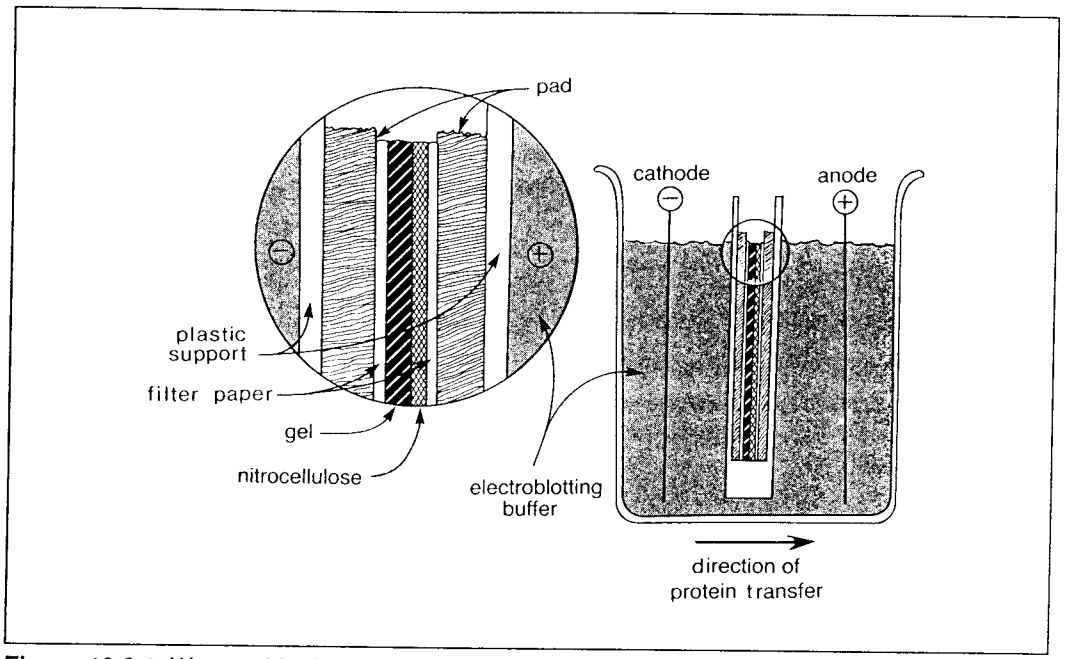

Figure 10.8.1 Western blotting. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of nitrocellulose precut to match the gel size. The nitrocellulose is overlaid with another sheet of filter paper. The filter paper containing the gel and nitrocellulose is sandwiched between Scotch-Brite pads. This sandwich is placed in a plastic support, and the entire assembly is placed in a tank containing transfer buffer. The voltage applied is typically 14 V for a period of 1 to 16 hr at 4°C. The nitrocellulose is positioned on the anode side of the gel. The negatively charged proteins are transferred electrophoretically from the gel onto the nitrocellulose membrane.

5. Place another piece of wetted Whatman filter paper on the anode side of the nitrocellulose and remove all air bubbles. Place another Scotch-Brite pad on top of this filter paper (Fig. 10.8.1).
6. Place this sandwich into a plastic support.

   It is important to orientate the sandwich so that the nitrocellulose faces the anodal (i.e., positively charged) side of the tank.

Transferring proteins from gel to nitrocellulose

7. Place the support containing the sandwich into the electroblotting apparatus in the correct orientation.
8. Fill the tank with electroblotting buffer.
9. Connect the leads of the power supply to the corresponding anodal and cathodal sides of the electroblotting apparatus.
10. Electrophoretically transfer the proteins from the gel to the nitrocellulose at 14 V constant voltage from 1 hr to overnight at 4°C in electroblotting buffer.

    Transfer time is dependent on the thickness of the gel and the size of the protein being transferred. Transfer can be monitored by silver staining the gel (UNIT 10.6) after transfer to ensure complete transfer of all materials to the nitrocellulose. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer is reliable and convenient.

Staining (reversible) of transferred proteins

11. Place nitrocellulose filter in Ponceau S solution 5 min to stain proteins.
12. Destain in water for 2 min. Photograph the filter if required and mark the molecular weight standard band locations with indelible ink. Completely destain the filter by soaking in water 10 min.

Blocking nonspecific antibody sites on the nitrocellulose

13. Place filter in a heat-sealable plastic bag with blocking solution. Seal the bag. Agitate the solution in the bag for 1 hr at room temperature with an orbital shaker or a rocking platform. Open the bag and pour out the blocking buffer.

    Usually 5 ml of buffer per 2 to 3 filters (10 × 15 cm size) is sufficient.

Probing electroblotted proteins with primary antibody

14. Dilute primary antibody (i.e., specific for the protein of interest) in blocking buffer.

    The primary antibody dilution is determined empirically but is usually 1:100 to 1:1000 for polyclonal antibody; 1:10 to 1:100 for hybridoma supernatants; and ≥1:1000 for murine ascites fluid for monoclonal antibodies.

15. Place filter in a heat-sealable plastic bag with *diluted* primary antibody 1 hr at room temperature using constant agitation with an orbital shaker or a rocking platform.

    Usually 5 ml of diluted primary antibody solution per 2 to 3 filters (10 × 15 cm size) is sufficient.

    Time may vary, depending on conjugate used.

Washing away nonspecifically bound primary antibody

16. Remove filter from plastic bag with gloved hand. Place filter in plastic box. Wash four times by agitating with 200 ml PBS each time.

Detecting bound antibody by HRPO–anti-Ig conjugate and formation of a DAB precipitate

17. Dilute HRPO conjugate in blocking buffer.
18. Repeat step 15 with *diluted* HRPO–anti-Ig conjugate.
19. Repeat step 16.
20. Place filter with 100 ml of freshly prepared DAB substrate solution in a plastic box. Time will vary, but color will usually develop within 2 to 3 min and often within seconds.

    CAUTION: *Handle DAB with care. It is a carcinogen.*

21. Stop the reaction by rinsing briefly with water.

Photographing the immunoblot

22. Photograph for permanent record.

    Original blot will fade after several hours when exposed to light.

SHORT PROTOCOL

WESTERN BLOTTING

1. Prepare sample and run SDS-PAGE.
2. Place gel in electroblot apparatus with nitrocellulose filter on anode side.
3. Transfer at 14 V constant voltage for 1 hr to overnight at 4°C, depending on the specific experiment.
4. Visualize transferred proteins by staining in Ponceau S solution 5 min. Destain in water for 2 to 3 min and mark molecular weight markers with indelible ink. Destain for 10 min.
5. Block filter with blocking buffer 1 hr at room temperature.
6. Dilute primary antibody in blocking buffer and incubate with filter 1 hr at room temperature.
7. Wash filter four times using 200 ml PBS for 15 min each wash.
8. Dilute HRPO–anti-Ig conjugate in blocking buffer and incubate with filter 1 hr at room temperature.
9. Wash filter four times using 200 ml PBS for 15 min each wash.
10. Add DAB substrate solution and develop.

    CAUTION: *Use gloves. DAB is a carcinogen.*
11. Stop staining reaction by washing with water.
12. Photograph stained filter for permanent record.

REAGENTS AND SOLUTIONS

NOTE: *Deionized, distilled water should be used to prepare all solutions.*

Blocking buffer

1 g instant nonfat dry milk in 100 ml PBS

Diaminobenzidine (DAB) substrate solution

50 mg 3,3′-diaminobenzidine
2 ml 1% $CoCl_2$ in H_2O
98 ml phosphate-buffered saline (PBS; *APPENDIX 2*)
0.1 ml 30% H_2O_2, added immediately prior to use
CAUTION: *Handle DAB with care. It is a carcinogen.*

Electroblotting buffer (20 mM Tris/150 mM glycine, pH 8)

Add 14.5 g Tris base (Sigma) and 67 g glycine to 4 liters of H_2O. Bring to pH 8, add 1200 ml methanol, and bring to 6 liters with H_2O.

Ponceau S solution (0.5% Ponceau S/1% acetic acid)

0.5 g Ponceau S
1 ml glacial acetic acid
Bring to 98.5 ml with H_2O

Horseradish peroxidase–anti-Ig conjugate (Cappel)

A variety of species-specific anti-Ig conjugates can be purchased. Dilute these as indicated by the manufacturer and store frozen in 0.025-ml aliquots until use.

COMMENTARY

Background Information and Literature Review

Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. Western blotting allows one to identify particular proteins by utilizing the specificity inherent in antigen:antibody recognition. This technique is extremely powerful, since it combines electrophoretic separation of proteins, glycoproteins, and lipopolysaccharides with immunological identification. Once such antigens have been detected, they can be further characterized by Western blotting. Both techniques can utilize either polyclonal or monoclonal antibodies.

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal. However, there are several problems inherent with immunoprecipitation, including (1) the requirement for radiolabeling of antigen, (2) coprecipitation of tightly associated macromolecules, (3) occasional difficulty in obtaining precipitating antibodies, and (4) insolubility of various antigens (Talbot et al., 1984). To circumvent these problems, electroblotting (Towbin et al., 1979), subsequently popularized as Western blotting (Burnette, 1981), was conceived. This procedure involves the solubilization and electrophoretic separation of macromolecules by SDS-PAGE or urea-PAGE followed by quantitative transfer and irreversible binding to either nitrocellulose or diazobenzyl-oxymethyl (DBM) paper. This technique has proved to be useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies, and it is highly sensitive (i.e., <1 ng of antigen can often be detected).

There are several problems associated with Western blotting. First, the antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS and urea). Some antibodies may not recognize the denatured form of the antigen transferred to the nitrocellulose filter. Second, transfer of some antigens from the gel to the blot paper may not be quantitative. Third, the results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents have been shown to restore the antigenicity of outer membrane proteins in Western blotting (Mandrell and Zollinger, 1984).

Critical Parameters

First and foremost, the antibody should recognize native and nonnative antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. The time of transfer and the primary antibody and conjugate dilutions should always be optimized.

Troubleshooting

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that current has flowed. Also, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the protein bands are diffuse, check the plastic holder and Scotch-Brite pads, because the filter may not have been firmly held in place. Rubber bands can be placed around the sandwich apparatus to reduce the diffusion of bands during the transfer.

If air bubbles are trapped between the filter and the gel, they will appear as clear white bubbles on the filter after blotting and staining. Take extra care to ensure that all bubbles are removed.

Nonspecific binding may occur and will show up as gray background on the filter. Try reducing the concentration of the primary or secondary antibody and/or the time of incubation to reduce the level of nonspecific binding.

Anticipated Results

A Western blot can be used to detect as little as 1 ng of a protein antigen that has been previously separated under denaturing conditions by SDS-PAGE, provided that an antibody that recognizes the denatured form of the protein is available. Either polyclonal or monoclonal antibodies may be used.

Time Considerations

The entire Western blot procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr to overnight on a regular gel and 1 hr on a minigel. Blocking, conjugate incubation, and washing each take an hour. Finally, substrate incubation requires 10 min.

Literature Cited

Burnette, W.H. 1981. Western Blotting: Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.

Mandrell, R.E. and Zollinger, W.D. 1984. Use of zwitterionic detergent for the restoration of antibody-binding capacity of electroblotted meningococcal outer membrane proteins. *J. Immunol. Meth.* 67:1-11.

Talbot, P.V., Knobler, R.L., and Buchmeier, M. 1984. Western and dot immunoblotting analysis of viral antigens and antibodies: Application to murine hepatitis virus. *J. Immunol. Meth.* 73:177-188.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76:4350-4354.

Key Reference

Salinovich, O. and Montelaro, R. 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by SDS-PAGE. *Anal. Biochem.* 156:341-347.

Describes the use of Ponceau S staining for Western blotting.

Contributed by Scott E. Winston,
Steven A. Fuller, and John G.R. Hurrell
Allelix, Inc.
Mississauga, Ontario