

Immunoblotting and Immunodetection

UNIT 6.2

Sean Gallagher,¹ Scott E. Winston (tank transfer systems),² Steven A. Fuller (tank transfer systems),² and John G.R. Hurrell (tank transfer systems; reversible staining of proteins)³

¹UVP, Inc., Upland, California

²Nabi, Rockville, Maryland

³FluorRx, Carmel, Indiana

ABSTRACT

Immunoblotting (western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. This unit provides protocols for all steps, starting with solubilization of the protein samples, usually by means of SDS and reducing agents. Following solubilization, the material is separated by SDS-PAGE and the antigens are electrophoretically transferred to a membrane, a process that can be monitored by reversible staining with Ponceau S. The transferred proteins are bound to the surface of the membrane, providing access to immunodetection reagents. After nonspecific binding sites are blocked, the membrane is probed with the primary antibody and washed. The antibody-antigen complexes are tagged with horseradish peroxidase or alkaline phosphatase coupled to a secondary anti-IgG antibody, and detected using appropriate chromogenic or luminescent substrates. Finally, membranes may be stripped and reprobed. *Curr. Protoc. Cell Biol.* 52:6.2.1-6.2.28. © 2011 by John Wiley & Sons, Inc.

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INTRODUCTION

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE (UNITS 6.1 & 6.4). The antigens are then electrophoretically transferred to a nitrocellulose, PVDF, or nylon membrane in a tank (see Basic Protocol 1) or semidry (see Alternate Protocol 1) transfer apparatus, or a dot or slot blotting apparatus (see Alternate Protocol 2). The transfer of proteins to the membrane can be monitored qualitatively or quantitatively by staining with Ponceau S (see Support Protocols 1 and 2). Proteins may also be transferred from previously stained gels (see Alternate Protocol 3).

The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining nonspecific binding sites are blocked by immersing the membrane in a solution containing a protein or detergent blocking agent. After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with a secondary anti-IgG antibody coupled to a horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzyme. The enzymes can be attached directly (see Basic Protocol 2) or via an avidin-biotin bridge (see Alternate Protocol 4) for increased sensitivity. Chromogenic or luminescent substrates (see Basic Protocol 3 and Alternate Protocols 5 and 6) are then used to visualize the enzyme activity. Finally, membranes may be stripped and reprobed (see Support Protocol 3).

NOTE: A video demonstration of this technique can be found at <http://www.jove.com/index/Details.stp?ID=759>.

Electrophoresis
and
Immunoblotting

6.2.1

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Supplement 52

PROTEIN BLOTTING WITH TANK TRANSFER SYSTEMS

In this procedure, blotting is performed in a tank of buffer with the gel in a vertical orientation, completely submerged between two large electrode panels. In some systems up to four gels can be transferred at one time. For difficult-to-transfer proteins (>100 kDa or hydrophobic; e.g., myosin), tank blotting is preferable to semidry systems because prolonged transfers are possible without buffer depletion. However, transfers >1 hr at high power require cooling using a heat exchanger and a circulating water bath that can maintain a constant transfer temperature of 10° to 20°C.

Materials

Samples for analysis

Protein molecular weight standards (Table 6.2.1; UNIT 6.1): prestained (Sigma or Bio-Rad), biotinylated (Vector Labs or Sigma), fluorescent (e.g., BenchMark Fluorescent Protein Standards; Invitrogen), or compatible with other colorimetric and fluorescent detection method (e.g., MagicMark and MagicMark XP Western Protein Standards; Invitrogen)

Transfer buffer (see recipe)

Powder-free gloves

Scotch-Brite pads (3M) or equivalent sponge

Whatman 3MM filter paper or equivalent

Transfer membrane: 0.45- μ m nitrocellulose (Millipore or Schleicher & Schuell), PVDF (Millipore Immobilon P), neutral nylon (Pall Biodyne A), or positively charged nylon (Pall Biodyne B; Bio-Rad Zetabind)

Electroblotting apparatus (Bio-Rad, Invitrogen, Amersham, or Hoefer; e.g., Figs. 6.2.1 and 6.2.2)

Indelible pen (e.g., Paper-Mate) or soft lead pencil

Additional reagents and equipment for one- and two-dimensional gel electrophoresis (UNITS 6.1 & 6.4) and staining proteins in gels (UNIT 6.6) and on membranes (see Support Protocol 1)

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

Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard-sized one- or two-dimensional gels (UNITS 6.1 & 6.4). Include prestained or biotinylated protein molecular weight standards in one or more gel lanes.

The protein markers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

Table 6.2.1 Protein Standards for Immunoblotting

Protein standard	Application
Unstained	Molecular weight calibration and transfer efficiency; can be visualized with total protein stains
Tagged	Molecular weight calibration and transfer efficiency; visualized during immunodetection steps; a variety of potential tags, including biotinylated and antibody-specific amino acid sequence engineered into standard proteins
Prestained	Excellent for checking transfer efficiency and visually inspecting the blot; typically does not produce as sharp a band as other standards, making precise molecular weight calculations difficult

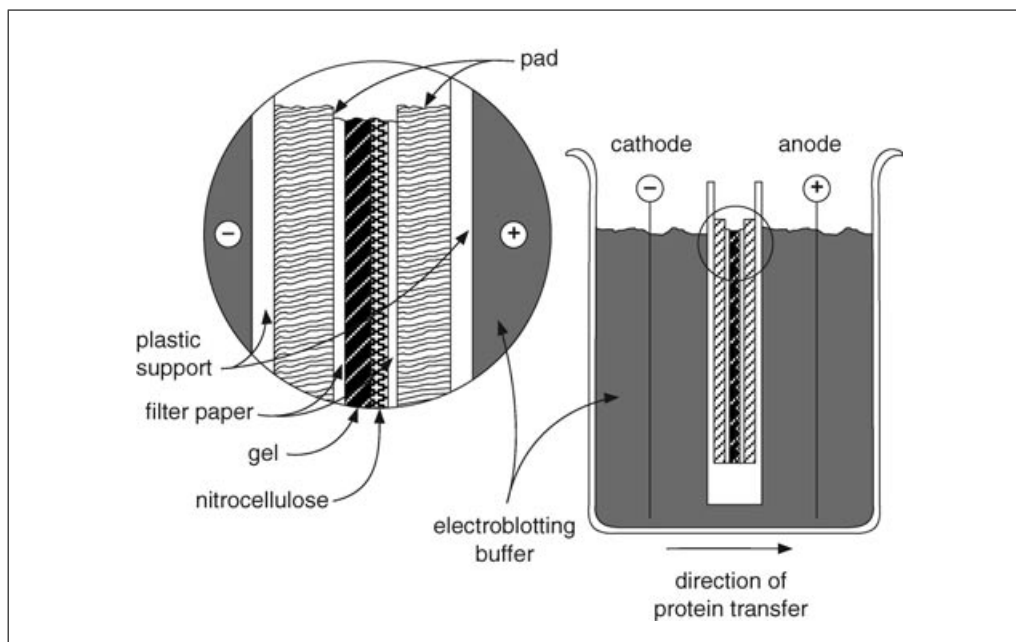


Figure 6.2.1 Immunoblotting with a tank blotting unit. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel plus 1 to 2 mm on each edge, then this membrane is overlaid with another sheet of filter paper. The filter paper containing the gel and membrane 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. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane. Transfer is achieved by applying a voltage of 100 V for 1 to 2 hr (with cooling) or 14 V overnight.



Figure 6.2.2 Minigel tank electrotransfer unit. Designed for smaller (8 × 10-cm) gels, these units will process four gels at a time. Note that the two outside panels hold the electrode grid.

6.2.3

MagicMark Western Protein Standards (Invitrogen) allow direct visualization of protein size standards on membrane blots without the need for protein modification or special detection reagents. The standard proteins are derived from E. coli cells containing a construct with repetitive units of a fusion protein forming the size variation and an IgG binding site. The proteins can be visualized with colorimetric, chemiluminescent, or fluorescent detection simply by probing the membrane for the specific protein. The IgG binding site will allow all the standards to react with the specific primary and secondary antibodies. The standards are in a ready-to-use format and do not have to be heated or reduced. Alternatively, BenchMark Fluorescent Protein Standards (Invitrogen) are visualized directly via UV transillumination on a UV transilluminator (UVP) when wet, or via overhead UV illumination (apparatus also available from UVP) when dry.

A variety of gel sizes and percentages of acrylamide can be used (UNIT 6.1). Most routinely used are either 14 cm × 14 cm × 0.75-mm gels or 8 cm × 10 cm × 0.75-mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

Assemble the immunoblot sandwich

2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate gel 30 min at room temperature in transfer buffer.

IMPORTANT NOTE: Use gloves when manipulating filter papers, gels, and membranes. Oil from hands blocks the transfer.

Match the appropriate transfer buffer to the membrane (see Reagents and Solutions).

Gel equilibration is required to prevent a change in the size of the gel during transfer. Any shift in gel dimension will result in a blurred transfer pattern.

3. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

The transfer cassette should be assembled under buffer to minimize trapping of air bubbles. Use Figure 6.2.1 as a guide to assembly.

4. On bottom half of plastic transfer cassette, place Scotch-Brite pad or sponge, followed by a sheet of filter paper cut to same size as gel, and prewet with transfer buffer.

5. Place gel on top of filter paper; the side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over surface of gel.

Any bubbles between the filter paper, gel, and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

6. Prepare transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle, so that the water wicks up into the membrane, wetting the entire surface. Equilibrate 10 to 15 min in transfer buffer.

If the membrane is placed into the water too quickly, air will be trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

7. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on top side of gel (i.e., anode side). To avoid bubbles, start by placing one corner of the membrane on the gel and slowly lower the rest of the membrane onto the gel. Remove all air bubbles as in step 5.

Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

The use of 0.2- μ m membranes may improve retention of smaller-molecular-weight proteins.

8. Wet another piece of Whatman 3MM filter paper, place on anode side of membrane, and remove all air bubbles. Place another Scotch-Brite pad or sponge on top of this filter paper.
9. Complete assembly by locking the top half of the transfer cassette into place (Fig. 6.2.1).

It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

10. Fill tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

11. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling or overnight at 14 V (constant voltage) in a cold room.

Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers >1 hr at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

12. Turn off the power supply and disassemble the apparatus. Remove membrane from blotting apparatus and note orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate pen.

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

Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

13. Stain gel for total protein with Coomassie blue (UNIT 6.6) to verify transfer efficiency. If desired, visualize transferred proteins on nitrocellulose or PVDF membranes by staining reversibly with Ponceau S (see Support Protocol 1), or irreversibly with Coomassie blue, India ink, naphthol blue, or colloidal gold.

These staining procedures are incompatible with nylon membranes.

If membrane shows significant staining on the backside, either the gel was heavily overloaded or the membrane has poor protein-binding capacity (see Troubleshooting). In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose, in particular, will show diminished binding capacity with age or poor storage conditions (e.g., high temperature

and humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

14. Proceed with immunoprob ing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 4 and 6).

ALTERNATE PROTOCOL 1

PROTEIN BLOTTING WITH SEMIDRY SYSTEMS

Even and efficient transfer of most proteins is also possible with semidry blotting, a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with transfer buffer, the gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 6.2.3), greatly reducing the amount of buffer required. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 1) should be used for proteins that require long blotting times for efficient transfer.

Additional Materials (also see Basic Protocol 1)

Six sheets of Whatman 3MM filter paper or equivalent, cut to size of gel and saturated with transfer buffer

Semidry transfer unit (Hoefer, Bio-Rad, or Invitrogen)

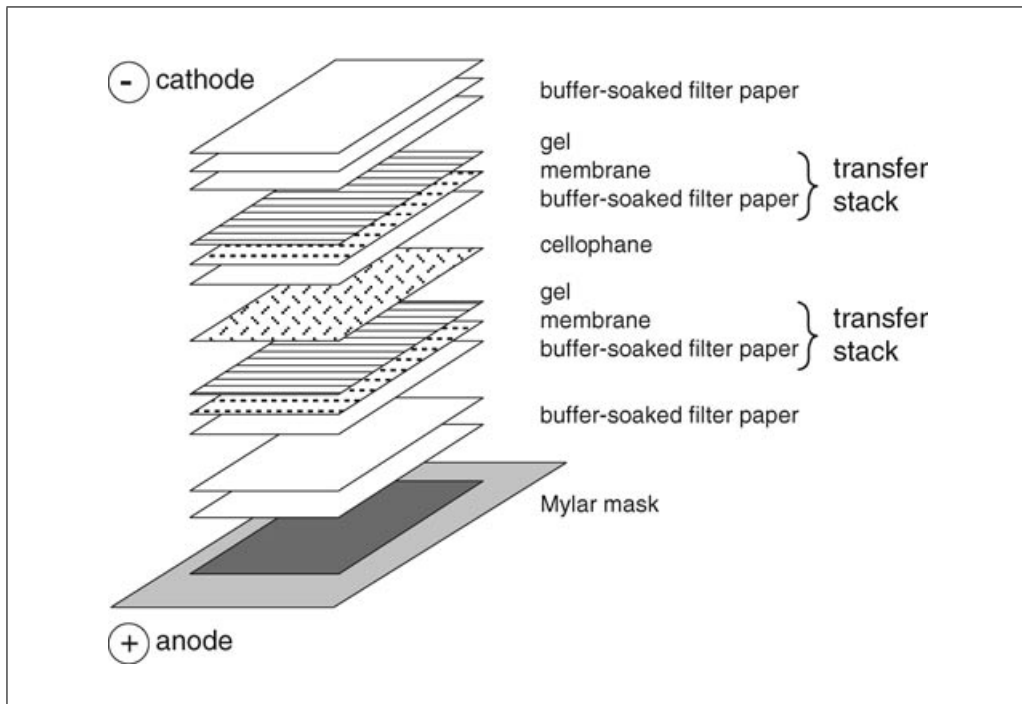


Figure 6.2.3 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer-soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm² of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm), this means 60 and 200 mA, respectively.

Assemble the immunoblot sandwich

1. Prepare samples and separate proteins using small or standard-sized one- or two-dimensional gels (UNITS 6.1 & 6.4).

Because transfer efficiency depends on many factors (e.g., gel concentration and thickness, protein size, shape, and net charge) results may vary. Below is a guideline for 0.75-mm-thick SDS-PAGE gels transferred by semidry blotting.

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency)
5–7	29–150 kD
8–10	14–66 kD
13–15	<36 kD
18–20	<20 kD

2. Prepare transfer membrane (see Basic Protocol 1, step 6).
3. Disassemble gel sandwich. Remove and discard stacking gel.

Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

4. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 6.2.3).

Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

CAPS transfer buffer, pH 10.5 (see recipe for transfer buffer) can be used in place of the Tris/glycine/methanol transfer buffer of Basic Protocol 1. CAPS buffer should be used if the protein is to be sequenced right on the membrane (Moss, 2001), as glycine will interfere with this procedure.

The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Hoefer) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

5. Place equilibrated transfer membrane on top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.

Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

6. Place gel on top of membrane. Gently roll a test tube over surface of gel to insure intimate contact between gel and membrane and to remove any interfering bubbles.

Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

7. Complete the transfer stack by putting the three remaining sheets of filter paper on top of gel. Roll out bubbles as described above.

Multiple gels can be transferred at the same time by simply placing a sheet of porous cellophane (Hoefer) or dialysis membrane (Bio-Rad) equilibrated with transfer buffer between the transfer stacks (Fig. 6.2.3). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit, with the most efficient transfer occurring in the stack closest to the anode. For critical applications, it is best to transfer one gel at a time.

Transfer proteins from gel to membrane

8. Place top electrode onto transfer stack.

Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

Once the transfer stack has been assembled with both electrodes, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane, distorting the transfer pattern.

9. Carefully connect high-voltage leads to the power supply (see UNIT 6.1 for safety precautions). Apply constant current to initiate protein transfer.

Transfers of 1 hr are generally sufficient.

In general, do not exceed 0.8 mA/cm² of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm) this means ~60 and 200 mA, respectively.

Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

10. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation as in step 12 of Basic Protocol 1. Proceed with staining and immunoprobings (see Basic Protocol 1, steps 13 and 14).

ALTERNATE PROTOCOL 2

SLOT AND DOT BLOTTING OF PROTEINS

Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single nitrocellulose or PVDF membrane for immunoblotting analysis. In slot and dot blotting, proteins are not separated by electrophoresis. Instead, the entire sample is applied directly to the membrane. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume routine quantitation of samples.

Additional Materials (also see Basic Protocol 1)

<10 µg protein sample in <100 µl water or TBS (no detergent)

Tris-buffered saline (TBS; APPENDIX 2A)

Slot or dot blotting apparatus (e.g., Hoefer, Bio-Rad, or Whatman; Fig. 6.2.4)

Vacuum source

1. Prewet the membrane in distilled water or 100% methanol as described in Basic Protocol 1, steps 6 and 7.
2. Prepare the slot or dot blot manifold according to manufacturer's instructions.
3. Apply samples under a low house vacuum according to manufacturer's instructions.

Typically, samples should contain <10 µg protein in <100 µl. Overloading the wells will prevent flow of liquid through the well.

Typical systems use a low house vacuum to pull liquid through each slot, binding the proteins to the membrane.

4. Follow sample application with two equivalent volumes of water or TBS, depending on which was used to dissolve the sample, to rinse the wells of unbound protein.

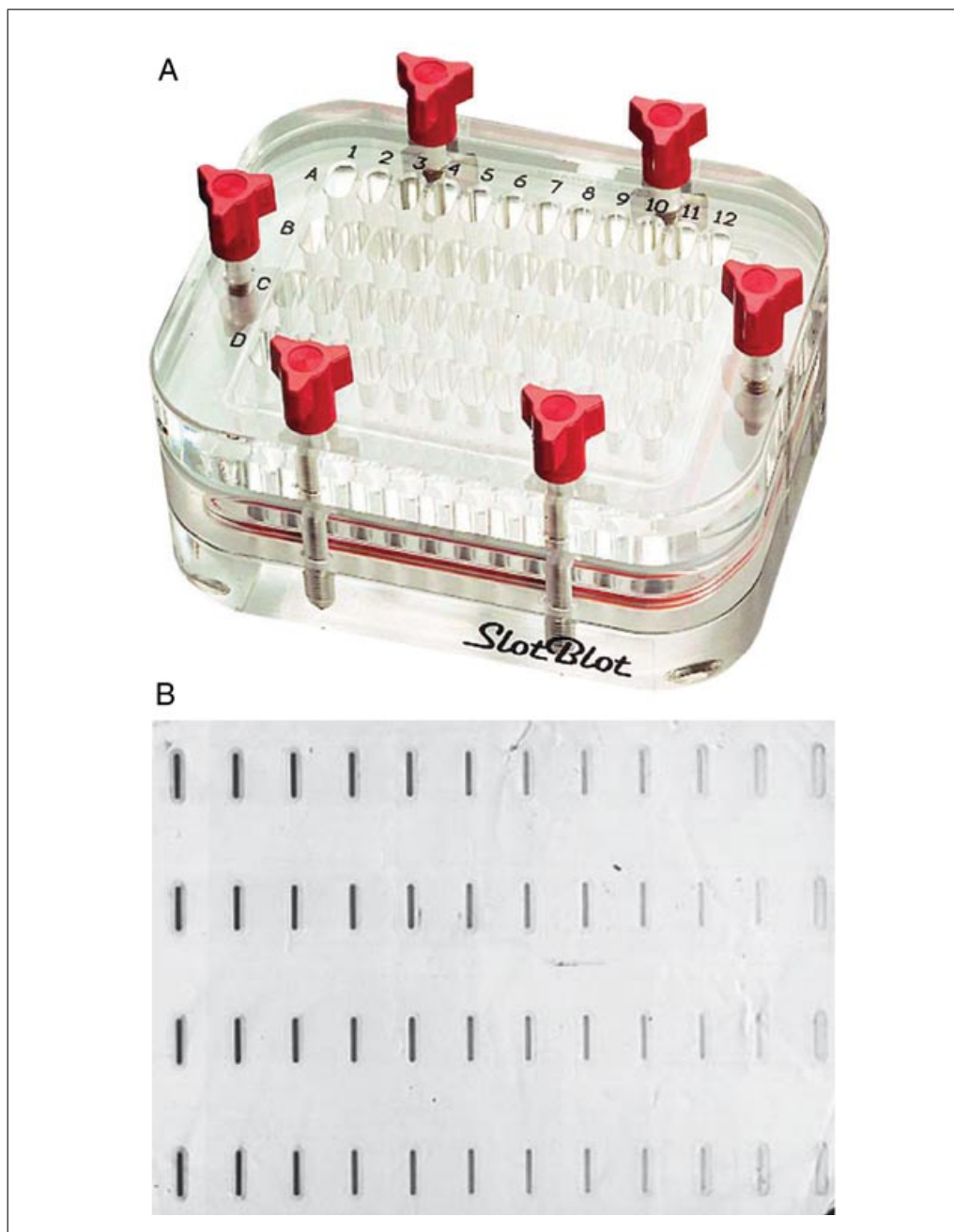


Figure 6.2.4 (A) Slot-blot unit. Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single nitrocellulose or PVDF membrane for immunoblotting. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume, routine quantitation of samples. (B) Dilution series of immunoprobed BSA developed with peroxidase-tagged secondary antibody and DAB/NiCl₂.

BLOTTING OF STAINED GELS

Gels stained with Coomassie blue R250 (UNIT 6.6) can be effectively immunoblotted by the following procedure, based on Perides et al. (1986) and Dionisi et al. (1995). Briefly, the stained gel is soaked in a series of solutions designed to increase the solubility of the proteins after staining and fixation. After transfer, the membranes are treated with 45% or 100% methanol to decrease the Coomassie blue bound to the membrane prior to processing for chromogenic development. For chemiluminescent development, removal of the Coomassie blue is generally not needed.

ALTERNATE PROTOCOL 3

Electrophoresis and Immunoblotting

6.2.9

Materials

Destained gel containing proteins of interest (UNIT 6.6)
25 mM Tris base/192 mM glycine/1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS with 6 M urea (optional)

1. Soak destained gel containing proteins of interest in distilled water for 15 min.
2. Equilibrate gel with 25 mM Tris base/192 mM glycine/1% SDS for 1 hr with gentle agitation.
3. Transfer gel to 25 mM Tris base/192 mM glycine/0.1% SDS and equilibrate 30 min with gentle agitation.
4. *Optional:* To increase transfer efficiency of larger proteins >50 kDa, transfer gel to the above solution with 6 M urea for an additional 30 min.

5. Proceed with transfer (see Basic Protocol 1, steps 2 to 12).

For the most efficient transfer and binding to the membrane, the transfer buffer should contain 0.1% (w/v) SDS.

6. After transfer, soak membranes for 10 to 30 min in 45% methanol (nitrocellulose) or 100% methanol (nylon or PVDF) to remove the bound Coomassie blue.

This step is not needed if using chemiluminescent reactions or radiolabeled protein A for immunodevelopment. Destaining of the nitrocellulose membrane is enhanced by adding a small ball of laboratory tissue to the methanol to absorb the Coomassie blue.

7. Proceed with immunoprob ing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 4 and 6).

SUPPORT PROTOCOL 1

REVERSIBLE STAINING OF TRANSFERRED PROTEINS WITH PONCEAU S

To verify transfer efficiency, nitrocellulose and PVDF membranes can be reversibly stained. This method will not work on nylon membranes.

Additional Materials (also see Basic Protocol 1)

Ponceau S solution (see recipe)
Additional reagents and equipment for photographing membranes (UNIT 6.6)

1. Following protein transfer to nitrocellulose or PVDF (see Basic Protocol 1 or Alternate Protocol 1), place membrane in Ponceau S solution for 5 min at room temperature.
2. Destain 2 min in water. Photograph membrane if required (UNIT 6.6) and mark the molecular-weight-standard band locations with indelible ink.
3. Completely destain membrane by soaking an additional 10 min in water.

SUPPORT PROTOCOL 2

QUANTITATION OF TRANSFERRED PROTEINS WITH PONCEAU S

In addition to qualitatively visualizing proteins on membranes after blotting, Ponceau S provides a convenient method for quantitating the amount of protein in a given lane. By eluting the dye off a strip of membrane and reading in a spectrophotometer (OD₅₂₅), an internal control value of protein on a lane is obtained. This value is used to correct for any differences in protein loading from lane to lane. Comparison of the Ponceau S value to the chemiluminescent or chromogenic immunodetection value determined by densitometry provides a straightforward correction for lane-to-lane variation. This method works best for complex mixtures where the immunodetected protein represents a small proportion of the total protein (Klein et al., 1995).

1. Following protein transfer to nitrocellulose, PVDF, or nylon (see Basic Protocol 1 or Alternate Protocol 1), stain membrane, photograph, and destain (see Support Protocol 1).

Membranes should be destained until the background becomes white.

2. Mark lanes with a soft pencil and cut lanes into strips.
3. Place each strip into 7 ml of distilled water for 7 min and remove the resulting solution. If any particulates are visible, centrifuge to remove them.
4. Read OD₅₂₅ in a 2-ml cuvette.

Any variation in gel-to-gel sample loading and blotting efficiency will be reflected in a change in OD of the sample lanes when compared to the control.

The change in OD can be calibrated to a known amount of protein loaded on the control lane. This will be a relative value, however, since the efficiency of transfer out of the gel and binding to the membrane is rarely 100%.

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

BASIC PROTOCOL 2

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, it is placed in a solution containing the antibody directed against the antigen (primary antibody). The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG for a rabbit primary antibody). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 3 and Alternate Protocol 5 or 6) of the enzyme bound to the membrane.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocols 1 to 3)

Blocking buffer appropriate for membrane and detection protocol (see recipes)

Primary antibody specific for protein of interest

TTBS (nitrocellulose or PVDF) or TBS (nylon; see APPENDIX 2A for recipes)

Secondary antibody conjugate: species-specific anti-Ig conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP; e.g., Cappel, Vector Labs, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25- μ l aliquots until use)

Heat-sealable plastic bag

Powder-free gloves

Plastic box

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

Usually 5 ml buffer is sufficient for two to three membranes (14 $\times$ 14-cm size). If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

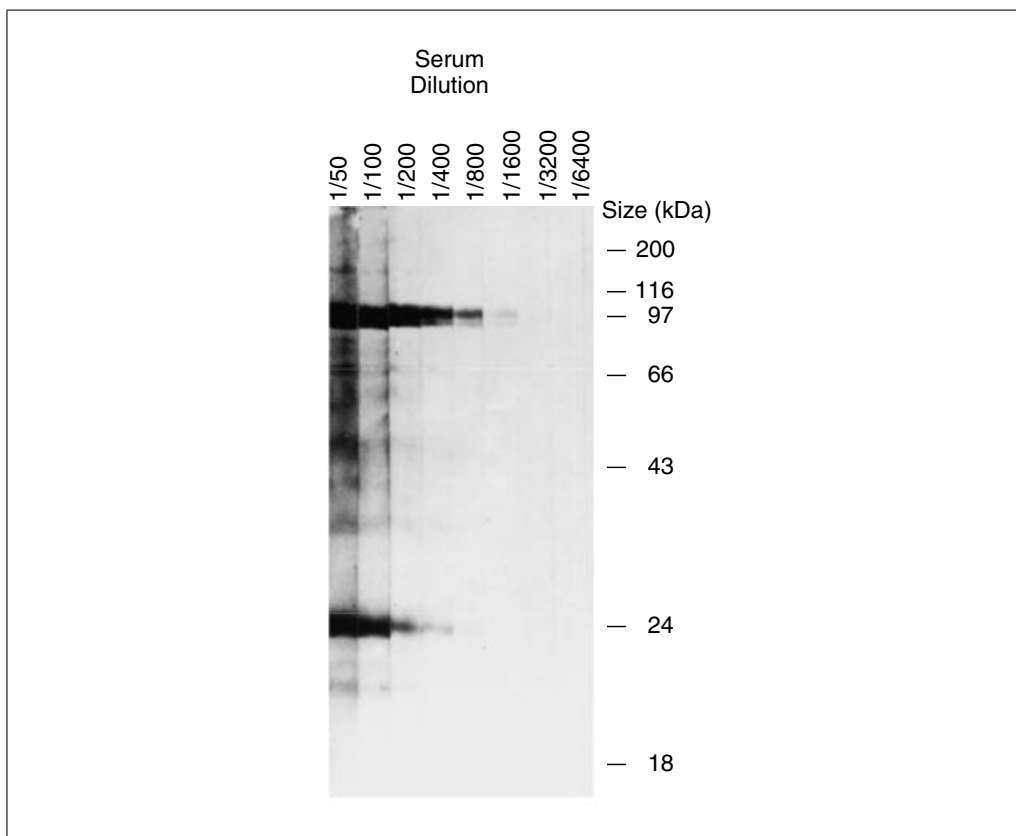


Figure 6.2.5 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. Blot was developed with HRPO-coupled avidin-biotin reagents according to Alternate Protocol 4 and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

2. Dilute primary antibody in blocking buffer.

Primary antibody dilution is determined empirically, but is typically 1/100 to 1/1000 for a polyclonal antibody, 1/10 to 1/100 for hybridoma supernatants, and $\geq 1/1000$ for murine ascites fluid. Ten- to one-hundred-fold higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate antibody concentration, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 6.2.5).

Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14-cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody conjugate in blocking buffer.

Commercially available enzyme-conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1999).

6. Place membrane in new heat-sealable plastic bag, add diluted secondary antibody conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

When using plastic incubation trays, see step 3 annotation for proper antibody volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 5 or 6).

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

ALTERNATE PROTOCOL 4

The following procedure is based on the Vectastain ABC kit from Vector Labs. It uses an avidin-biotin complex to attach the enzyme to a biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody.

Additional Materials (also see Basic Protocol 2)

Vectastain ABC (HRP) or ABC-AP (AP) kit (Vector Labs) containing the following: reagent A (avidin), reagent B (biotinylated HRP or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥ 2 hr at 37°C.

TTBS is well suited for avidin-biotin systems. For nylon, protein-binding agents are recommended. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it must be used in the blocking step only. If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. The correct dilution should give low background and high specificity (Fig. 6.2.5).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14-cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough

TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14-cm membrane.

6. Transfer membrane to fresh plastic bag containing secondary antibody solution. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

When using plastic incubation trays, see step 3 annotations for proper antibody volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRP or -AP complex. Mix two drops Vectastain reagent A and two drops reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity.

Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; Vector Labs).

8. Transfer membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.
9. Develop membrane according to the appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 5 or 6).

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

Bound antigens are typically visualized with chromogenic enzyme substrates. The substrates 4CN, DAB/NiCl₂, and TMB are commonly used with HRP, while BCIP/NBT is recommended for AP (see Table 6.2.2). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins and probed with antibody-enzyme complex (see Basic Protocol 2 or Alternate Protocol 4)

TBS (APPENDIX 2A)

Chromogenic visualization solution (Table 6.2.2)

Additional reagents and equipment for gel photography (UNIT 6.6)

1. If final membrane wash was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution.

Bands should appear in 10 to 30 min.

3. Terminate reaction by washing membrane in distilled water. Air dry and photograph (UNIT 6.6) for a permanent record.

Table 6.2.2 Chromogenic and Luminescent Visualization Systems^a

System	Reagent ^b	Reaction/detection	Comments ^c
Chromogenic			
HRP-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light
	DAB/NiCl ₂ ^d	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB ^e	Forms dark purple stain	More stable, less toxic than DAB/NiCl ₂ ; may be somewhat more sensitive ^e ; can be used with all membrane types; kits available from Kirkegaard & Perry, Invitrogen, Sigma-Aldrich, and Vector Labs
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRP-based	Luminol/H ₂ O ₂ / <i>p</i> -iodophenol	Oxidized luminol substrate gives off blue light; <i>p</i> -iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen, Lumi-Phos 530 ^f)	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting); kits available from GE Life Sciences, ABI, Pierce, Vector Labs, Beckman Coulter, Perkin Elmer.

^a Abbreviations: AMPPD or Lumigen, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-naphthol; CSPD, AMPPD with substituted chlorine moiety on adamantane ring; DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3',5,5'-tetramethylbenzidine.

^b Recipes and suppliers are listed in Reagents and Solutions except for TMP, for which use of a kit is recommended.

^c See Commentary for further details.

^d DAB/NiCl₂ can be used without the nickel enhancement, but it is much less sensitive.

^e McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.

^f Lumi-Phos 530 contains dioxetane phosphate, MgCl₂, CTAB, and fluorescent enhancer in a pH 9.6 buffer.

VISUALIZATION WITH LUMINESCENT SUBSTRATES

Bound antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for HRP or dioxetane phosphate for AP (Table 6.2.2). Film contact exposure or CCD-based digital imaging is used to capture the chemiluminescent signal. Exposures range from a few seconds to several hours, although typically signals appear within a few seconds or minutes.

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Additional Materials (also see Basic Protocol 3)

Luminescent substrate buffer: 50 mM Tris-Cl, pH 7.5 (for HRP; APPENDIX 2A) *or* dioxetane phosphate substrate buffer (for alkaline phosphatase; see recipe)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Applied Biosystems) in dioxetane phosphate substrate buffer, prepared just before use
Luminescent visualization solution (Table 6.2.2)
Clear plastic wrap
Additional reagents and equipment for autoradiography

NOTE: See Troubleshooting section for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with 50 ml substrate buffer.
For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.
2. *For AP reactions using nitrocellulose or PVDF membranes:* Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer (volumes as in step 1).

Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen concentrate. It is required for nitrocellulose and recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRP reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to visualization solution (volumes as in step 1). Soak 30 sec (HRP) to 5 min (AP).

Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step .

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure and quickly place against film (step 5a) or in CCD imaging system (step 5b).

To insure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

- 5a. *For film:* In a darkroom, place membrane face down onto film and expose film for a few seconds to several hours.

Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that insures a tight fit.

Typically, immunoblots produce very strong signals within a few seconds or minutes. Weak signals may require several hours to overnight exposure (see Troubleshooting).

- 5b. *For CCD imaging:* Place the membrane in the imager, focus, and close the door. Preview the exposure with a high pixel binning (4×4 to 8×8). Once satisfied with the preview image, capture at 1×1 (highest resolution) to 4×4 (lower resolution, but faster more sensitive capture).

Exposures with cooled CCD cameras fitted with low-light optics are rarely longer than 10 min. Once captured, archive the image for later analysis (Fig. 6.2.6; Medberry et al., 2004).

6. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Basic Protocol 3).

Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First probe membrane

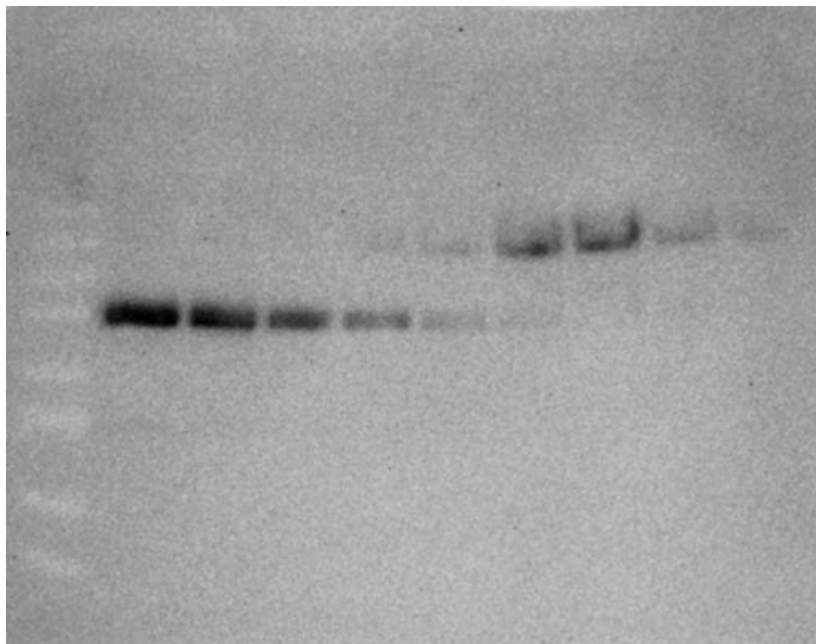


Figure 6.2.6 Chemiluminescent blot captured with a 5-min exposure using a 1-megapixel cooled (-100°C) CCD scientific camera. In this case the reaction had decayed to a point where film was not able to record an image, while the CCD camera shows the dilution series. The blot, a two-fold IgG dilution series from 500 to 2 ng, was probed using peroxidase-conjugated affinity-purified sheep anti-mouse IgG (H&L; Rockland Immunochemicals) and visualized with luminol.

with the chemiluminescent reactions to record on film. If stripping and reprobing are needed, process by wetting and NaOH treatment (see Support Protocol 3). For the last reaction, use chromogenic development to produce a permanent visual record of the blot. Alternatively, once the film record of the chemiluminescent blot is recorded, the blot can be rinsed briefly with distilled water and placed in the appropriate chromogenic solution for chromogenic development of the blot. This results in a permanent reference stain on the blot for comparison to the more easily scanned and quantitated film record.

SIGNAL AMPLIFICATION WITH AVIDIN-BIOTIN VISUALIZATION REAGENTS

Specialized kits are available that provide significant signal amplification and can accommodate the chromogenic, chemiluminescent, or fluorescent development of a substrate. The Vectastain ABC-AmP Western Blotting Immunodetection Kit (Vector Labs) features a preformed complex between streptavidin and biotinylated alkaline phosphatase. The substrate is either the chromogenic substrate BCIP/NBT, which produces a blue-purple precipitate, or a chemiluminescent/fluorescent substrate, DuoLux, that provides very high sensitivity and has sustained light-emission characteristics. Blots can be exposed to film several times over an 8-hr period for the optimization of band intensities or resolution. In addition to providing an amplified signal, this approach produces a very low background on both nitrocellulose and PVDF membranes. These kits have been developed to produce maximum sensitivity with minimal background staining.

Materials

- Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocols 1 to 3)
- Primary antibody (from mouse or rabbit) specific for protein of interest
- Vectastain ABC-AmP Chromogenic or Chemiluminescent Western Blotting Immunodetection Kit (Vector Labs) containing:

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10× casein solution (250 ml)
 Biotinylated secondary antibody (0.25 ml): anti-mouse IgG (for mouse primary antibodies) *or* anti-rabbit IgG (for rabbit primary antibodies)
 Reagents A and B (0.5 ml each)
 Substrate: chemiluminescent/fluorescent substrate (DuoLux; 100 ml) or BCIP/NBT chromogenic substrate kit (stock reagents for 200 ml)
 PBS: 10 mM sodium phosphate buffer, pH 7.5 (APPENDIX 2A) containing 150 mM NaCl
 0.1 M Tris·Cl, pH 9.5 (APPENDIX 2A)
 Staining trays
 X-ray film (e.g., Kodak BioMax)
 UV transilluminator or UV imaging trans- and epi-CCD acquisition system (UVP, Inc.)

NOTE: The components supplied in each Vectastain ABC-AmP Western Blotting Immunodetection Kit provide sufficient reagents to develop approximately twenty 100-cm² blots. The volumes of the reagents in the protocol below are optimized for the development of a 100-cm² membrane. Volumes may be proportionally adjusted for blots of a different size. All kit reagents may be used immediately following dilution. For optimal results, it is recommended that all diluted reagents from the kit be used the same day that they are prepared. Vectastain ABC-AmP Kit stock reagents should be stored under refrigeration and kept in the box in which they are supplied.

Block membrane

1. For a 100-cm² (10 × 10-cm) blot, prepare 120 ml of 1× casein solution by adding 12 ml of 10× casein solution (provided with Vectastain kit) to 108 ml distilled water. Block membrane by incubating in 10 ml of 1× casein solution for 5 min at room temperature with gentle agitation. Save remaining 1× casein to be used in subsequent steps.

In rare cases, some nonspecific bands may develop. This can usually be eliminated by increasing the concentration of NaCl to 0.3 to 0.5 M in the Vectastain ABC-AmP reagent (see step 5, below). Some enzymes isolated from tissues may have covalently attached biotin as a cofactor. If high salt does not prevent the Vectastain ABC-AmP reagent from binding to particular bands, use an avidin/biotin blocking step (Vector Laboratories, cat. No. SP-2001) between steps 1 and 2 in this protocol.

2. Incubate the membrane for 30 min (or for a time established to be optimal for the concentration of primary antibody used) with gentle agitation at room temperature in an appropriate concentration of primary antibody diluted in PBS.
3. Wash the membrane in 10 ml of 1× casein solution three times, each time for 4 min at room temperature with gentle agitation.
4. Prepare the biotinylated secondary antibody solution by adding 10 µl of biotinylated anti-mouse IgG or anti-rabbit IgG to 10 ml of 1× casein solution (final concentration 1.5 µg/ml). Incubate membrane 30 min at room temperature with gentle agitation in 10 ml of the biotinylated secondary antibody solution. At end of incubation, wash in 1× casein solution as in step 3.
5. Prepare the Vectastain ABC-AmP reagent by adding 20 µl reagent A and 20 µl reagent B to 10 ml of 1× casein solution. Incubate the membrane in 10 ml reagent for 10 min at room temperature with gentle agitation. At end of incubation, wash with 1× casein solution as in step 3.

Incubate membrane in substrate solution

It is recommended that the membrane be transferred to a different staining tray for the substrate development step.

To detect chemiluminescent and/or fluorescent signal

- 6a. Equilibrate membrane for 5 min in 0.1 M Tris·Cl, pH 9.5. Remove excess buffer by holding membrane vertically and touching edge of membrane to absorbent paper.
- 7a. Place membrane target-side-up on plastic wrap on a level surface. Pipet 5 ml of substrate on to membrane surface. Incubate 5 min under subdued light or in dark.
- 8a. Rinse membrane in 0.1 M Tris·Cl, pH 9.5, for a few seconds, then remove excess buffer by holding membrane vertically and touching edge of membrane to absorbent paper.

If necessary, background may be further reduced by washing membrane in 0.1 M Tris·Cl, pH 9.5, for 5 min at room temperature and removing excess buffer before exposure to film.
- 9a. Place membrane between two pieces of acetate, plastic wrap, or clear sheet protector. Expose membrane to X-ray film (e.g., KODAK BioMax film) for the appropriate time.
- 10a. *Optional:* To obtain fluorescent signal, following step 9a, wash membrane in 0.1 M Tris·Cl, pH 9.5, for 5 min at room temperature. Remove excess buffer. Place membrane target-side-up on a UV transilluminator (254 to 365 nm) or UV imaging trans- and epi-CCD acquisition system, and observe results.

To detect chromogenic signal

- 6b. Equilibrate membrane for 5 min in 0.1 M Tris·Cl, pH 9.5.
- 7b. Prepare chromogenic reagent by adding 4 drops of reagent from each of the three dropper bottles in the BCIP/NBT substrate kit into 10 ml of 0.1 M Tris·Cl, pH 9.5. Incubate membrane in the substrate solution at room temperature with gentle agitation until the appropriate density of colored bands develops.

Incubation times may vary from 30 min to several hours.
- 8b. Briefly rinse the membrane in PBS and air dry. Store blot protected from light.

STRIPPING AND REUSING MEMBRANES

This stripping procedure works with blotted membranes from one- and two-dimensional gels, including previously stained gels (Suck and Krupinska, 1996). Reprobing PVDF membranes that have been developed with chemiluminescent reagents is simple and straightforward. All residual antibodies are removed from the membrane by first rewetting it in water and then briefly treating it with NaOH. Although repeated reprobing can lead to loss of signal, up to five reprobing is generally feasible. The blot should have been blocked with 5% nonfat dry milk prior to treatment.

Chromogenic development leaves a permanent stain on the membrane that is difficult to remove, and should not be used when reprobing. The stain can interfere with subsequent analysis if reactive bands from sequential immunostainings are close together.

Materials

0.2 M NaOH

1. Wash blot 5 min in distilled water.

In order to effectively reprobe the membranes, casein (for AP systems) or nonfat dry milk must be used as the blocking agent.

2. Transfer to 0.2 M NaOH and wash 5 min.

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3. Wash blot 5 min in distilled water.
4. Proceed with immunoprob ing procedure (see Basic Protocol 2 and Alternate Protocol 4).

Casein or nonfat dry milk is recommended as blocking agent when reprobing membranes.

REAGENTS AND SOLUTIONS

Deionized, distilled water should be used to prepare all solutions. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX. For selection of appropriate chromogenic or luminescent solutions, and for definition of abbreviations, see Table 6.2.2.

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5 (APPENDIX 2A)
 100 mM NaCl
 5 mM MgCl₂

BCIP/NBT visualization solution

NBT stock: 50 mg/ml nitroblue tetrazolium in 70% *N,N*-dimethylformamide (DMF). Store <1 year at 4°C.

BCIP stock: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100% DMF. Store <1 year at 4°C.

Mix 33 µl NBT stock and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 µl BCIP stock and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1999). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, and Vector Labs.

Blocking buffer

For colorimetric detection:

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in Tris-buffered saline (TTBS; APPENDIX 2A). Store up to 1 week at 4°C.

For neutral and positively charged nylon: Tris-buffered saline (TBS; APPENDIX 2A) containing 10% (w/v) nonfat dry milk. Prepare just before use.

For luminescent detection:

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): With constant mixing, add 0.2% casein (e.g., Hammarsten grade or I-Block; Applied Biosystems) to warm (65°C) TTBS (APPENDIX 2A). Stir for 5 min. Cool before use. Prepare just before use.

For positively charged nylon: With constant mixing, add 6% (w/v) casein and 1% (v/v) polyvinyl pyrrolidone (PVP) to warm (65°C) TTBS (APPENDIX 2A). Stir for 5 min. Cool before use. Prepare just before use.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN). Separately, mix 60 µl of 30% H₂O₂ with 100 ml TBS (APPENDIX 2A) at room temperature. Rapidly mix the two solutions and use immediately.

DAB/NiCl₂ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
 100 µl 3,3'-diaminobenzidine (DAB) stock (40 mg/ml in H₂O, stored in 100-µl aliquots at -20°C)
 25 µl NiCl₂ stock (80 mg/ml in H₂O, stored in 100-µl aliquots at -20°C)

continued

15 μ l 3% H_2O_2
Mix just before use

CAUTION: Handle DAB carefully, wearing gloves and mask, as it is a carcinogen.

Suppliers of peroxidase substrates are Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Dioxetane phosphate substrate buffer

1 mM MgCl_2
0.1 M diethanolamine
0.02% sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Traditionally, the AMPPD substrate buffer has been a solution containing 1 mM MgCl_2 and 50 mM sodium carbonate/bicarbonate, pH 9.6 (Gillespie and Hudspeth, 1991). The use of diethanolamine results in better light output (Applied Biosystems Western Light instructions).

Alternatively, 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/5 mM MgCl_2 can be used (Sandhu et al., 1991).

Dioxetane phosphate visualization solution

Prepare 0.1 mg/ml AMPPD or CSPD (Applied Biosystems) or Lumigen (see Table 6.2.2) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use.

This concentration (240 μM) of AMPPD substrate is the minimum recommended by Applied Biosystems Western Light. Ten-fold lower concentrations can be used but require longer exposures.

Lumi-Phos 530 (Beckman Coulter; Lumigen, Perkin Elmer) is a ready-to-use solution and can be applied directly to the membrane.

Luminol visualization solution

0.5 ml 10 $\times$ luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10 $\times$ *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
25 μ l 3% H_2O_2
 H_2O to 5 ml
Prepare just before use

*Recipe is from Schneppenheim et al. (1991). Alternative recipes are also available (Haan and Behrmann, 2007). Premixed luminol substrate mix (Amersham ECL; Perkin Elmer; Kirkegaard & Perry LumiGLO) may also be used. *p*-Iodophenol is an optional enhancing agent that increases light output. Luminol and *p*-iodophenol stocks can be stored for ≤ 6 months at -20°C .*

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. The pH of the solution is ~ 8.3 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

CAPS transfer buffer can also be used. Add 2.21 g cyclohexylaminopropane sulfonic acid (CAPS; free acid), 0.5 g DTT, 150 ml methanol, and water to 1 liter. Adjust to pH 10.5 with NaOH and chill to 4°C . For proteins > 60 kDa, reduce methanol content to 1% (Moss, 2001).

COMMENTARY

Background Information

Immunoblotting

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal. However, there are several problems inherent in this method, including the requirement for radiolabeling of antigen, co-precipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979; Kurien and Scofield, 2006)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE (UNIT 6.1) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon. This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive (1 ng of antigen can be detected).

Protein blotting has many important research and clinical applications. For example, it is the confirmatory test for human immunodeficiency virus Type 1 (HIV-1). SDS-PAGE-separated virus proteins are blotted onto nitrocellulose and processed with patient sera. After washing and probing with an anti-human IgG coupled to HRP or alkaline phosphatase, the antigen-antibody complexes are identified by chromogenic development, indicating the presence of anti-HIV antibodies in the serum. For other selected applications, see Table 6.2.3.

Electroblotting of previously stained gels is a convenient way to visualize and document the gel prior to immunoblotting. Transfer efficiencies at all molecular weights will be lower with fixed and stained gels. This is particularly true of proteins >50 kDa (Perides et al., 1986). The additional incubation in 6 M urea will significantly increase transfer efficiency of all proteins and is required for proteins >50 kDa.

Ponceau S staining provides an easy method for calibrating and quantitating the amount of material on a nitrocellulose or

PVDF blot. An alternative to this method is to use an internal protein control with a separate antibody probe, but these tend to be expensive and time-consuming to use. Other applications for Ponceau S calibration include monitoring transfer efficiency under varied conditions for optimization of tank and semidry blotting.

Desired antigens are probed using indirect immunochemistry with secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). Signal amplification can be achieved by adding a biotin-avidin step using Vectastain ABC kits (Vector Labs). In Alternate Protocol 4, avidin acts as a bridge between biotinylated secondary antibody and biotinylated enzyme (HRP or AP). Sensitivity is improved because the multiple avidin-biotin binding sites increase the amount of enzyme per molecule of secondary antibody. Higher sensitivity is achieved using the Vectastain ABC-Amp kit (Alternate Protocol 6), which uses a preformed complex of streptavidin and biotinylated alkaline phosphatase.

Detection methods

Immunoblotted proteins can be detected using chromogenic or luminescent enzyme substrates (see Basic Protocol 3 and Alternate Protocol 5; see Table 6.2.2 for comparison). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRP and phosphatase systems without the need for radioisotopes (for alkaline phosphatase, see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely go more than 10 min. Depending on the system, the luminescence can last for several hours, permitting multiple exposures of the same blot.

AP-based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted. The procedure described in Alternate Protocol 5 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Alternate Protocol 6 describes an amplification protocol that can be used in chromogenic, chemiluminescent, or fluorescent detection schemes. Chemiluminescent and fluorescent detection are achieved using Vectastain's

Table 6.2.3 Applications of Immunoblotting

Application	Comment
Antibody development and characterization	Characterize antibody specificity
Subcellular localization of an expressed protein	Analyze isolated organelles (e.g., Golgi apparatus, plasma membrane, nucleus) for presence of specific proteins; probe intact cells and tissue for subcellular localization using visible light, fluorescence, and electron microscopy
Protein purification	Demonstrate enrichment of well-characterized antibody by sampling, electrophoresing, and blotting at various stages of purification
Diagnostics	Separate and blot a viral lysate to test serum for antibodies that bind key viral proteins, indicating presence of antibodies against an infection (e.g., HIV testing)
Gene expression	Detect presence or absence and amount of a specific protein during gene expression; track markers and reporter genes (e.g., luciferase) in transgenic organisms to get a complete picture of transcription and translation
Post-translational modifications	Phosphoproteomics: determine phosphorylation status of the protein complement in the cell or tissue using antibodies specific for phospho amino acids
Protein sequencing by mass spectrometry	Characterize the readily available proteins blotted onto membranes using sensitive methods, e.g., peptide sequencing by MALDI (Carr and Annan, 1996)

DuoLuX Chemiluminescent/Fluorescent Substrate, a novel acridan-based substrate that can be used not only in immunoblots, as described here, but also in nucleic acid detection methods (e.g., Southern and northern blotting, colony lifts, ELISAs). The substrate is available for either AP or HRP development. Generally, signal development is faster when using HRP. Thus, HRP may be preferred when digital imaging systems are used or when abundance of target reduces the need for a high signal-to-noise ratio. However, for applications where optimal sensitivity is required, AP is recommended, as it will provide a higher signal-to-noise ratio than HRP.

The DuoLuX Chemiluminescent/Fluorescent Substrate has very high sensitivity and prolonged light-emission characteristics. This enables image documentation with either film or digital imaging systems (see below). Unlike some chemiluminescent substrates, blots can be re-exposed to film as often as necessary over many hours. Because many digital imaging systems require a longer exposure time than film, the faster signal development of HRP relative to AP may be preferred when using these systems. PVDF, nitrocellulose, or nylon membranes can be used, although the chemiluminescent signal develops faster on nylon and PVDF.

In addition to its chemiluminescent properties, the reaction product is also fluorescent. Fluorescence can be recorded with a digital imaging system or a conventional camera months after chemiluminescence has faded. For fluorescence detection, nitrocellulose is recommended due to the intrinsic fluorescence of nylon. Acquisition of fluorescent signal requires a much shorter exposure time than chemiluminescence, often a fraction of a second.

Imaging methods

Film contact exposure or CCD-based digital imaging can be used to capture the signal from chemiluminescent blots. Film-based capture is inexpensive to get started and requires very little in the way of equipment: film, processing chemicals, and cassettes that hold the blot close to the film. Film produces a physical record that is robust, and the film can be scanned for quantitation. However, in spite of the high initial cost, CCD-based imaging hardware has a number of advantages over film, including speed, ease of use, and data quality. First, CCD cameras with appropriate optics, cooled to minimize thermal noise, are much more sensitive than film (Fig. 6.2.6). Second, CCD image capture requires many fewer steps. The camera's preview function gives an

accurate estimate of exposure time for capture of the image. Trial exposures and developing are not needed as with film. Finally, CCD capture yields higher-quality data with a wider linear range compared to film, and digital files can be automatically date stamped and archived on backup systems.

Scientific CCD cameras, cooled from ambient temperature to -20°C to -100°C , are needed for the range of exposures required for chemiluminescent imaging. Cooling lowers the dark noise on the CCD sensor, so that longer exposures (up to 60 min or more) are possible without a significant increase in background noise (Medberry et al., 2004).

In addition to cooling, CCD binning, where groups of pixels are imaged at one time, is important for high-sensitivity low-light imaging. By grouping pixels into a single large pixel, signal is collected much more quickly, giving a higher signal-to-noise ratio (Medberry et al., 2004). The disadvantage of this technique is that the output resolution is also reduced. By using a typical configuration of 4×4 binning, 16 pixels on the sensor become effectively one, lowering output resolution.

Successful digital imaging of chemiluminescent immunoblots depends not only on the camera sensor and cooling, but also on the optics, which determine how much light is collected and imaged onto the CCD sensor. The standard designation of light-gathering capacity in the lens is the f number or f -stop. Typical f -stops range from $f/22$, letting in the least light, to $f/1.2$ or lower, letting in the most light (aperture blades wide open). Lenses designed for low-light imaging typically have designations at or below $f/1.2$.

Image quantitation is accomplished with image analysis software. Internal standards can be added to the separation (a probe or protein of known amount) to serve as a relative reference. Luminescent blots can be easily erased and reprobed because the reaction products are soluble and do not deposit on the membrane. Compared to chromogenic development, the luminescent image is easier to photograph and to quantitate by densitometry.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and primary antibody and conjugate dilutions should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1999). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C . Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink. However, even with the application of such standard blocking procedures as 5% to 10% milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. If a secondary antibody detection is used, the blocking protein (i.e., normal serum) should be from the same species as the secondary antibody. Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing Immobilon-P membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a ten-fold increase in the primary antibody (serum) concentration to achieve an adequate background-free antigen signal.

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents, optimizing antibody concentrations, letting the reaction proceed for several minutes before imaging, or simply limiting the exposure time of the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents ten-fold. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species.

Currently, due to a combination of low background and ease of processing, PVDF and nitrocellulose membranes are the most

commonly used membranes for protein blotting. Nylon membranes, typically used for nucleic acid blotting applications, are robust and can be used for protein blotting as well. However, nylon requires more stringent blocking to minimize background, limiting its popularity for protein blotting applications. Two types of nylon membrane are used for protein transfer—neutral (e.g., Pall Biotodyne A) and positively charged (e.g., Pall Biotodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to give a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes have been popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% nonfat dry milk in TBS is recommended for chromogenic development. During luminescence development, however, background is a more significant problem. Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP). Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are done without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use. Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

If the primary procedure is problematic due to loss of sensitivity or an increase in background, two possible alternative procedures for stripping membranes are recommended. The first uses 2-mercaptoethanol and SDS (Kaufmann et al., 1987; Tesfagzi et al., 1994). Briefly, the membranes are incubated in 2% SDS/100 mM Tris-Cl, pH 7.4/100 mM 2-mercaptoethanol for 30 min at 70°C, effectively removing primary and secondary anti-

bodies. As with the primary procedure recommended above, repeated probing should be done with caution due to the potential loss of detection signal, and 5% nonfat dry milk is required as a blocking agent. The milk blocking agent facilitates antibody removal from the blot (Kaufmann et al., 1987). The second uses guanidine-HCl. For nylon and PVDF membranes (do *not* use with nitrocellulose), incubate the immunoblot in 7 M guanidine-HCl for 10 min at room temperature. (The short wash time is critical, as guanidine-HCl is a very strong denaturant, so do not leave the filter in this solution >15 min.) Pour off excess guanidine-HCl and then rinse the membrane several times in 1× TTBS. Reblock the membrane and proceed with the standard immunoblotting procedure. Membranes stripped using this procedure can generally be reused three or four times.

In Alternate Protocol 6, extensive washing will reduce signal strength, so the wash time should not be extended unless high background is observed. If background is excessive, use a wash time of 5 to 10 min in step 8a (may require optimization). The long emission lifetime of the substrate allows the blot to be re-exposed until an optimal signal-to-noise ratio is achieved. Blotting can be done onto either nylon or nitrocellulose. Nylon requires shorter exposure times, and is therefore preferred for chemiluminescent applications. However, because of nylon's intrinsic fluorescence, nitrocellulose is preferred for fluorescence detection.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents (e.g., Zwittergent 3-14 from EMD Chemicals, <http://www.emdbiosciences.com>) have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can also be performed.

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and

discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS to the transfer buffer at a concentration of 0.1% improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kDa in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers (Pluskal et al., 1986). SDS and methanol should not be used in the transfer buffer for nylon (Peluso and Rosenberg, 1987).

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette.

Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

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

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 6.2.5).

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse image on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the alkaline phosphatase substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Applied Biosystems Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% casein, 1% polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN₃, 10 mM EDTA, and PBS, pH 6.8. Prior to use, the casein must be heated to 65°C to reduce alkaline phosphatase activity in the casein itself. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the

sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., using patient sera against viral proteins in AIDS western blot test), multiple bands will be visualized.

For immunoblot or protein dot blot chemiluminescent applications, the sensitivity using HRP is ~1 pg of target protein. For chemiluminescent blots, DuoLuX Chemiluminescent/Fluorescent Substrate (see Alternate Protocol 6) can be used on either nitrocellulose or PVDF membranes.

Time Considerations

The entire immunoblotting 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 (high-power transfer) to overnight. Blocking, antibody probing, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence).

For the DuoLuX Chemiluminescent/Fluorescent Substrate (see Alternate Protocol 6), optimal exposure times can vary from 5 sec to 15 min. It is recommended that initial exposures be taken between 1 and 5 min. Band intensities or resolution can then be optimized by lengthening or shortening exposure times based on the initial results.

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