Immunoblot Detection

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 in selected cases with reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME); some antibody epitopes are destroyed if reducing conditions are used. Following solubilization, the material is separated by SDS-PAGE (using either one- or two-dimensional gels; *UNIT B3.1*). The antigens are then electrophoretically transferred in a tank or a semidry electroblotting unit to a nitrocellulose, polyvinylidene difluoride (PVDF), or nylon membrane (*UNIT B3.2*). When nitrocellulose or PVDF membranes are used, the process can be monitored by a reversible staining procedure with Ponceau S (*UNIT B3.3*). After staining, protein bands on the membrane can be photographed and/or the positions of the detected proteins can be marked with indelible ink (e.g., Paper-Mate pen). The membrane is then completely destained by soaking in water for an additional 10 min.

At this point the transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After being probed with primary antibody, the membrane is washed and the antibody-antigen complexes are identified using horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes coupled to the secondary anti-immunoglobulin-G (anti-IgG) antibody (e.g., goat anti-rabbit IgG). The enzymes are attached directly (Basic Protocol) or via an avidin-biotin bridge (Alternate Protocol) to the secondary antibody. Chromogenic or luminescent substrates (Support Protocols 1 and 2) are then used to visualize the activity.

BASIC PROTOCOL

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

After electrophoretic transfer to the membrane (*UNIT B3.2*), the immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is first immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, the membrane is placed in a solution containing an antibody directed against the antigen (primary antibody). The blot is washed and then exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Support Protocols 1 and 2) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is a common alternative to protein blocking agents for use with nitrocellulose or PVDF filters.

Materials

Membrane with transferred proteins (*UNIT B3.2*)
Blocking buffer (see recipe) appropriate for membrane and detection protocol
Primary antibody specific for protein of interest
TTBS (nitrocellulose or PVDF) *or* TBS (neutral or positively charged nylon; see recipes for both solutions)
Secondary antibody conjugate: horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-anti-Ig (Cappel, Vector, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25-µl aliquots until use)

Contributed by Sean Gallagher
Current Protocols in Food Analytical Chemistry (2001) B3.4.1-B3.4.11
Copyright © 2001 by John Wiley & Sons, Inc.

Heat-sealable plastic bags
Powder-free gloves
Plastic box

Additional reagents and equipment for chromogenic or luminescent visualization (see Support Protocol 1 or see Support Protocol 2)

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 × 14–cm size).

   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. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. B3.4.1; Cooper and Paterson, 1995; Andrew and Titus, 1991a,b,c, 1993), 1/10 to 1/100 for hybridoma supernatants (Yokoyama, 1991a), and ≥1/1000 for murine ascites fluid containing monoclonal antibodies (Yokoyama, 1991b). Ten- to one-hundred-fold higher dilutions can be used with alkaline phosphatase– or luminescence–based detection systems. 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.

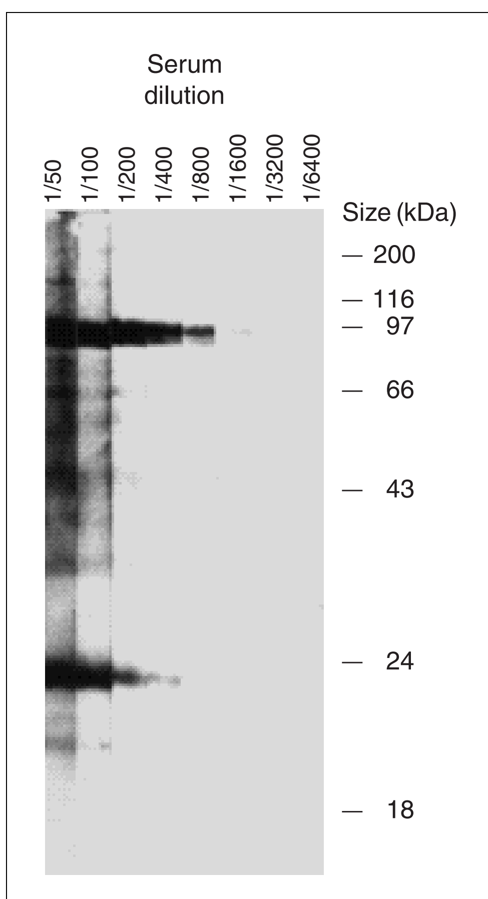

Figure B3.4.1 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. The blot was developed with HRP-coupled avidin-biotin reagents according to the Alternate Protocol and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

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 4 times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody HRP- or AP-anti-Ig conjugate in blocking buffer.

   Commercially available enzyme-conjugated secondary antibody is usually diluted 1/200 to 1/2000 (i.e., 20 µl/ml to 2 µl/ml) prior to use (Harlow and Lane, 1988).

6. Place membrane in fresh heat-sealable plastic bag, add diluted HRP- or AP-anti-Ig 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 solution volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Support Protocol 1 or see Support Protocol 2).

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

ALTERNATE PROTOCOL

The following procedure is based on the Vectastain ABC kit from Vector (SUPPLIERS APPENDIX). It uses an avidin-biotin complex to attach horseradish peroxidase (HRP) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity because multiple reporter enzymes are bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein blocking agents when using nitrocellulose or PVDF membranes.

Additional Materials *(also see Basic Protocol)*

Vectastain ABC (HRP) *or* ABC-AP (AP) kit (Vector) 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 that will interfere with the immunoassay, its use must be restricted to the blocking step only.

   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 1/10,000. 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. To determine the appropriate concentration of primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., with a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher & Schuell or 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. B3.4.1).

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

   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 3 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 2 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 annotation for proper antibody solution volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRP or -AP complex. Mix 2 drops Vectastain reagent A and 2 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. Azide is a peroxidase inhibitor and should not be used as a preservative for long-term storage of the antibody solution. Casein, nonfat dry milk, serum, and some grades of bovine serum albumin (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; see also instructions from Vector).

8. Transfer membrane to fresh plastic bag containing avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.

   Hybridization in a plastic bag requires 5 to 10 ml avidin-biotin-enzyme solution. Membrane strips require 5 to 10 ml/strip, whereas blots from standard-sized gels (i.e., 14 × 16 cm) require 50 ml for convenient handling in a tray.

9. Develop membrane according to the appropriate visualization protocol (see Support Protocol 1 or see Support Protocol 2).

SUPPORT PROTOCOL 1

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

After incubation with primary and secondary antibody conjugates (see Basic Protocol or see Alternate Protocol), bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with horseradish peroxidase (HRP)–based immunodetection procedures, whereas BCIP/NBT is recommended for alkaline phosphatase (AP)–based procedures (see Table B3.4.1). 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 or see Alternate Protocol)
TBS (see recipe)
Chromogenic visualization solution (Table B3.4.1; see recipes)

Additional reagents and equipment for gel photography

1. If final membrane wash (see Basic Protocol, step 7, or see Alternate Protocol, step 9) was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

   The Tween 20 in the 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 for a permanent record.

Table B3.4.1 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 on exposure to light
	$DAB/NiCl_2$[d]	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane is easily scanned
	TMB[e]	Forms dark purple stain	More stable and less toxic than $DAB/NiCl_2$; may be somewhat more sensitive[e]; can be used with all membrane types
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_2O_2/ *p*-iodophenol	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction is detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane phosphates (e.g., AMPPD, CSPD, Lumigen-PPD, 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)

[a]Abbreviations: AMPPD or Lumigen-PPD, 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-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.

[b]Recipes and suppliers for all reagents except TMB are listed in Reagents and Solutions. Kits containing TMB are available from Kirkegaard & Perry, TSI Center for Diagnostic Products, and Vector.

[c]See Commentary for further details.

[d]$DAB/NiCl_2$ can be used without the nickel enhancement, but sensitivity is greatly reduced.

[e]First treating nitrocellulose filters with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0) causes TMB to precipitate onto the membrane with a sensitivity much greater than that seen for 4CN or DAB and equal to or better than that for BCIP/NBT (McKimm-Breschkin, 1990).

[f]Lumi-Phos 530 contains dioxetane, $MgCl_2$, cetyltrimethylammonium bromide (CTAB), and fluorescent enhancer in a pH 9.6 buffer.

SUPPORT PROTOCOL 2

VISUALIZATION WITH LUMINESCENT SUBSTRATES

After incubation with primary and secondary antibody conjugates (see Basic Protocol and see Alternate Protocol), antigens can also be visualized with luminescent substrates. Detection with light offers both greater speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRP) systems, or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap and placed firmly against film. Exposures range from a few seconds to several hours, although typically strong signals appear within a few seconds or minutes.

Additional Materials *(also see Support Protocol 1)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (APPENDIX 2A; HRP), *or* dioxetane phosphate substrate buffer (see recipe; AP)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Applied Biosystems) in dioxetane phosphate substrate buffer (see recipe), prepared just before use
Luminescent visualization solution (Table B3.4.1; see recipes)
Clear plastic wrap

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

1. Equilibrate membrane in two 15-min washes with substrate buffer each.

   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.

   For blots of whole gels, use 50 ml Nitro-Block solution and substrate buffer; for strips, use 5 to 10 ml/strip.

   Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen-PPD 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–based reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to luminescent visualization solution. Soak 30 sec (HRP reactions) to 5 min (AP reactions).

   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 5.

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane and seal with tape to form a liquid-tight enclosure.

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

5. In a darkroom, place membrane face down onto film.

   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 ensures a tight fit.

6. Expose film for a few seconds to several hours.

   Typically, immunoblots produce very strong signals within a few seconds or minutes; however, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr and, if needed, overnight (see Troubleshooting).

7. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Support Protocol 1).

   It is possible to develop the same membrane with chromogenic substrates after luminescent visualization.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

Blocking buffer

Colorimetric detection

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; see recipe).

For neutral and positively charged nylon: Tris-buffered saline (TBS; see recipe) containing 10% (w/v) nonfat dry milk.

TTBS can be stored up to 1 week at 4°C. Prepare blocking buffer containing nonfat dry milk immediately prior to use, as the milk blocking solution is not stable.

Luminescent detection

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): 0.2% (w/v) casein (e.g., Hammarsten grade or I-Block; Applied Biosystems) in TTBS (see recipe).

For positively charged nylon: 6% (w/v) casein/1% (v/v) polyvinylpyrrolidone (PVP) in TTBS (see recipe).

For each solution: With constant mixing, add casein and PVP to warm (65°C) TTBS. Stir for 5 min, then cool. Prepare each solution just before use.

Chromogenic visualization solutions

BCIP/NBT visualization solution: Mix 33 µl NBT stock [100 mg NBT in 2 ml 70% (v/v) dimethylformamide (DMF), stored <1 year at 4°C] and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 µl BCIP stock (100 mg BCIP in 2 ml 100% DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1988). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, and Vector (SUPPLIERS APPENDIX).

4CN visualization solution: Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN). Separately mix 60 µl of 30% (w/v) H_2O_2 with 100 ml TBS (see recipe) at room temperature. Rapidly mix the two solutions and use immediately.

$DAB/NiCl_2$ visualization solution:
5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 µl DAB stock (40 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
15 µl 3% (w/v) H_2O_2
Mix just before use

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

Suppliers of chromogenic HRP substrates (4CN and $DAB/NiCl_2$) are Sigma, Kirkegaard & Perry, Moss, and Vector (SUPPLIERS APPENDIX). For selection of appropriate chromogenic solutions, and for definition of abbreviations, see Table B3.4.1.

Dioxetane phosphate substrate buffer

1 mM $MgCl_2$
0.1 M diethanolamine
0.02% (w/v) 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 (Western Light instructions; Applied Biosystems).

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

Luminescent visualization solutions

Dioxetane phosphate visualization solution: Prepare 0.1 mg/ml AMPPD or CSPD (Applied Biosystems) *or* 0.1 mg/ml Lumigen-PPD (Lumigen) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use. Lumi-Phos 530 (Boehringer Mannheim or Lumigen) is a ready-to-use solution and can be applied directly to the membrane.

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

Luminol visualization solution:

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml dimethyl sulfoxide (DMSO)]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
25 µl 3% (w/v) H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). Premixed luminol substrate mix (Mast Immunosystems, Amersham, Du Pont NEN Renaissance, or Kirkegaard & Perry Lumi-GLO) may also be used. For selection of appropriate luminescent solutions, and for definition of abbreviations, see Table B3.4.1.

p-Iodophenol is an optional enhancing agent that increases light output. Luminol and p-iodophenol stocks can be stored ≤6 months at −20°C.

Tris-buffered saline (TBS)

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.9% (w/v) NaCl
Store up to several months at 4°C

Tween 20/TBS (TTBS)

0.1% (v/v) Tween 20 in Tris-buffered saline (TBS; see recipe)
Store up to several months at 4°C

COMMENTARY

Background Information

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal. However, there are several problems inherent to immunoprecipitation, including the need to radiolabel the antigen, coprecipitation 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)—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 or urea-PAGE (*UNIT B3.1*), followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon membranes (*UNIT B3.2*). 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).

Immunoblotted proteins can be detected by chromogenic or luminescent assays (see Table B3.4.1 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRP and AP systems without the need for radioisotopes. Substrates for the latter have only recently been applied to protein blotting (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 exceed 1 hr. Depending on the system, the luminescence can last up to 3 days, permitting multiple exposures of the same blot. Furthermore, the signal is detected by film, and varying the exposure can result in more or less sensitivity. 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, a luminescent image recorded on film is easier to photograph and to quantitate by densitometry.

AP–based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted (see Reagents and Solutions). The procedure described in Support Protocol 2 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

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 dilutions of primary antibody and conjugate should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1988). 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, 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.

Two types of nylon membranes are used for transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to have 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 are popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% (w/v) nonfat dry milk in TBS is recommended for chromogenic development. During development of luminescence, however, background is a more significant problem. When compared to dry milk, purified casein has minimal endogenous AP activity (such 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-40). Because nonfat dry milk and casein may contain biotin, which 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.

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 have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can sometimes preserve antigenicity.

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).

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune sera 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. B3.4.1).

Because of 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 weaker signals nearby 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 it to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the AP 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% (w/v) casein, 1% (v/v) polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN_3, 10 mM EDTA, and phosphate-buffered saline (PBS), pH 6.8. Prior to use, the casein must be heated to 65°C to reduce AP 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 one is simultaneously testing multiple antibodies directed against a complex protein mixture, multiple bands will be visualized.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on the 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 (for high-power transfer) to overnight. Blocking, 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).

Literature Cited

Andrew, S.A. and Titus, J.A. 1991a. Purification of immunoglobulin G. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.7.1-2.7.12. John Wiley & Sons, New York.

Andrew, S.A. and Titus, J.A. 1991b. Fragmentation of immunoglobulin G. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.8.1-2.8.10. John Wiley & Sons, New York.

Andrew, S.A. and Titus, J.A. 1991c. Fragmentation of immunoglobulin M. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.10.1-2.10.4. John Wiley & Sons, New York.

Andrew, S.A. and Titus, J.A. 1993. Purification of immunoglobulin M. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.9.1-2.9.3. John Wiley & Sons, New York.

Bjerrum, O.J., Larsen, K.P., and Heegaard, N.H.H. 1988. Nonspecific binding and artifacts—specificity problems and troubleshooting with an atlas of immunoblotting artifacts. *In* CRC Handbook of Immunoblotting of Proteins, Vol. I: Technical Descriptions (O.J. Bjerrum and N.H.H. Heegaard, eds.) pp. 227-254. CRC Press, Boca Raton, Fla.

Blake, M.S., Johnston, K.H., Russell-Jones, G.J., and Gotschlich, E.C. 1984. A rapid, sensitive method for detection of alkaline phosphatase–conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.

Bronstein, I., Voyta, J.C., Murphy, O.J., Bresnick, L., and Kricka, L.J. 1992. Improved chemiluminescent Western blotting procedure. *BioTechniques* 12:748-753.

Burnette, W.N. 1981. Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.

Cooper, H.M. and Paterson, Y. 1995. Production of polyclonal antisera. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.4.1-2.4.9. John Wiley & Sons, New York.

Gillespie, P.G. and Hudspeth, A.J. 1991. Chemiluminescence detection of proteins from single cells. *Proc. Natl. Acad. Sci. U.S.A.* 88:2563-2567.

Harlow, E. and Lane, D. 1988. Immunoblotting. *In* Antibodies: A Laboratory Manual, pp. 471-510. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Harper, D.R. and Murphy, G. 1991. Nonuniform variation in band pattern with luminol/horseradish peroxidase Western blotting. *Anal. Biochem.* 192:59-63.

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. Methods* 67:1-11.

McKimm-Breschkin, J.L. 1990. The use of tetramethylbenzidine for solid phase immunoassays. *J. Immunol. Methods* 135:277-280.

Sandhu, G.S., Eckloff, B.W., and Kline, B.C. 1991. Chemiluminescent substrates increase sensitivity of antigen detection in Western blots. *BioTechniques* 11:14-16.

Schneppenheim, R., Budde, U., Dahlmann, N., and Rautenberg, P. 1991. Luminography—a new, highly sensitive visualization method for electrophoresis. *Electrophoresis* 12:367-372.

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. Methods* 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.

Yokoyama, W. 1991a. Production of monoclonal antibodies. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.5.1-2.5.17. John Wiley & Sons, New York.

Yokoyama, W. 1991b. Monoclonal antibody supernatant and ascites fluid. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 2.6.1-2.6.7. John Wiley & Sons, New York.

Key References

Gillespie and Hudspeth, 1991. See above.

Describes alkaline phosphatase–based luminescent detection methods.

Harlow and Lane, 1988. See above.

Details alternative detection methods.

Schneppenheim et al., 1991. See above.

Details horseradish peroxidase–based luminescent detection methods.

Contributed by Sean Gallagher
Motorola, Inc.
Tempe, Arizona