Table of Contents

Introduction

This Guide contains a collection of protein blotting protocols for research scientists and others who use microporous surfaces for biochemical analysis.

These protocols all use Immobilon-P transfer membrane, a polyvinylidene fluoride (PVDF) microporous material for binding proteins transferred from a variety of gel matrices. This hydrophobic membrane offers a uniformly controlled pore structure with a high binding capacity for biomolecules. When compared to nitrocellulose, Immobilon-P membrane has improved handling characteristics and staining capabilities, increased solvent resistance, and a higher signal-to-noise ratio for enhanced sensitivities.

Immobilon-P membrane has a pore size of 0.45 µm and is useful for blotting proteins > 20 kDa. The open pore structure makes it easy for probe molecule permeation and removal from the background.

It is an ideal substrate for immunodetection. Using the Millipore Rapid Immunodetection Method, researchers can reduce detection time by up to 2 hours. The unique membrane properties of Immobilon-P not only eliminate the need for blocking in western blots; they dramatically reduce the number and length of washes required, without compromising specificity or sensitivity. The substrate is compatible with chemiluminescent and chemifluorescent detection of proteins.

Immobilon-P Transfer Membrane Specifications

Applications
- Western blotting
- Binding assays
- Amino acid analysis
- N-terminal protein sequencing
- HIV detection
- Dot / slot blotting
- Glycoprotein visualization
- Lipopolysaccharide analysis

Detection Methods
- Chromogenic
- Radioactive
- Fluorescent
- Chemiluminescent

Protein Binding Capacity [(a)]
- Adsorption
- Insulin: 85 µg / cm^2
- BSA: 131 µg / cm^2
- Goat IgG: 294 µg / cm^2

Compatible Stains
- Coomassie® brilliant blue
- Amido black
- India ink
- Ponceau S red
- Colloidal gold
- Transillumination[(b)]
- CPTS [(c)]
- Immobilon-CD Stain

Composition, Pore Size and Phobicity
- PVDF, 0.45 µm
- Hydrophobic

Primary Binding Mechanisms
- Electrostatic
- Hydrophobic

Format
- Discs
- Sheets
- Roll

(a) Adsorption: Membrane discs were incubated for 2 hours in 1 mL of phosphate-buffered saline containing 1 mg of trace-labeled protein. After washing, bound protein was quantified by measuring the amount of bound radioactivity.

(b) Transillumination: See Reig, J., and Klein, D.C., *Applied and Theoretical Electrophoresis,* 1:59-60. 1988.

(c) CPTS: copper phthalocyanine 3,4', 4", 4"'-tetrasulfonic acid; see Bickar, D., and Reid, P.D., *Anal. Biochem.,* 203:109-115, 1992.

Membrane Handling Considerations

- Do not remove membrane from protective interleaves until ready to be wet with alcohol.
- Wear powder-free gloves at all times.
- Cut the membrane with clean scissors, clean razor blade, or clean paper cutter.
- To label, use a dull No. 2 pencil to write along the edge in an area where protein will not be transferred. Sharp pencils can tear the membrane and ink can introduce contaminants. Note that writing on the membrane crushes the microporous structure.
- Handle the membrane at the edges with flat-tipped forceps (e.g., Millipore XX62 000 06). Alternatively, hold the membrane lightly at the corners with gloved hands.
- Hydrophobic membrane is first wet in methanol; membrane changes from opaque to translucent. Methanol is exchanged with water, and water is exchanged with buffer.

Helpful Hints

- Prepare all solutions using high-quality reagents and deionized water from a well-maintained source (18.2MΩ resistivity). Low levels of contaminants can cause big problems. For example pyrogens inhibit the enzyme activity of horseradish peroxidase.
- As an indicator of overall transfer efficiency, run prestained protein markers as a control.
- Stain the gel after transfer to make sure proteins transferred — especially valuable if developing a new protocol, changing any step of a protocol, or encountering new sample types.
- It is possible to exceed the binding capacity of the membrane, so run a series of dilutions.
- A sufficient volume of solution is required for incubation / washing / etc. The membrane should move freely in the solution. If there is too little solution, the membrane may stick to the container and / or rub against it causing bald spots or uneven exposure. If there is too much solution, the membrane won't move with the solution and won't receive adequate circulation of fresh solution. Small plastic bags do not provide adequate circulation. Plastic dishes or boats are preferable. Of course, use a clean container for each step to avoid undesired cross-reactions.
- When designing a new experiment, work backwards. Consider the detection method first, then choose antibodies, buffers, etc. that are compatible (and don't cross-react).
- Make sure that the entire membrane is wet or dry, depending upon requirements. The surface may look wet / dry while the interior is still dry / wet – resulting in incomplete or uneven results.
- Particles in incubation buffers can cause undesireable backgrounds, if necessary filter the blocking buffer, washing buffer, and antibody diluent to low binding Durapore® filter before use.

Preparation of Blots

A blot, any membrane with proteins immobilized on it, can be prepared by:

- **Filtration** – direct application of the sample onto a specialized membrane which binds proteins but allows other components to pass through; or
- **Electrotransfer** – separating the proteins on a polyacrylamide gel and then applying an electrical field to transfer them to the membrane.

After the proteins have been immobilized on the membrane, they are available for analysis by a variety of techniques.

Filtration

Filtration is a direct method of applying proteins onto a membrane. A dissolved sample is pulled through the membrane by applying a vacuum; proteins bind to the membrane and the other sample components pass through. The proteins on the membrane are then available for analysis.

Dissolve sample in appropriate buffer

▼

Place membrane in blotting unit

▼

Place sample in blotting unit wells

▼

Apply vacuum

▼

Proteins bind to membrane

This technique can be used either as a qualitative method for rapid screening of a large number of samples or as a quantitative technique. It is especially useful for testing the suitability of experimental design parameters.

When preparing blots by filtration, keep in mind the following:

- Detergents can inhibit the binding of proteins to the membrane. Buffers used for sample dissolution and washing should contain no more than 0.05% detergent, if required.
- Choose a sample volume large enough to cover the exposed membrane in each well, but be careful not to exceed the binding capacity of the membrane.
- Samples with high particulate loads or viscosity will reduce the flow rate and clog the membrane. Centrifuge samples with particulates, and apply only the supernatant to the membrane. Dilute viscous samples in buffer.

The following protocol describes a typical procedure for filtering proteins onto an Immobilon-P membrane. Please review the instructions supplied with your blotting unit for additional information.

Required Equipment and Solutions

Two sheets of Immobilon-P membrane, cut to size for blotting unit*

Filter paper, cut to size for blotting unit

Methanol, 100%

Ultrapure water (produced by the Milli-Q® System)

Buffer, for sample loading and wash

Blotting unit, dot blot or slot blot format

* To ensure that the microporous structure of the membrane is not compressed when placed in the blotting unit, it is recommended that a second sheet of membrane be placed between the filter paper and the primary membrane.

Set Up

1. Prepare Immobilon-P membrane:
   a. Wet the membrane by laying it on the surface of methanol for 15 seconds. Do not immerse. The membrane should uniformly change from opaque to semi-transparent.
   b. Carefully place the membrane in ultrapure water and soak for 2 minutes.
   c. Carefully place the membrane in buffer and let equilibrate for at least 5 minutes.
2. Dissolve the sample in buffer.
   a. If the sample solution is cloudy, centrifuge to remove particles.
   b. If the sample is viscous, dilute with additional buffer.

Assemble the Blotting Unit

Note: See manufacturer's instructions for detailed assembly instructions.

1. Place one sheet of moistened filter paper on unit. (Note: Some units may require more than one sheet.)
2. Place two sheets of Immobilon-P membrane on top of the filter paper.
3. Close the unit.
4. Connect to vacuum line.

Filter

1. Briefly apply the vacuum to remove excess buffer.
2. With the vacuum off, carefully pipette samples into the wells.
3. Apply vacuum to the blotting unit.
4. When all of the samples have filtered through the membrane, turn off the vacuum.
5. Add buffer to each well to wash down the sides.
6. When all of the wash buffer has filtered through the membrane, turn off the vacuum.

Remove the Blot

1. Open the blotting unit.
2. Using forceps, carefully remove the membrane.
3. Place the membrane on a piece of clean filter paper to dry. For additional drying methods, see **Membrane Drying Methods** later in this section.

Note: Do not allow the membrane to dry out if analysis of the bound protein requires the native conformation or enzyme activity.

For information regarding protein visualization and immunodetection techniques, see the appropriate chapters later in this guide.

For possible causes and remedies to general symptoms which may occur during the preparation of blots, see the tables below:

Troubleshooting

Filtration

Symptom	Possible Cause	Remedy
Solution does not filter through membrane or filters slowly.	Inadequate vacuum.	Make sure that blotting unit is closed properly and seal is intact. Make sure vacuum pump (or other source) is operating properly.
		Use a high quality laboratory tape to seal off any open wells in blotting unit.
		If possible, ensure that all samples filtered at the same time have similar compositions.
	Membrane pores clogged.	Centrifuge samples to remove particulates.
		Dilute viscous samples.
		Increase vacuum level.
		Be patient.
Staining indicates little or no protein.	Not enough protein applied to membrane.	Minimize sample dilution. Filter more sample through membrane.
	Detergents (e.g., SDS) may inhibit protein binding (especially for low molecular weight proteins).	Eliminate detergents if possible.
	Stain not sensitive enough.	Use more sensitive staining technique.
Stained blot is not uniform.	Membrane structure was compressed by filter paper.	Place second membrane in the blotting unit to protect the membrane receiving the samples.
	Air bubbles trapped in interior of membrane.	Pre-wet membrane by laying it on the surface of methanol. Immersing the membrane can entrap air.
	Membrane not pre-wet in methanol.	Membrane must be pre-wet with methanol; entire membrane should change from opaque to semi-transparent.
	Air bubbles in sample.	Carefully pipette samples into wells to avoid formation of air bubbles on surface of membrane.
	Not enough sample.	Sample must cover entire exposed membrane area.
Protein smeared across top of membrane.	Sample leaked between wells.	Make sure that blotting unit is properly assembled and closed prior to filtration.
Protein smeared across back of membrane.	Binding capacity of membrane was exceeded.	Reduce the amount of protein loaded.

Electrotransfer

Electrotransfer is a method of transferring proteins from a gel to a membrane by applying an electrical field. The proteins on the membrane are then available for analysis.

Resolve proteins on a polyacrylamide gel

▼

Place gel in contact with membrane, and stack along with other supporting filters

▼

Place stack in electrotransfer device

▼

Apply electrical field perpendicular to the plane of the gel, causing proteins to move onto the membrane

▼

Proteins bind to membrane

The two commonly used electrotransfer techniques are:

- Tank transfer
- Semi-dry transfer

Both are based on the same basic principles and differ only in the mechanical device used to hold the gel / membrane stack and apply the electrical field.

Tank transfer is the traditional technique where the gel / membrane stack is immersed in a buffer reservoir and then the current is applied. It is an effective but slow (approximately 2 hours) technique. Also, it can be difficult to set up a tank and buffer to accommodate large gels.

Semi-dry transfer replaces the buffer reservoir with layers of filter paper soaked in buffer. This technique is as effective, but far quicker (15 - 45 minutes) than tank transfer.

Tank Transfer

The following protocol describes the standard procedure for transferring proteins from a polyacrylamide gel (SDS-PAGE) onto an Immobilon-P membrane using a tank transfer system. Please review the instructions supplied with your tank transfer system for additional information.

Required Equipment and Solutions

Polyacrylamide gel containing the resolved proteins

Immobilon-P membrane, cut to the same dimensions as the gel (including notched corner for orientation purposes)

Two sheets of Whatman™ 3MM filter paper or equivalent, cut to the same dimensions as the gel

Two foam pads (for example, Scotch Brite® pads)

Tris / glycine transfer buffer

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Tank transfer system large enough to accommodate gel

Glass pipette or stirring rod

Set Up

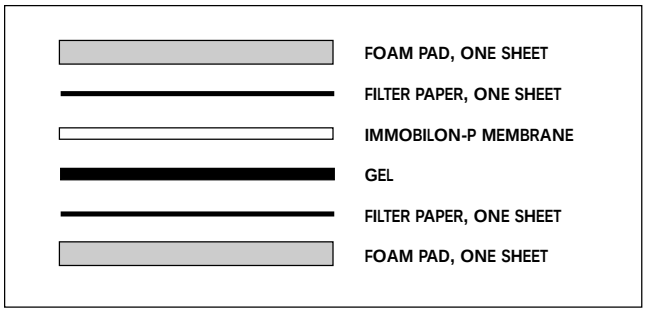
1. Prepare sufficient Tris / glycine transfer buffer to fill the transfer tank, plus an additional 200 mL to equilibrate the gel and membrane, and wet the filter paper. The transfer buffer consists of:
   - 25 mM Tris
   - 192 mM glycine
   - 10% (v / v) methanol
   - No pH adjustment
2. Remove the gel from its glass cassette; trim away any stacking gel.
3. Immerse gel in transfer buffer for 15 to 30 minutes.
4. Soak filter paper in transfer buffer for at least 30 seconds.
5. Prepare Immobilon-P membrane:
   a. Wet the membrane in methanol for 15 seconds. Membrane should uniformly change from opaque to semi-transparent.
   b. Carefully place membrane in ultrapure water and soak for 2 minutes.
   c. Carefully place membrane in transfer buffer and let equilibrate for at least 5 minutes.

Assemble the Transfer Stack

Important! To ensure an even transfer, remove air bubbles between layers by carefully rolling a pipette or a stirring rod over the surface of each layer in the stack. Do not apply excessive pressure or you may damage the membrane and gel.

1. Remove the cassette holder from the transfer tank and open it.
2. Place a foam pad on one side of the cassette.
3. Place one sheet of filter paper on top of the pad.
4. Place the gel on top of the filter paper.
5. Place Immobilon-P membrane on top of the gel.
6. Place a second sheet of filter paper on top of the stack.
7. Place second foam pad on top of the filter paper.
8. Close the cassette holder.

The completed transfer stack should look like this:



Transfer the Proteins

1. Place the cassette holder in the transfer tank so that the gel side of the cassette holder is facing the cathode (-) and the membrane side is facing the anode (+).
2. Add adequate buffer to the tank to cover the cassette holder.
3. Insert the black cathode lead (-) into the cathode jack and the red anode lead (+) into the anode jack.
4. Connect the anode lead and cathode lead to their corresponding power outputs.
5. If available, set up the cooling unit on the tank transfer unit according to the manufacturer's instructions.
6. Turn on the system for 1 to 2 hours at 6 to 8 V/cm interelectrode distance.

Remove the Blot

1. Remove the cassette holder from the tank.
2. Using forceps, carefully disassemble the transfer stack.
3. Place the membrane on a piece of clean Whatman 3MM paper to dry. For additional drying methods, see **Membrane Drying Methods** later in this section.

Note: Do not allow the membrane to dry out if analysis of the bound protein requires the native conformation or enzyme activity.

For information regarding protein visualization and immunodetection techniques, see the appropriate chapters later in this guide.

Semi-Dry Transfer

The following protocol describes the standard procedure for transferring proteins from a polyacrylamide gel (SDS-PAGE) onto an Immobilon-P membrane using a semi-dry transfer system. It is specific for semi-dry transfer devices with the anode plate serving as the base. For devices having the cathode plate as the base, consult the instruction manual for recommended buffers and transfer stack assembly.

Gels can be transferred individually or multiple gels can be transferred in a single stack.

Required Equipment and Solutions

Polyacrylamide gel containing the resolved proteins

Immobilon-P membrane, cut to the same dimensions as the gel (including notched corner)

Six pieces of Whatman 3MM filter paper or equivalent, cut to the same dimensions as the gel

Anode buffer I

Anode buffer II

Cathode buffer

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Semi-dry transfer system large enough to accommodate gel

Glass pipette or stirring rod

For multiple gel transfers only:

Dialysis membrane, cut to the same dimensions as the gel and wet with ultrapure water. The membrane should have a molecular weight exclusion small enough to retain the lowest molecular weight protein in the gel.

Set Up

1. Prepare 200 mL of each anode buffers and 400 mL of cathode buffer:
   a. Anode buffer I
      - 0.3 M Tris, pH 10.4
      - 10% (v/v) methanol
   b. Anode buffer II
      - 25 mM Tris, pH 10.4
      - 10% (v/v) methanol
   c. Cathode buffer
      - 25 mM Tris
      - 40 mM 6-amino-n-caproic acid (Note: glycine may be substituted)
      - 10% (v/v) methanol
      - pH 9.4

2. Remove the gel from its glass cassette; trim away any stacking gel.
3. Immerse the gel in cathode buffer for 15 minutes.
4. Soak two pieces of filter paper in anode buffer I for at least 30 seconds.*
5. Soak one piece of filter paper in anode buffer II for at least 30 seconds.*
6. Soak three pieces of filter paper in cathode buffer for at least 30 seconds.*
7. Prepare Immobilon-P membrane:
   a. Wet the membrane in methanol for 15 seconds. The membrane should uniformly change from opaque to semi-transparent.
   b. Carefully place the membrane in ultrapure water and soak for 2 minutes.
   c. Carefully place the membrane in anode buffer II and let equilibrate for at least 5 minutes.

* Additional pieces will be required for transfer of multiple gels.

Assemble the Transfer Stack

Important! To ensure an even transfer, remove air bubbles between layers by carefully rolling a pipette or stirring rod over the surface of each layer in the stack. Do not apply excessive pressure or you may damage the membrane and gel.

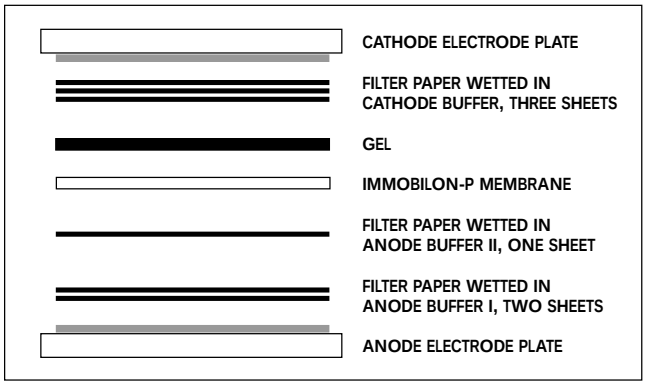
Refer to the manufacturer's specific operating instructions for your semi-dry transfer system.

Single transfer:

1. Place the anode electrode plate on a level bench top.
2. Place the two pieces of filter paper soaked in anode buffer I in the center of the plate.
3. Place the filter paper soaked in anode buffer II on top of the first two sheets.
4. Place Immobilon-P membrane on top of the filter papers.
5. Place the gel on top of the membrane.
6. Place the three pieces of filter paper soaked in cathode buffer on top of the membrane.
7. Place the cathode electrode plate on top of the stack.

Caution: Do not bump the cathode plate cover since it could disturb the alignment of the transfer stack and cause inaccurate results.

The completed transfer stack should look like this:

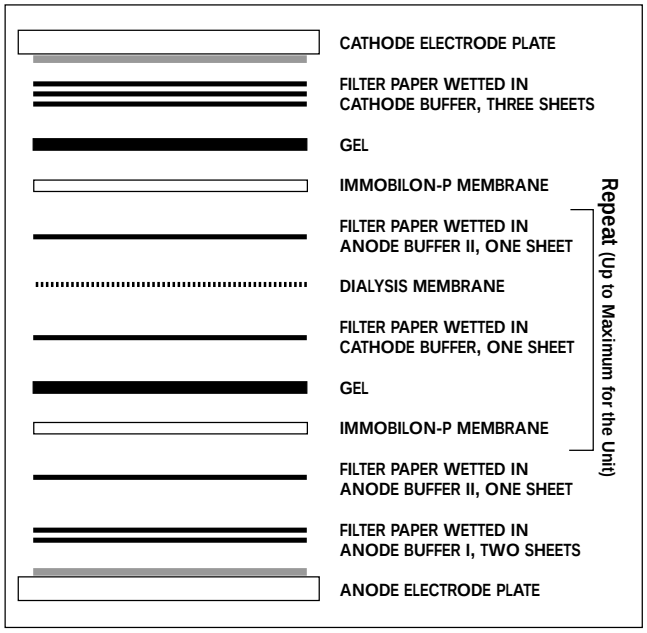


Multiple transfers:

1. Place the anode electrode plate on a level bench top.
2. Place the two pieces of filter paper soaked in anode buffer I in the center of the plate.
3. Place the filter paper soaked in anode buffer II on top of the first two sheets.
4. Place the Immobilon-P membrane on top of the filter papers.
5. Place the gel on top of the membrane. For the last gel, go to step 10.
6. Place a piece of filter paper soaked in cathode buffer on top of the gel.
7. Place a piece of dialysis membrane on top of the filter paper.
8. Place a piece of filter paper soaked in anode buffer II on top of the dialysis membrane.
9. Repeat steps 4 through 8 until all gels (up to the maximum for the unit) have been incorporated into the stack.
10. Place three pieces of filter paper soaked in cathode buffer on top of the last gel.
11. Place the cathode electrode plate on top of the stack.

Caution: Do not bump the cathode plate cover since it could disturb the alignment of the transfer stack and cause inaccurate results.

The completed transfer stack should look like this:



Transfer the Proteins

1. Insert the black cathode lead (-) into the cathode plate jack.
2. Insert the red anode lead (+) into the anode plate jack.
3. Connect the anode lead and cathode lead to their corresponding power supply outputs.
4. Turn on the power supply.
5. Set the current and let it run for the time indicated in the following chart:

Current Density	Time Limit
0.8 mA / cm^2 *	1 to 2 hours
1.2 mA / cm^2	1 hour
2.5 mA / cm^2	30 to 45 minutes
4.0 mA / cm^2	10 to 30 minutes

* The surface area (cm^2) is determined from the dimensions of the footprint of the stack on the anode plate. This value is independent of the number of gels in the stack.

Removing the Blot

1. Turn off power supply when the transfer is complete.
2. Disconnect the system leads.
3. Remove the cover.
4. Remove and discard the filter papers.

Note: For graphite plates, graphite particles from the anode electrode plate occasionally appear on the filter paper. These particles do not affect operation.

5. Remove the gel.
6. Remove the blotted membrane with a pair of forceps.
7. Place the membrane on a piece of clean Whatman 3MM paper to dry. For additional drying methods, see **Membrane Drying Methods** later in this section.

Note: Do not allow membrane to dry out if analysis of the bound protein requires the native conformation or enzyme activity.

For information regarding protein visualization and immunodetection techniques, see the appropriate chapters later in this guide.

Membrane Drying Methods

The membrane must be dried before continuing on to transillumination or rapid immunodetection procedures. As the blotted membrane dries, it becomes opaque.

The table below details four drying methods and required times. Always wait the full length of drying time to ensure that all liquid has evaporated from within the membrane's pore structure.

Drying Method	Required Time*
Soak in 100% methanol for 10 seconds. Remove from methanol and place on piece of filter paper until dry.	15 minutes
Secure between two sheets of filter paper and place in a vacuum chamber.	30 minutes
Incubate at 37°C.	1 hour
Place on lab bench and let dry at room temperature.	2 hours

* Longer times required in higher humidity environments.

Troubleshooting

Symptom	Possible Cause	Remedy
Bands smeared / distorted.	Membrane not uniformly wetted.	Membrane must be pre-wet with methanol; entire membrane should change from opaque to semi-transparent.
	Air bubbles under membrane, or between other layers in stack.	Using a pipette or stirring rod, gently roll out any trapped air bubbles.
	Too much heat generated during transfer. Temperature should not exceed 20°C.	For tank transfer; pre-chill buffer or place in cold room during transfer. For semi-dry transfer; shorten the run time, increase the number of filter papers or reduce the current.
	Proteins transferred too rapidly, forming build-up of protein on membrane surface.	Reduce strength of electrical field.
	Filter paper dried out during semi-dry transfer	Make sure filter paper is "sopping" wet prior to transfer, or use additional sheets. Complete assembly of stack in <15 minutes.
Poor transfer. Proteins pass through membrane.	Membrane not pre-wet in methanol.	Membrane must be pre-wet with methanol; entire membrane should change from opaque to semi-transparent.
	SDS may inhibit protein binding (especially for low molecular weight proteins).	Remove SDS from gel by equilibrating in transfer buffer for at least 15 minutes. Eliminate SDS if possible.
	Methanol concentration in transfer buffer too low to facilitate removal of SDS.	Methanol concentration should be increased to 15% to 20% (v / v).
Poor transfer. Proteins retained in the gel.	Methanol concentration in transfer buffer too high, preventing transfer due to aggregation of high molecular weight proteins in the gel.	Methanol concentration should be 10% (v / v) or less. Supplement the transfer buffer with 0.05% (v / v) SDS.
	Isoelectric point of protein is higher than buffer pH.	Adjust buffer pH.
	Transfer time too short.	Increase transfer time.
	Transfer voltage too low.	Increase voltage.
	Gel must be oriented toward cathode (-) and membrane toward anode (+) (unless transferring from acid-urea gels).	Reorient stack.

Protein Visualization Techniques

Protein visualization, or staining, is a simple technique to make protein bands visible on a blot. Staining can be used to:

- Verify that proteins have transferred onto the membrane
- Evaluate the overall efficiency of the transfer
- Evaluate the transfer efficiency for a new buffer system or protein sample

Many types of stains are available and they fall into two general categories:

- Reversible
- Non-reversible

Non-reversible stains generally exhibit the best sensitivity but can interfere with or prevent further analysis of the proteins. Although less sensitive, reversible stains allow assessment of the blot and then can be washed from the membrane. However, there is a risk that some proteins may undergo chemical modification during the process or be washed from the membrane.

While not technically a stain, transillumination may be the most valuable of the visualization techniques. It provides a sensitivity similar to Coomassie® brilliant blue R without the risk of losing or modifying the proteins.

The most important factor in choosing a stain is how the proteins on the blot are to be analyzed. For example, if immunodetection analysis will follow, non-reversible stains are not recommended.

Gels should be stained whenever a change has been made to the transfer protocol or a new sample type is encountered. This procedure can verify that the proteins have indeed eluted from the gel. Follow the protocol below for Coomassie brilliant blue R with the exception of increasing the staining time to 1 hour and the destaining time to 1–3 hours.

Staining Protocols

The following protocols describe typical procedures for staining proteins immobilized on an Immobilon-P membrane.

Note: When examining a stained blot, the degree of contrast is best while the membrane is still wet. For photographic purposes, use a wet blot and light transmitted through the membrane.

Coomassie Brilliant Blue R

This non-reversible stain produces dark bands on a light background.

Important! This stain will interfere with immunodetection.

Required Equipment and Solutions

Stain: 0.1% (w/v) Coomassie brilliant blue R in 50% (v/v) methanol, 7% (v/v) acetic acid

Destain solution I: 50% (v/v) methanol, 7% (v/v) acetic acid

Destain solution II: 90% (v/v) methanol, 10% (v/v) acetic acid

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Shallow tray, large enough to hold membrane

Stain the Blot

1. If the blot is dry, re-wet in 100% methanol.
2. Fill the tray with enough stain to cover the blot.
3. Place the blot in stain and agitate for 2 minutes.
4. Remove the blot and rinse briefly with ultrapure water.
5. Place the blot in destain solution I (50% methanol, 7% acetic acid) and agitate for 10 minutes to remove excess stain.
6. Place the blot in destain solution II (90% methanol, 10% acetic acid) and agitate for 10 minutes to completely destain the background.

Amido Black

This non-reversible stain produces dark bands on a light background.

Important! This stain will interfere with immunodetection.

Required Solutions

Stain: 0.1% (w/v) amido black in 25% (v/v) isopropanol, 10% (v/v) acetic acid

Destain solution: 25% (v/v) isopropanol, 10% (v/v) acetic acid

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Shallow tray, large enough to hold membrane

Stain the Blot

1. If the blot is dry, re-wet in 100% methanol.
2. Fill the tray with enough stain to cover the blot.
3. Place the blot in stain and agitate for 10 minutes.
4. Remove the blot and rinse briefly with ultrapure water.
5. Place the blot in destain solution and agitate for 5 to 10 minutes to remove excess stain.

Ponceau-S Red

This stain is reversible and produces pinkish bands on a light background.

Required Solutions

Stain: 0.2% Ponceau-S red, 1% acetic acid
[Prepared by diluting the stock solution (2% dye in 30% (w/v) trichloroacetic acid and 30% (w/v) sulfosalicylic acid) 1 to 10 in 1% (w/v) acetic acid.]

Methanol, 100%

0.1 N NaOH

Ultrapure water (produced by the Milli-Q System)

Shallow tray, large enough to hold membrane

Stain the Blot

1. If the blot is dry, re-wet in 100% methanol.
2. Fill the tray with enough stain to cover the blot.
3. Place the blot in stain and agitate for 1 minute.
4. Remove the blot and rinse thoroughly with ultrapure water until the desired contrast has been achieved.
5. To remove the stain completely, wash the blot with 0.1 N NaOH.

CPTS (Copper phthalocyanine tetrasulfonic acid)

This stain is reversible and produces turquoise blue bands on a light background.

Required Equipment and Solutions

Stain: 0.05% (w/v) CPTS in 12 mM HCl

Destain solution I: 12 mM HCl

Destain solution II: 20% (v/v) methanol

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Protein destain solution: 0.5 M $NaHCO_3$

Shallow tray, large enough to hold membrane

Stain the Blot

1. If the blot is dry, re-wet in 100% methanol.
2. Fill the tray with enough stain to cover the blot.
3. Place the blot in stain and agitate for 1 minute.
4. Place the blot in destain solution I to remove excess stain and achieve the desired contrast.
5. To remove stain from the background completely, wash the blot with destain solution II.
6. To completely destain the proteins, agitate the blot in protein destain until the stain has been removed.

Transillumination

Transillumination is a nondestructive, reversible technique. This method is based on the change of PVDF from opaque to semi-transparant when wet in methanol. 20% methanol will wet out regions of the membrane coated with protein, but not areas without protein.

The protein bands appear as clear areas when placed on a light box. Detection sensitivity is comparable to Coomassie brilliant blue R stain when used in conjunction with photography.

Required Equipment and Solutions

20% (v/v) methanol

Shallow tray, large enough to hold membrane

White light box

"Stain" the Blot

1. Dry the blot completely using one of the drying methods in the **Preparation of Blots** section.
2. Fill the tray with enough 20% methanol to cover the blot.
3. Place the blot in 20% methanol for 5 minutes.
4. Place the blot on a light box and mask the areas around the blot with a sheet of black paper.
5. The bands appear as clear areas against an opaque background.
6. If a permanent record is desired, photograph the wet blot.
7. When all the methanol has dried, the blot will return to its original appearance.

Troubleshooting

For possible causes and remedies to general symptoms which may occur during visualization of proteins, see the table below:

Symptom	Possible Cause	Remedy
Blot stains weakly or unevenly.	Blot was dry prior to staining with Coomassie blue, Amido black, Ponceau S stain, or CPTS.	Re-wet blot in 100% methanol.
	Protein transfer to membrane was incomplete.	Stain gel to check for residual proteins. If transfer was not complete, review transfer technique. See **Preparation of Blots**.
Bands don't show when using transillumination.	Blot saturated with water only.	Blot must be saturated with 20% methanol.
	Blot prepared using nitrocellulose, Immobilon-P^{SQ} or -CD membrane.	Technique works best with Immobilon-P membrane.
	Blot was not dry prior to immersion in 20% methanol.	Dry the blot completely.
High background staining.	Residual stain on membrane.	Continue destaining until the desired contrast is reached.
	Non-specific protein binding to membrane.	Check the buffers used in electrotransfer for protein contamination. Use high quality reagents and ultrapure water.

Immunodetection is a method of protein detection using a specific antibody to identify the location of the membrane-bound protein. The specificity of antibody-antigen binding permits the identification of a single protein in a complex sample.

Block unoccupied membrane sites to prevent non-specific binding of antibodies

▼

Incubate membrane with primary antibody, which binds the protein of interest

▼

Wash to remove any unbound antibodies

▼

Incubate with secondary antibody, which binds the first antibody, and carries an enzyme conjugate

▼

Wash to remove any unbound antibodies

▼

Incubate with substrate which reacts with labeled antibody to reveal location of protein

When developing procedures for your own samples, carefully consider all components and their interactions. Experiment with antibody concentrations, buffer composition, blocking agents and incubation times to determine the best conditions. And don't forget the importance of water quality in all steps – small impurities can cause big problems. For instance, the enzyme activity of horseradish peroxidase is inhibited by pyrogens, a common contaminant of even high purity water.

The following paragraphs provide important information regarding the immunodetection procedure. Understanding these basic concepts will help you optimize protocols for your specific samples.

Buffers

The two most commonly used buffers are phosphate buffered saline (PBS) and Tris buffered saline (TBS). Many variations on the compositions of these buffers have been published. The key feature is that the buffer must preserve the biological activity of the antibodies. Thus, the ionic strength and pH should be fairly close to physiological conditions. We have found that PBS formulations with 10 mM total phosphate work well with a wide array of antibodies and substrates.

Blocking

For meaningful results, the antibodies must bind only to the protein of interest – not to the membrane. Non-specific binding of antibodies can be reduced by blocking the unoccupied membrane sites with an inert protein or non-ionic detergent.

The blocking agent should:

- fill all unoccupied binding sites;
- not displace or bind to epitopes on the protein of interest;
- have a greater affinity for the membrane than the antibodies; and
- not cross-react with the chosen antibodies.

The most common blocking agents are BSA (bovine serum albumin), non-fat milk and dilute solutions of Tween® 20 (<0.05% v/v). The blocking agent is usually dissolved in an appropriate buffer, such as PBS (phosphate buffered saline).

There are risks associated with blocking; a poorly selected blocking agent or excessive blocking can displace or obscure the protein of interest. For a protocol which does not require blocking, see **Rapid Immunodetection Method**.

Antibodies

After blocking, the blot is incubated with one or more antibodies. The first antibody binds to the protein of interest and a secondary antibody binds to the first. The secondary antibody is labeled with an enzyme which will be used to indicate the location of the protein.

Although the primary antibody may be labeled directly, using a secondary antibody has distinct advantages. The same labeled secondary antibody (enzyme-antibody conjugate) can be used for a large number of primary antibodies of different specificities, thereby eliminating the need to purify and label numerous primary antibodies. Also, because more than one molecule of the secondary antibody may be able to bind to the primary antibody, a secondary antibody can enhance the signal.

Either polyclonal or monoclonal antibodies may be used. Polyclonal antibodies may be in the form of antiserum or affinity purified antibody. Monoclonal antibodies may be in the form of ascites fluid or tissue culture fluid. Antibodies are diluted in buffer and blocking solution to prevent non-specific binding to the membrane. The antibody diluent also normally contains trace amounts of Tween 20 or another detergent to prevent non-specific aggregation of the antibodies. Many published protocols for chemiluminescence call for 0.1% (v/v) Tween 20 in the blocking solution and antibody diluent. It is important to recognize that concentrations above 0.05% (v/v) have the potential to wash blotted proteins from the membrane.

In addition to being specific for the protein of interest, the antibodies used must:

- not recognize the blocking proteins or any other proteins on the membrane;
- not cross-react with components of the blocking buffer; and
- be pure, because impurities in the form of other proteins or aggregates can cause unreliable results.

Washing

Washing the blot removes from the membrane any unbound antibodies which could cause a high background and poor detection. A dilute solution of Tween 20 (0.05% v/v) in buffer is commonly used, especially when the antibody preparations are comparatively crude or used at high concentrations. More concentrated detergent solutions could elute the protein of interest from the membrane. For highly purified antibodies, buffer alone is often sufficient for washing.

The amount of washing required is best determined experimentally. Too little washing will yield an excessive background, while too much may elute the antibody or protein and reduce the signal.

Detection Substrates

After the final wash, the blot is incubated with an appropriate detection substrate. The enzyme-antibody conjugate already on the blot catalyzes a reaction with the substrate, producing a visible compound at the site of the protein.

Chromogenic detection uses the enzyme to catalyze a reaction resulting in the deposit of a colored precipitate. This technique is easy to perform and requires no special equipment for analysis, however:

- production of the precipitate can interfere with enzyme activity and limit sensitivity; and
- the precipitate can be difficult to strip from membrane, limiting reuse of the blot for detection of other proteins.

Chemiluminescent detection uses the enzyme to catalyze a reaction resulting in the production of visible light. This technique combines the speed and safety of chromogenic detection with the sensitivity of radioisotopic detection. Reprobing is possible with chemiluminescent substrates. See the **Membrane Stripping** section.

Substrates should be prepared immediately before using. Most substrates are supplied complete with preparation instructions.

Rapid Immunodetection Method

Rapid immunodetection takes advantage of the fact that antibodies cannot bind to the hydrophobic surface of the Immobilon-P membrane, but will bind to a protein immobilized on the membrane. Rapid immunodetection is compatible with both chromogenic and chemiluminescent substrate detection.

The major advantage of rapid immunodetection is that blocking is not required, saving time and eliminating the risks involved. Also, because excess antibody won't bind to a dry membrane, the amount of washing required is reduced. As a result, the total time for analysis is under 2 hours, as opposed to over 4 hours for the standard method. All without loss of sensitivity.

The following protocol employs chromogenic detection. Good results have been achieved using alkaline phosphatase labeled secondary antibodies and BCIP / NBT (5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium) as the substrate. A dark purple precipitate indicates the location of the protein. Immobilon-P is also compatible with horseradish peroxidase labeled secondary antibodies and its many substrates.

For rapid immunodetection with chemiluminescent detection see the protocol in **Chemiluminescent Detection** later in this section.

Very Important! The blot must be thoroughly dry before beginning rapid immunodetection. Refer to the drying methods in the **Preparation of Blots** section.

Required Equipment and Solutions

Primary antibody (specific for protein of interest)

Secondary antibody (specific for primary antibody), labeled with alkaline phosphatase

Substrate: BCIP / NBT (5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium)

Phosphate buffered saline (PBS): 10 mM sodium phosphate, pH 7.2, 0.9% (w / v) NaCl

Tween 20

BSA (bovine serum albumin)

Ultrapure water (produced by the Milli-Q System)

Shallow trays, large enough to hold blot

Set Up

1. Dry the blot completely using one of the drying methods in the **Preparation of Blots** section.
2. Prepare antibody diluent in PBS:
   - 1% (w / v) BSA
   - 0.05% (v / v) Tween 20

Enough solution should be prepared to allow for 0.1 mL of antibody diluent (primary and secondary) per cm^2 of membrane.

3. Dilute primary antibody to the normal working concentration.
4. Dilute secondary antibody to the normal working concentration.

Antibody Incubations

1. Place the blot in the primary antibody solution and incubate with agitation for 1 hour. The solution should move freely across the surface of the membrane.
2. Place the blot in PBS and wash for 10 seconds. Repeat with fresh buffer.
3. Place the blot in the secondary antibody solution and incubate with agitation for 30 minutes.
4. Place blot in PBS and wash for 10 seconds. Repeat with fresh buffer.

Protein Detection

1. Prepare the substrate according to supplier's instructions.
2. Place the blot in a clean container and add substrate to completely cover the surface of the membrane. Incubate for 10 minutes or until signal reaches desired contrast.
3. Rinse the blot with ultrapure water to stop the reaction.
4. Store the blot out of direct light to minimize fading.

Standard Immunodetection Method

Standard immunodetection is the traditional technique requiring wetting of the blot in methanol followed by blocking of the unoccupied membrane binding sites. The drawbacks of this method are the need for blocking and the total time requirement of over 4 hours. The advantage is that standard immunodetection may require less optimizing for new sample types.

Standard immunodetection is compatible with both chromogenic and chemiluminescent substrate detection.

The following protocol employs chromogenic detection. Good results have been achieved using alkaline phosphatase or horseradish peroxidase labeled secondary antibodies and their corresponding substrates. Many protocol variations are possible, as discussed above.

Important: The membrane must be wet in methanol prior to standard immunodetection.

Required Equipment and Solutions

Primary antibody (specific for protein of interest)

Secondary antibody (specific for primary antibody), labeled with alkaline phosphatase or horseradish peroxidase

Substrate appropriate to the enzyme conjugate

Phosphate buffered saline (PBS): 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl

Tween 20

BSA (bovine serum albumin)

Methanol, 100%

Ultrapure water (produced by the Milli-Q System)

Shallow trays, large enough to hold blot

Set Up

1. Thoroughly re-wet blot in methanol for 5 minutes.
2. Prepare blocking solution and antibody diluent in PBS:
   - 1% (w/v) BSA
   - 0.05% (v/v) Tween 20

Enough solution should be prepared to allow for 1 mL of blocking solution per cm^2 of membrane and 0.1 mL of antibody solution (primary and secondary) per cm^2.

3. Dilute primary antibody to the normal working concentration.
4. Dilute secondary antibody to the normal working concentration.

Antibody Incubations

1. Place the blot in the blocking solution and incubate with agitation for 1 hour.
2. Place the blot in the primary antibody solution and incubate with agitation for 1 hour. The solution should move freely across the surface of the membrane.
3. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
4. Place the blot in the secondary antibody solution and incubate with agitation for 1 hour.
5. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.

Protein Detection

1. Prepare the substrate according to supplier's instructions.
2. Place the blot in a container and add substrate to completely cover the surface of the membrane. Incubate for 10 minutes or until the signal reaches the desired contrast.
3. Rinse the blot with ultrapure water to stop the reaction.
4. Store the blot out of direct light to minimize fading.

Rapid Chemiluminescent Detection Method

Chemiluminescent detection is based on the oxidation of luminol in the presence of peroxide and is catalyzed by horseradish peroxidase. The oxidation of luminol emits light which is detected by exposing the blot to X-ray film.

For best results, a low background is important. Therefore, use more dilute antibodies and slightly longer wash times than for rapid chromogenic detection. If converting a standard chromogenic method to rapid chemiluminescent detection, start with primary and secondary antibody concentrations that are three to five times lower and a Tween 20 concentration of 0.01% (v/v).

This protocol is based on the Rapid Immunodetection Method and was optimized for the detection of transferrin in human serum. The primary antibody used was goat anti-(human transferrin) and the secondary antibody horseradish peroxidase-labeled rabbit anti-(goat IgG).

Required Equipment and Solutions

Primary antibody

Secondary antibody, labeled with horseradish peroxidase

Enhanced chemiluminescent substrate: Amersham ECL™

Phosphate buffered saline (PBS): 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4 (Sigma 1000-3)

Casein

Tween 20

Ultrapure water (produced by the Milli-Q System)

Shallow trays, large enough to hold blot

Glass plates

Plastic wrap (e.g., Saran™)

Autoradiography film and cassette

Dark room

Set Up

1. Dry the blot completely using one of the drying methods in the **Preparation of Blots** section.
2. Prepare antibody diluent in PBS:
   - 1% (w/v) casein
   - 0.04% (v/v) Tween 20

   Heat to approximately 50°C for 5 to 10 minutes to dissolve casein.
3. Dilute primary antibody to the desired working concentration.
4. Dilute secondary antibody to the desired working concentration.

Antibody Incubations

1. Place the blot in the primary antibody solution and incubate with agitation for 1 hour.
2. Rinse the blot quickly with PBS, then place in PBS and wash for 5 minutes. Repeat wash twice with fresh buffer.
3. Place the blot in the secondary antibody solution and incubate with agitation for 30 minutes.
4. Rinse blot quickly with PBS, then place in PBS and wash for 5 minutes. Repeat wash twice with fresh buffer.

Protein Detection

1. Prepare the substrate according to supplier's instructions.
2. Place the blot in container and add substrate to completely cover surface of membrane. Incubate for 1 minute.
3. Drain excess substrate.
4. Place the blot on a clean piece of glass and wrap in plastic wrap.
5. Gently smooth out any air bubbles.
6. In a dark room, place the wrapped membrane in a film cassette.
7. Place a sheet of autoradiography film on top and close the cassette.
8. Expose film. Multiple exposures of 15 seconds to 30 minutes should be run to determine the optimum exposure time; 2 to 5 minutes is common.

Membrane Stripping

A single blot can be sequentially analyzed with multiple antibodies by stripping the first antibody from the blot and incubating with another. The stripping process disrupts the antigen-binding capacity of the antibody and dissolves it into the surrounding buffer.

Two protocols are presented below. The first is applicable to any chemiluminescent substrate system and uses a combination of detergent and heat to release the antibodies. The second is commonly used for applications where antibodies have to be separated from an antigen and employs low pH to alter the structure of the antibody in such a way that the binding site is no longer active.

Neither method will remove the colored precipitates generated from chromogenic detection systems (e.g., BCIP, 4CN, DAB and TMB). However, it is still possible to analyze the blot with another antibody.

Important: The blot should not be allowed to dry between rounds of immunodetection. Any residual antibody molecules will bind permanently to the membrane if it is allowed to dry.

Method #1 – Heat/Detergent

Applicable to any chemiluminescent substrate system.

Required Equipment and Solutions

Stripping solution: 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7

Buffer (same as used in initial immunodetection)

Shallow tray, large enough to hold membrane

Stripping

1. Place the blot in stripping solution and agitate for 30 minutes at 50°C.
2. Place the blot in buffer and agitate for 10 minutes. Repeat with fresh buffer.
3. (OPTIONAL) Repeat the initial detection protocol (omitting the primary antibody step) to make sure that the antibodies have been inactivated or stripped from the membrane.
4. Place the blot in buffer and agitate for 10 minutes.
5. Proceed to the blocking step for the next round of detection.

Method #2 – Low pH

May be used for any application where antibodies have to be separated from an antigen in such a way that the antibody binding site is no longer active.

Required Equipment and Solutions

Stripping solution: 25 mM glycine-HCl, pH 2, 1% (w/v) SDS

Buffer (same as used in initial immunodetection)

Shallow tray, large enough to hold membrane

Stripping

1. Place the blot in stripping solution and agitate for 30 minutes.
2. Place the blot in buffer and agitate for 10 minutes. Repeat with fresh buffer.
3. Proceed to the blocking step for the next round of detection.

Troubleshooting

For possible causes and remedies to general symptoms which may occur during immunodetection, see the table below:

General (applies to all immunodetection methods)

Symptom	Possible Cause	Remedy
High background.	Impurities in polyclonal antibody(s).	Use affinity purified antibodies.
	Unbound antibodies not thoroughly washed from blot.	Increase wash times. Decrease antibody concentrations.
	Antibody bound to membrane sites.	Increase blocking time. Change blocking agent.
	Substrate incubation time too long (chromogenic).	Decrease incubation time.
	Film exposure time too long (chemiluminescence).	Decrease exposure time.
Low sensitivity.	Incomplete reaction of antibody and antigen.	Increase antibody incubation times.
	Blocking agent has affinity for protein of interest.	Try a different blocking agent.
	Antibody concentration too low.	Increase the antibody concentration.
	Substrate outdated.	Use fresh substrate that has been properly stored.
Uneven / splotchy results.	Insufficient solution in incubation or washing container.	Use container large enough for solution to move freely across the blot. Do not use "seal-a-meal" type bags. Do not incubate more than one blot at a time.
	Contamination.	Make sure all buffers and other reagents are fresh and free of contaminants and particulates. Filtration of buffers and centrifugation of antibody stocks may be necessary. Make sure deionized water supply is pure and well-maintained.
	Air bubbles trapped on surface of blot during incubation.	Gently pull membrane across edge of container to remove bubbles.
	Immobilized protein not exposed to incubation solutions.	Make sure the blot is placed in the container with the protein side facing upwards.
Contrast poor (chromogenic detection).	Blot has dried.	Re-wet blot in water.

Standard Immunodetection Method

Symptom	Possible Cause	Remedy
Uneven / splotchy results.	Blot was not wet with methanol prior to standard immunodetection.	Rewet blot in 100% methanol.
Low sensitivity.	Blocking agent has obscured proteins.	Too much blocking agent used or exposure time too long.

Rapid Immunodetection Method

Symptom	Possible Cause	Remedy
High background.	Blot was not completely dry prior to rapid immunodetection.	Blot must be dry. See **Membrane Drying Methods** in **Preparing the Blot** section.
	Membrane wet out during incubations.	After electrotransfer, rinse the blot in ultrapure water to wash out SDS carried over from the gel. Reduce the Tween 20 concentration in the antibody diluent to <0.04% (v / v). Agitate membrane more gently. Excessive friction may cause wetting.

Rapid Chemiluminescent Detection

Symptom	Possible Cause	Remedy
High background.	Blot was not completely dry prior to rapid immunodetection.	Blot must be dry. See **Membrane Drying Methods** in **Preparing the Blot** section.
	Antibody concentrations too high.	Decrease primary and secondary antibody concentrations.
	Washing incomplete.	Increase wash times and volumes.
Low sensitivity.	Antibody concentrations too low.	Increase primary and secondary antibody concentrations.
	Non-specific binding of antibody.	Gradually increase Tween 20 concentration (no more than 0.04% (v / v)).

Glossary

Blocking
Technique used to reduce non-specific binding of antibodies during immunodetection; unoccupied membrane sites are blocked with an inert protein or non-ionic detergent.

Blotting
Process of transferring proteins or nucleic acids from a gel to a membrane. A membrane with proteins immobilized on it is called a blot.

Chemiluminescent detection
Immunodetection technique which results in the production of visible light at the site of the protein of interest.

Chromogenic detection
Immunodetection technique which results in the deposit of a colored substance at the site of the protein of interest.

Electrotransfer
Common method for the transfer of proteins from a gel to a membrane. Proteins move from the gel and onto the membrane in an electrical field applied perpendicular to the plane of the gel.

Filtration
Direct application of sample onto a membrane. A dissolved sample is pulled through the membrane by applying a vacuum; proteins bind to the membrane and the other sample components pass through.

Gel
The substrate, usually polyacrylamide, on which sample proteins have been separated.

Immunodetection
Method of protein detection using a specific antibody to identify the location of a membrane-bound protein. The specificity of antibody-antigen binding permits the identification of a single protein in a complex sample.

Rapid Immunodetection
Faster method of immunodetection which eliminates the need for (and risks of) blocking.

Semi-dry transfer
Electrotransfer technique where the traditional buffer reservoir is replaced by layers of filter paper soaked in buffer. An equally effective, but far quicker technique than tank transfer.

Staining
Technique used to make protein bands visible on a blot. The colored stain may be reversible or non-reversible.

Stripping
Process of antibody removal from a membrane prior to a subsequent round of immunodetection.

Tank transfer
Traditional electrotransfer technique where the gel and membrane are immersed in a reservoir of buffer; an effective but slow technique.

Transillumination
Non-destructive, reversible technique used to make protein bands visible on a blot. The protein bands appear as clear areas when placed on a light box.

References

Beisiegel, U. "Protein Blotting." *Electrophoresis*. 7:1-18. 1986.

Gershoni, J. "Protein Blotting: A Manual." *Methods of Biochemical Analysis*. Wiley, New York. 33:1-58.

Immobilon-P Transfer Membrane User Guide. Millipore Product Lit. No. P15372.

Kyhse-Anderson, J. "Electroblotting of Multiple Gels: A Simple Apparatus Without Buffer Tank for Rapid Transfer of Proteins from Polyacrylamide to Nitrocellulose." *J. Biophys. Biochemistry Methods.* 10, 203-209. 1984.

LeGendre, N. "Immobilon-P Transfer Membrane: Applications and Utility in Protein Biochemical Analysis." *BioTechniques.* 9, 788. 1990.

Mansfield, M. "Protein Blotting Using Polyvinylidene Fluoride Membranes." *Protein Blotting: A Practical Approach*. Bonnie Dunbar, ed. pp. 33-52. IRL Press, Oxford. 1994.

Mansfield, M. "Rapid Immunodetection of Polyvinylidene Fluoride Membrane Blots Without Blocking." *Analytical Biochemistry.* 229, 140-143. 1995.

Rapid Immunodetection of Blotted Proteins Without Blocking. Millipore Application Note No. RP562. 1995.

Rapid Immunodetection Method on Immobilon™-P Using Chemiluminescence. Millipore Technical Note No. TN051. 1997.

Reig, J.A. and D.C. Klein. "Submicron Quantities of Unstained Proteins Are Visualized on Polyvinylidene Difluoride Membranes by Transillumination." *Applied and Theoretical Electrophoresis*. 1:59-60. 1988.

Stott, D.I. "Immunoblotting and Dot Blotting." *J. of Immunological Methods*. 119:153-187. 1989.

Towbin, H., Staehelin, T. and Gordon, J. "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications." *Proc. Natl. Acad. Sci*. USA 76, 4350-4354. 1979.

Ordering Information

Immobilon-P (PVDF) Transfer Membranes (0.45 µm pore size)

Description	Qty/pk	Catalogue No.
82 mm discs	50	IPVH 082 50
137 mm discs	50	IPVH 137 50
9 cm x 12 cm sheets	10	IPVH 091 20
10 cm x 10 cm sheets	10	IPVH 101 00
15 cm x 15 cm sheets	10	IPVH 151 50
20 cm x 20 cm sheets	10	IPVH 202 00
26 cm x 26 cm sheets	10	IPVH 304 F0
26.5 cm x 3.75 m roll	1	IPVH 000 10

To Place an Order or to Receive Technical Assistance

For additional information call the nearest number listed below:

In the U.S. and Canada, call toll-free: 1-800-MILLIPORE (1-800-645-5476).
In the U.S., Canada, and Puerto Rico, fax orders toll-free: 1-800-MILLIFX (1-800-645-5439).
On the Internet http://www.millipore.com/immobilon
E-mail: tech_service@millipore.com

Millipore Worldwide

AUSTRALIA
Millipore Australia Pty. Ltd.
9A Byfield Street
Locked Bag 2044
North Ryde NSW 2113
Tel: 1 800 222 111 (toll free) or
(02) 888 8999
Fax: (02) 878 0788

AUSTRIA, CENTRAL EUROPE, C.I.S., AFRICA, MIDDLE EAST AND GULF
Millipore Ges.m.b.H.
Hietzinger Hauptstrasse 145
A-1130 Wien, Austria
Tel: (43) 1 877-8926
Tlx: 131464 miliv a
Fax: (43) 1 877-1654

BALTIC COUNTRIES
Millipore Oy
Lanttikatu 2
FIN-02770 Espoo, Finland
Tel: 358-9-804 5110
Fax: 358-9-859 6616

BELGIUM AND LUXEMBOURG
Millipore S.A.-N.V.
Rue de la Fusée 60
Raketstraat 60
B-1130 Brussels
Tel: (02) 726 88 40
Fax: (02) 726 98 84

BRAZIL
Millipore Indústria e
Comércio Ltda.
Rua Prof. Campos de
Oliveira, 430
CEP 04675-100
Saõ Paulo-SP
Tel: (011) 548-7011
Tlx: (011) 57751 MILY BR
Fax: (011) 548-7923

CANADA
Analytical Division
Millipore (Canada) Ltd.
36 Antares Drive, Suite 700
Nepean, Ontario K2E 7W5
Tel: 1-800-MILLIPORE
(1-800-645-5476)
Fax: (613) 225-9366
BioProcess Division
Millipore (Canada) Ltd.
109 Woodbine Downs Blvd.
Unit 5
Etobicoke, Ontario M9W 6YI
Tel: (416) 675-1598
Fax: (416) 675-1846

CHINA, PEOPLE'S REPUBLIC OF
Millipore China Ltd.
Room 302 Office Bldg.
Asia Jinjiang Hotel
8 Xinzhong Xijie
Gongti Bei Lu
Beijing 100027
Tel: (86-10) 65008063
Fax: (86-10) 65007372

Millipore Asia/China Ltd.
1406 City Plaza 4
12 Taikoo Wan Road
Hong Kong
Tel: (852) 2803-9111
Fax: (852) 2513-0313

Millipore China Ltd.
Shanghai Office
Suite 2806 Union Bldg.
100 Yan An Road (E)
Shanghai 20002
Tel: (86-21) 63737256
Fax: (86-21) 63200236

CZECH REPUBLIC
Millipore spol. s.r.o.
Ricanova 21
16900 Praha 6
Tel: 02-205 138 41
02-205 138 42
Fax: 02-35 23 75

DENMARK
Millipore A/S
Baldersbuen 46
DK-2640 Hedehusene
Tel: 46 59 00 23
Fax: 46 59 13 14

FINLAND
Millipore Oy
Lanttikatu 2
FIN-02770 Espoo
Tel: (90) 804 5110
Fax: (90) 859 6616

FRANCE
Millipore S.A.
BP 307
F-78054 Saint-Quentin
Yvelines CEDEX
Tel: (01) 30.12.70.00
Fax: (01) 30.12.71.80

GERMANY
Millipore GmbH
Postfach 5647
D-65731 Eschborn
Tel: (06196) 494-0
Fax: (06196) 494 163

HUNGARY
Millipore Kft.
Andor u. 47-49
1119 Budapest
Tel: 1-205 9784
Fax: 1-205 9792

INDIA
Millipore (India) Pvt. Ltd.
50A 2nd Phase Ring Road
Peenya
560 058 Bangalore
Tel: (91) 80-839 63 20
(91) 80-839 46 57
Fax: (91) 80-839 63 45

ITALY
Millipore S.p.A.
Via XI Febbraio, 99
20090 Vimodrone (Milano)
Tel: (02) 250781
Fax: (02) 2650324

Millipore S.p.A.
Via D. Sansotta 100
I-00144 Roma
Tel: (06) 5203600
Fax: (06) 5295735

JAPAN
Nihon Millipore Ltd.
Mita Kokusai Bldg.
4-28, Mita 1-Chome
Minato-ku
Tokyo 108
Tel: (03) 5442-9716
Fax: (03) 5442-9736

KOREA
Millipore Korea Co. Ltd.
13 FL, Yuksung Building
706-25, Yoksam-dong
Kangnam-ku
Seoul 135-080
Tel: (82-2) 538-6215
Fax: (82-2) 563-6253

MALAYSIA
Millipore Asia Ltd.
Malaysia Branch
B4- 05, PJ. Industrial Park
Section 13, Jalan Kemajuan
46200 Petaling Jaya
Selangor
Tel: (60) 3-7571322
Fax: (60) 3-7571711

MEXICO
Millipore S.A. de C.V.
Ings. Militares 85-PB
Col. Argentina Poniente
11230 México, D.F.
Tel: (525) 576-9688
Fax: (525) 576-8706

THE NETHERLANDS
Millipore B.V.
Penningweg 33
Postbus 166
NL-4870 AD Etten-Leur (N.B.)
Tel: 076-5022000
Fax: 076-5022436

NORWAY
Millipore AS
Postboks 39 Simensbraten
N-1107 Oslo
Tel: 22 67 82 53
Fax: 22 68 53 15

POLAND
Millipore (Austria) Ges.m.b.H.
Oddzial w Warszawie
ul. Jasnodworska 7
01745 Warszawa
Tel: 22-669 12 25
22-663 70 31
Fax: 22-663 70 33

PUERTO RICO
Millipore Cidra Inc.
P.O. Box 11977
Cidra, P.R. 00739-1977
Tel: (787) 273-8495
Fax: (787) 747-8449

SINGAPORE
Millipore Asia Ltd.
#07-01 Jumbo Industrial Bldg.
19 Kim Keat Road
Singapore 328804
Tel: (65) 253 2733
Fax: (65) 254 4056

SPAIN AND PORTUGAL
Millipore Iberica, S.A.
Avda. Llano Castellano,13
E-28034 Madrid
Tel: (91) 729 03 00
Fax: (91) 729 29 09

Millipore Ibérica, S.A.
Balmes, 89-91
E-08008 Barcelona
Tel: (93) 451 70 00
Fax: (93) 451 60 48

SWEDEN
Millipore AB
Box 7090
S-172 07 Sundbyberg
Tel: 08-628 69 60
Fax: 08-628 64 57

SWITZERLAND
Millipore AG
Chriesbaumstrasse 6
CH-8604 Volketswil
Tel: (01) 908-30-60
Fax: (01) 908-30-80

TAIWAN
Millipore Asia Ltd.
Unit #707, 7th fl
136 Sec.3 Jen-Ai Road
Taipei Taiwan R.O.C.
Tel: (886-2) 7001742
Fax: (886-2) 7553267

U.K. AND IRELAND
Millipore (U.K.) Ltd.
The Boulevard
Blackmoor Lane
Watford, Hertfordshire
WD1 8YW
Tel: (01923) 816375
Fax: (01923) 818297

U.S.A.
Millipore Corporation
80 Ashby Road
Bedford, MA 01730-2271
Tel: (617) 275-9200
Fax: (617) 275-5550

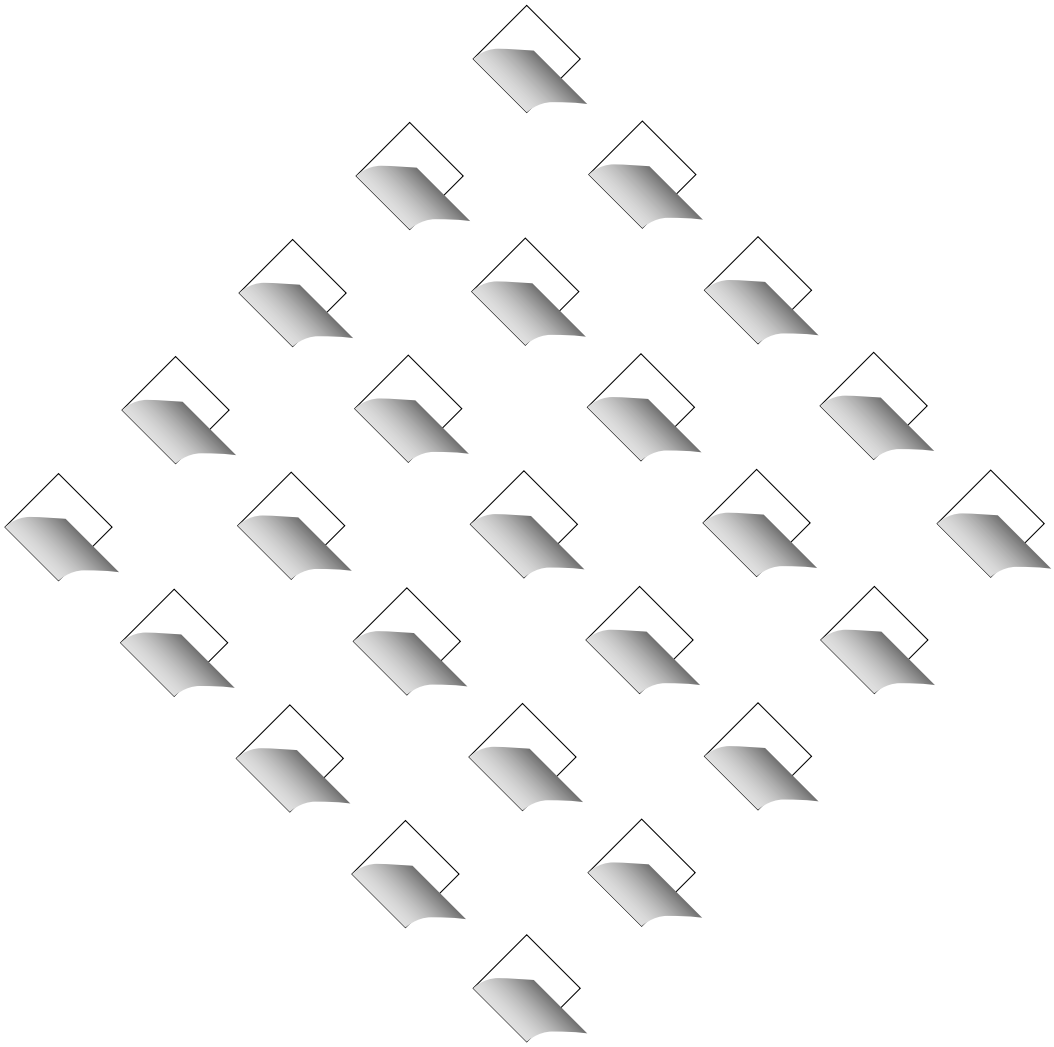
IN ALL OTHER COUNTRIES
Millipore Intertech
P.O. Box 255
Bedford, MA 01730 U.S.A.
Tel: +1 (617) 275-9200
Fax: +1 (617) 533-8630

MILLIPORE

Lit. No. TP001 Printed in U.S.A. Revised 6/97

‰ Printed with soy ink on recycled paper.

PROTEIN BLOTTING APPLICATIONS GUIDE



Â