Protein Blotting Handbook
Tips and Tricks

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About the Sixth Edition

With the publication of the sixth edition of the Protein Blotting Handbook, Merck Millipore continues to keep researchers up to date on innovations in protein detection. We've added more comprehensive guidance on optimizing antibody concentrations and reducing background, along with expanded data and protocols for fluorescence detection.

This handbook represents the collective experience of our application scientists, who are actively engaged in advancing the science of protein blotting and detection. It also includes many of the most common recommendations provided by our technical service specialists who are contacted by scientists worldwide for assistance.

Better membranes, better blots.

Merck Millipore has been a leading supplier of transfer membranes for nearly four decades. E.M. Southern used these membranes to develop the first nucleic acid transfer from an agarose gel in 1975[1]. The first 0.45 µm PVDF substrate for Western blotting, Immobilon®-P membrane, was introduced in 1985, and the first 0.2 µm PVDF substrate for protein blotting and sequencing, Immobilon®-P^{SQ} membrane, was introduced in 1988.

In addition to Immobilon® membranes and reagents, Merck Millipore provides a wide selection of other tools for protein research, including gentle protein extraction kits, rapid protein isolation with PureProteome™ magnetic beads, and fast, effective concentration with Amicon® Ultra centrifugal filters.

Where to Get Additional Information

If you have questions or need assistance, please contact an Merck Millipore technical service specialist, or pose your question online at: **www.merckmillipore.com/techservice**

You'll also find answers to frequently asked questions (FAQs) concerning Western blotting and other related methods at: **www.merckmillipore.com/wb_faqs**

1. Southern E. J Mol Bio 1975;98:503.

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Introduction

Since its introduction in 1979 (Towbin et al., 1979), protein blotting has become a routine tool in research laboratories. It is traditionally used to detect low amounts of proteins in complex samples or to monitor protein expression and purification. The simplest protein blotting procedure, known as dot blot or slot blot, uses vacuum filtration to transfer protein onto a microporous membrane. While this method may provide qualitative information about total protein expression levels and can be performed on multiple samples in parallel, it lacks information on protein molecular weight. Also, specificity can be compromised as protein degradation products or post-translationally modified isoforms may be detected along with the intact protein.

A more complex procedure, Western blotting, involves the separation of a protein mixture by gel electrophoresis with subsequent electrotransfer to a suitable membrane (e.g., PVDF). After proteins have been transferred onto a PVDF membrane, they can be stained for visualization and directly identified by N-terminal sequencing, mass spectrometry or immunodetection. Immunodetection involves the identification of a protein through its reaction with a specific antibody. Through spatial resolution, this method provides molecular weight information on individual proteins and distinguishes isoforms, alternate processing products, and other post-translationally modified forms.

In the clinical laboratory, immunoblotting has proven useful in fields such as infectious and autoimmune diseases, allergy and others (Towbin et al., 1989; Stahl et al., 2000). Western blotting is considered to be a reliable confirmatory diagnostic test following a repeatedly reactive ELISA over the course of viral infection, and is a sensitive, unequivocal and simple assay, providing high complexity of information (Bauer, 2001; Mylonakis et al., 2000; Heermann et al., 1988).

Examples of Western blotting applications include analysis of protein expression in yeast by quantitative Western analysis (Ghaemmadami et al., 2003), determination of protein copy number and compartmentalization (Rudolph et al., 1999), study of competitive protein kinase inhibition by ATP (Wang and Thompson, 2001), and detection of genetically modified organisms in crops and foods (Ahmed, 2002).

Clearly, protein blotting remains the platform of choice for exploratory research, and is still the standard by which new antibodies and other protein detection assays (such as ELISA, bead-based assays, flow cytometry and immunohistochemistry) are evaluated. However, the need to analyze more proteins simultaneously to characterize complex networks and the associated need to conserve valuable samples has driven ongoing research into improving the sensitivity and speed of blotting techniques. A "double blotting" method (Lasne, 2001, 2003) eliminates false positives due to strong nonspecific interactions between the blotted proteins and unrelated secondary antibodies. Far-Western blotting enables the detection of specific protein-protein interactions (Grasser, 1993) and Southwestern blotting is used to identify proteins that interact with specific DNA sequences (Silva, 1987). Multistrip Western blotting has proved to increase throughput while minimizing inter-blot variability (Aksamitiene, 2007). A new generation of blotting technologies features reductions in the amounts of protein required to produce a signal (Swank, 2006) and methods to improve the quantitative power of Western blotting (Schilling, 2005a; 2005b).

1 Membrane Selection

The type of membrane used for blotting can influence the following factors:

- Protein binding capacity
- Requirement for prewetting with alcohol
- Ability to perform multiple stripping and reprobing experiments
- Protein visualization
- Long-term blot storage
- Signal-to-noise ratio

Polyvinylidene fluoride (PVDF) and nitrocellulose are the two membrane types most commonly used in Western blotting applications.

There are many advantages to electroblotting onto PVDF membranes rather than onto nitrocellulose membranes. PVDF membranes offer better protein retention, physical strength and broad chemical compatibility (Pluskal, et al., 1986). The higher mechanical strength and superior chemical resistance of PVDF membranes make them ideal for a variety of staining applications and reprobing in immunodetection. Another advantage of using PVDF membranes is that replicate lanes from a single gel can be used for various applications, such as staining with Coomassie® Blue dye followed by band excision and N-terminal sequencing, proteolysis/peptide separation/internal sequencing, and immunodetection (Kurien, et al., 2003). Typical binding capacity of commercially available nitrocellulose membranes is 80 – 100 µg/cm^2 while PVDF membranes offer a binding capacity of 100 – 200 µg/cm^2.

In a direct comparison of PVDF vs. nitrocellulose membranes in detecting human immunodeficiency virus (HIV) antigens in serum, PVDF membrane was shown to have better retention of total HIV antigens and improved detection of antibodies to glycosylated envelope antigens (Lauritzen and Pluskal, 1988). See Table 1 for a comparison of nitrocellulose and PVDF membrane attributes.

Table 1. Comparison of PVDF and nitrocellulose membrane attributes and applications.

Attributes/Applications	Nitrocellulose	PVDF
Physical strength	Poor	Good
Protein binding capacity	80 – 100 µg/cm^2	100 – 300 µg/cm^2
Solvent resistance	No	Yes
Western transfer	Yes	Yes
Total protein stain	Colloidal gold	Colloidal gold
	Ponceau-S red	Ponceau-S red
	Amido black	Amido black
	India ink	India ink
	Sypro® blot stains	Coomassie® Blue dye
Detection	Chromogenic	Chromogenic
	Chemiluminescent	Chemiluminescent
	Fluorescent	Fluorescent
	Radioactive	Chemifluorescent
		Radioactive
Double-blotting method	No	Yes
Rapid immunodetection	No	Yes
Western reprobing	Yes	Yes
Edman sequencing	No	Yes
Amino acid analysis	Yes	Yes
Binding in the presence of SDS	Poor	Good
On-membrane digestion or mass spectrometry	No	Yes
Direct MALDI-TOF MS analysis	No	Yes
Data can be archived	No	Yes

Immobilon® PVDF Transfer Membranes

Merck Millipore offers three PVDF membranes:

- **Immobilon®-P membrane** (0.45 µm) is a versatile substrate that is well suited for common immunoblotting applications
- **Immoblion®-P^{SQ}** membrane (0.2 µm) is ideal for protein sequencing and immunoblotting of low molecular weight proteins. It has a higher protein binding capacity and a higher retention than 0.45 µm membranes.
- **Immobilon®-FL membrane** (0.45 µm) was developed for fluorescence-based immunodetection. It has very low background fluorescence across a wide range of excitation and emission wavelengths.

Immobilon® transfer membranes are available in rolls and cut sheets. Precut membranes are compatible with all precast gels and most commercially available gel running systems. See Table 2 for properties of Immobilon® transfer membranes. See Table 3 (page 8) to match sizes of precut Immobilon® membrane with the most commonly used electrophoresis systems. See Table 4 (page 9) to match Immobilon® membrane cut sizes with available precast gels.

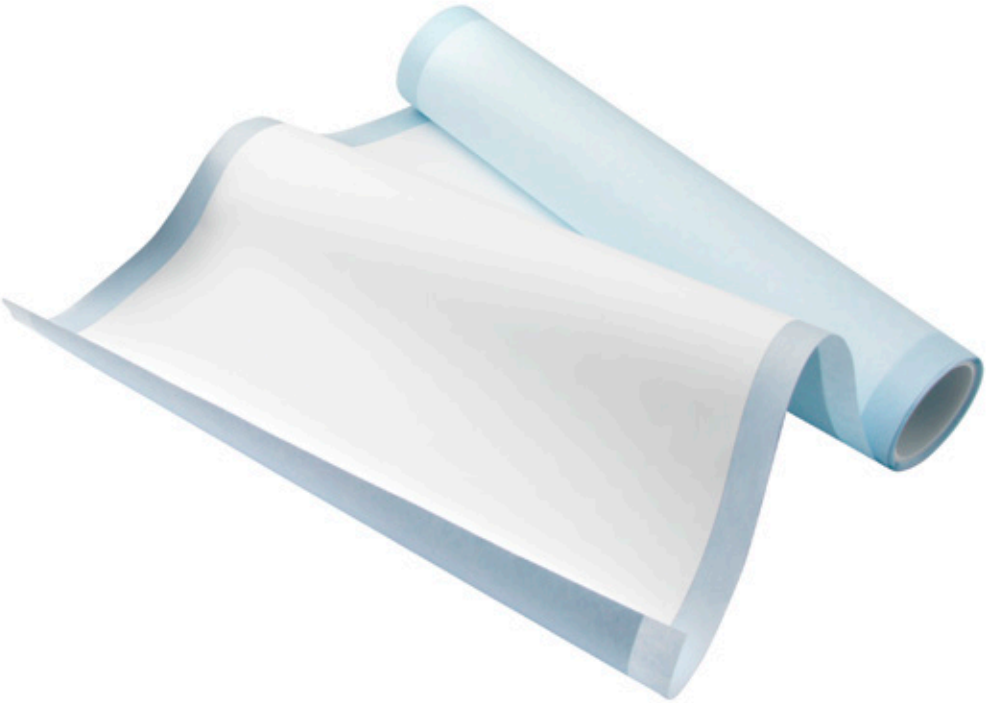


Table 2. Properties and applications of Immobilon® PVDF transfer membranes.

	Immobilon®-P transfer membrane	Immobilon®-P^{SQ} transfer membrane	Immobilon®-FL transfer membrane
Description	Optimized to bind proteins transferred from a variety of gel matrices	Uniform pore structure results in superior binding of proteins with MW <20 kDa	Optimized for fluorescence immunodetection applications
Composition	PVDF	PVDF	PVDF
Pore size	0.45 µm	0.2 µm	0.45 µm
Phobicity	Hydrophobic	Hydrophobic	Hydrophobic
Applications	Western blotting	Low molecular weight	Western blotting
	Binding assays	Western blotting	Dot/slot blotting
	Amino acid analysis	Amino acid analysis	Fluorescence immunodetection
	N-terminal protein sequencing	Mass spectrometry	
	Dot/slot blotting	N-terminal protein sequencing	
	Glycoprotein visualization		
	Lipopolysaccharide analysis		
	Mass spectrometry		
Detection methods	Chromogenic	Chromogenic	Fluorescent
	Chemifluorescent	Fluorescent	Chromogenic
	Chemiluminescent	Chemifluorescent	Chemifluorescent
	Radioactive	Chemiluminescent	Chemiluminescent
		Radioactive	
Protein binding capacity	Insulin: 160 µg/cm^2	Insulin: 262 µg/cm^2	Insulin: 155 µg/cm^2
	BSA: 215 µg/cm^2	BSA: 340 µg/cm^2	BSA: 205 µg/cm^2
	Goat IgG: 294 µg/cm^2	Goat IgG: 448 µg/cm^2	Goat IgG: 300 µg/cm^2
Compatible stains reversible	Transillumination	Transillumination	Transillumination
	Ponceau-S	Ponceau-S	Ponceau-S
	CPTS	CPTS	CPTS
	Toluidine blue	Toluidine blue	Sypro® blot stains
	Sypro® blot stains	Sypro blot stains	
Compatible stains irreversible	Coomassie® Brilliant Blue dye	Coomassie® Brilliant Blue dye	Coomassie® Brilliant Blue dye
	Amido black	Amido black	Amido black
	India ink	India ink	
	Colloidal gold	Colloidal gold	

Table 3. Immobilon® PVDF transfer membrane cuts and matching electrophoresis systems.

Manufacturer	Vertical Gel Box	Gel size (cm)	Immobilon® Size (cm)	Immobilon®-P 0.45 µm	Immobilon®-P^{SQ} 0.2 µm	Immobilon®-FL 0.45 µm*
GE Healthcare	SE 250 Mighty Small™	8 x 7	8.4 x 7	IPVH07850	ISEQ07850	
	SE 260 Mighty Small™	8 x 9.5	8 x 10	IPVH08100	ISEQ08100	
	miniVE	8 x 9.5	8 x 10	IPVH08100	ISEQ08100	
	miniVE	10 x 10	10 x 10	IPVH10100	ISEQ10100	IPFL10100
	SE 400	14 x 16	15 x 15	IPVH15150	ISEQ15150	
	SE 600	14 x 16	15 x 15	IPVH15150	ISEQ15150	
	SE 600	14 x 8	13.5 x 8.5	IPVH08130	ISEQ08130	
Bio-Rad	Mini-PROTEAN® 3, Mini-PROTEAN® 3 Dodeca™	8.3 x 7.3	8.4 x 7	IPVH07850	ISEQ07850	
	Criterion™, Criterion™ Dodeca™	13.3 x 8.7	13.5 x 8.5	IPVH08130	ISEQ08130	
	PROTEAN® II xi	16 x 16	15 x 15	IPVH15150	ISEQ15150	
	PROTEAN® II xi	16 x 20	26.5 x 375	IPVH00010	ISEQ00010	IPFL00010
	PROTEAN® II XL	19.3 x 18.3	20 x 20	IPVH20200	ISEQ20200	IPFL20200
	PROTEAN® Plus Dodeca™	20 x 20.5	26 x 26	IPVH304F0	ISEQ304F0	
	Mini-PROTEAN® II	8.3 x 7.3	8.4 x 7	IPVH07850	ISEQ07850	
Invitrogen	XCell SureLock™ Mini-Cell, XCell6™ MultiGel	8 x 8	8.4 x 7	IPVH07850	ISEQ07850	
Thermo Scientific	P81 Puffin™, P82 Wolverine™, P8DS Emperor Penguin™	10 x 10	10 x 10	IPVH10100	ISEQ10100	IPFL10100
	P8DS Emperor Penguin™	8 x 10	8 x 10	IPVH08100	ISEQ08100	
	P9DS Emperor Penguin™	16 x 16	15 x 15	IPVH15150	ISEQ15150	
	P10DS Emperor Penguin™	20 x 20	20 x 20	IPVH20200	ISEQ20200	IPFL20200

Table 4. Immobilon® PVDF transfer membrane cuts and matching pre-cast gels.

Manufacturer	Precast Gel Name	Gel size (cm)	Immobilon® Size (cm)	Immobilon®-P 0.45 µm	Immobilon®-P^{SQ} 0.2 µm	Immobilon®-FL 0.45 µm*
Bio-Rad	Ready Gel®	8.3 x 7.3	8.4 x 7	IPVH07850	ISEQ07850	
	Criterion™	13.3 x 8.7	13.5 x 8.5	IPVH08130	ISEQ08130	
	PROTEAN Ready Gel®	16 x 16	15 x 15	IPVH15150	ISEQ15150	
	PROTEAN Ready Gel®	19.3 x 18.3	20 x 20	IPVH20200	ISEQ20200	IPFL20200
	PROTEAN Ready Gel®	20 x 20.5	26 x 26	IPVH304F0	ISEQ304F0	
Cambrex	PAGEr®	9 x 10	8 x 10	IPVH08100	ISEQ08100	
	PAGEr®	10 x 10	10 x 10	IPVH10100	ISEQ10100	IPFL10100
Gradipore	MicroGel®	8 x 2.5	8 x 10 (cut in 1/2)	IPVH08100	ISEQ08100	
	igels™	8 x 5.8	8 x 10 (cut in 1/2)	IPVH08100	ISEQ08100	
	LongLife Gels	8 x 5.8	8 x 10 (cut in 1/2)	IPVH08100	ISEQ08100	
Invitrogen	NuPAGE®	8 x 8	8.4 x 7	IPVH07850	ISEQ07850	
	Novex®	8 x 8	8.4 x 7	IPVH07850	ISEQ07850	
	Zoom®	8 x 8	8.4 x 7	IPVH07850	ISEQ07850	
	E-Gel®	13.5 x 10.8	13.5 x 8.5	IPVH08130	ISEQ08130	
Thermo Scientific	Precise Protein Gels	8 x 5.8	8 x 10 (cut in 1/2)	IPVH08100	ISEQ08100	

*All sizes can be cut from rolls of Immobilon®-FL membrane (IPFL00010).

2 Protein Binding

PVDF is an inherently hydrophobic polymer and will not wet out in aqueous solutions. In order to use PVDF membrane with aqueous buffers and systems, it must first be wet in a 50% (v/v) or greater concentration of alcohol. Methanol, ethanol, and isopropanol are suitable to wet the membrane. Complete wetting is evident by a change in the membrane's appearance from opaque to semi-transparent. The alcohol must be removed from the membrane by extensive rinsing in water, and the membrane can then be directly equilibrated in transfer buffers.

Binding Differences between Immobilon®-P and Immobilon®-P^{SQ} Transfer Membranes

Once the membrane is wet, protein binding can be achieved by simply bringing the protein into contact with the membrane. Because binding occurs throughout the depth of the membrane, the binding capacity is determined by the internal surface area of the pores (Mansfield, 1994). Immobilon®-P^{SQ} transfer membrane has approximately three times the internal surface area of Immobilon®-P transfer membrane, resulting in higher adsorptive capacity (Table 2, page 7). The values listed in Table 2 represent upper limits for protein binding after saturation of the membrane surface in a non-denaturing buffer. In any given application, Immobilon®-P^{SQ} transfer membrane can be expected to bind more protein than Immobilon®-P transfer membrane. However, the maximum binding that can be achieved will depend on the specific protocols employed, due to variations in the structural conformation of the proteins, the chemical nature of the buffers used, and the limitations of the methods used to apply the sample.

An example of the binding difference between Immobilon®-P and Immobilon®-P^{SQ} transfer membranes is shown in Figure 1, where protein samples were electrotransferred from a polyacrylamide gel. A fraction of the proteins passed through the Immobilon®-P transfer membrane and were captured on a second membrane placed behind the first one. In contrast, all of the proteins were bound to the Immobilon®- P^{SQ} membrane without passing through it. In this case, the tighter pore structure and higher internal surface area of polymer facilitated complete adsorption of all of the transferred protein. However, immunodetection on Immobilon®-P^{SQ} transfer membrane can result in a higher background and can require more stringent washing conditions. Thus, the choice of membrane is dictated by the goal of the experiment: use Immobilon®-P transfer membrane for high sensitivity detection of >20 kDa proteins, but switch to Immobilon®-P^{SQ} transfer membrane if smaller proteins are being analyzed or 100% protein capture is necessary for peptide sequencing.

Factors Affecting Protein Binding

At the molecular level, protein adsorption results, at least in part, from the interaction of hydrophobic amino acid side chains and hydrophobic domains with the polymer surface. Matsudaira (1987) observed an 80% decline in sequencing efficiency of small peptides after hydrophobic residues were cleaved, presumably due to washout of the peptide remnants. Also, in peptide digestions, it has been observed that peptides characterized as hydrophobic often do not elute from the membrane as efficiently as more hydrophilic peptides (e.g., Iwamatsu, 1991; Fernandez et al., 1992). McKeon and Lyman (1991) demonstrated that addition of Ca^{2+} ions to the transfer buffer enhanced the binding of calmodulin to Immobilon®-P transfer membrane. Binding of the calcium causes formation of a hydrophobic pocket in the molecule's structure.

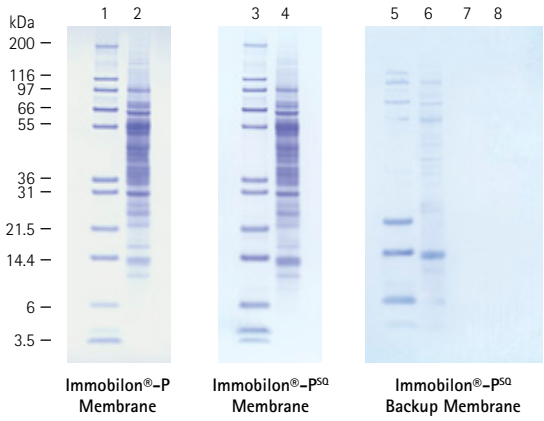


Figure 1.

Prolonged electrotransfer of proteins using Immobilon®-P and Immobilon®-P^SQ transfer membranes. Molecular weight standards (lanes 1,3,5,7) and calf liver lysate (lanes 2,4,6,8) were transferred to Immobilon®-P or Immobilon®-P^SQ membranes by the tank transfer method and stained with Coomassie® Blue. A sheet of Immobilon®-P^SQ transfer membrane was placed behind the primary membranes to capture proteins that passed through them. (Lanes 5 and 6 behind Immobilon®-P; lanes 7 and 8 behind Immobilon®-P^SQ.)

3 Blotting Methods: Principles and Optimization

Filtration

Filtration is a direct method of applying proteins onto a membrane. A dissolved sample is filtered through the membrane by applying vacuum. Proteins adsorb onto the membrane, and the other sample components are pulled through by the vacuum (Figure 2). Alternatively, the sample can be spotted directly onto the surface and allowed to dry. The proteins immobilized on the membrane can then be analyzed.

Dot blotting (Figure 3) and slot blotting are two variations of the filtration method, employing manifolds that permit application of samples to the membrane in dot or slot patterns. These techniques can be used as qualitative method for rapid screening of a large number of samples or as a quantitative technique for analysis of similar samples. It is especially useful for testing the suitability of experimental design parameters to be used in more complex analyses.

Another variation of the filtration method is grid immunoblotting, a technique useful for highly parallel sample analysis when the amount of sample is extremely limited and analysis cannot be performed by conventional techniques such as ELISA. For example, grid immunoblotting has been used in the characterization of allergen-specific antibody response with minimal amounts of patient serum (Reese et al., 2001).

When preparing blots by filtration, consider the following:

- Detergents can inhibit the adsorption of proteins to the membrane. Buffers used for sample dissolution and washing should contain no more than 0.05% detergent, and only if required.
- The sample volume should be large enough to cover the exposed membrane in each well but should not contain protein in excess of the binding capacity of the membrane.
- Samples with high particulate loads may clog the membrane, while those with high viscosity will reduce the flow rate. Particles should be removed by prefiltration or centrifugation, and only the supernatant should be applied to the membrane. Viscous samples should be diluted in buffer.

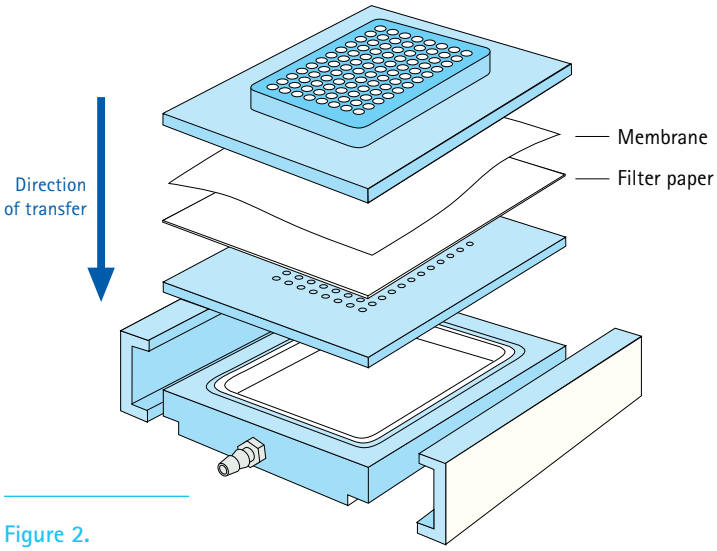


Figure 2.

A blotting system using filtration.

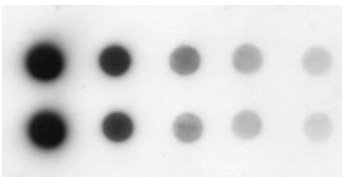


Figure 3.

Detection of transferrin on a spot blot of human serum (See Protocol 1.4. Spot Blotting, page 40) using Immobilon® Western Chemiluminescent HRP Substrate. Transferrin was detected in duplicate serial dilutions of serum with goat anti-transferrin antibody (dilution 1:10,0000) and rabbit anti-goat HRP-conjugated secondary antibody (dilution 1:50,000) on Immobilon®-P membrane.

Western Blotting

Western blotting comprises the following steps:

- Resolution of a complex protein sample in a polyacrylamide gel.
- Transfer of the resolved proteins onto a membrane.
- Identification of a specific protein on the membrane.

For a successful Western blot, four requirements must be met:

- Elution from the gel — the protein must elute from the gel during transfer. If it is retained in the gel, it will not be available for analysis on the blot.
- Adsorption to the membrane — the protein must adsorb to the membrane during the transfer process. If the protein is not adsorbed, it will not be available for analysis on the blot.
- Retention during processing — the protein must remain adsorbed to the membrane during post-transfer processing of the blot.
- Accessibility during processing — the adsorbed protein must be available to the chemistries used to detect it. If the protein is masked, it cannot be detected.

The sections that follow discuss theoretical and practical considerations of the protocols involved in Western blotting.

Separation of Complex Protein Mixtures in 1-D or 2-D Gels

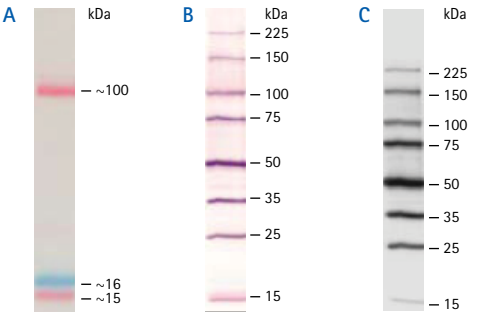
The most common way of separating complex protein mixtures prior to the blotting is one-dimensional (1-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), where proteins are separated on the basis of their molecular weight (Figure 4). In some cases, non-denaturing conditions are used to separate native proteins. Although this method usually lacks the resolution of denaturing electrophoresis, it may be particularly useful when the primary antibody recognizes only non-denatured proteins or when the protein's biological activity has to be retained on the membrane.

Two-dimensional (2-D) gel electrophoresis is the technique of choice for analyzing protein composition of cell types, tissues and fluids, and is a key technology in proteomics. Immunoblotting of 2-D gels provides information on molecular weight and isoelectric point and can be useful to discriminate protein isoforms generated by post-translational modifications (Celis and Gromov, 2000). In some cases, protein phenotyping can be achieved by immunoblotting after only a 1-D separation by isoelectrofocusing (Poland, et al., 2002; Eto et al., 1990). An example of a 2-D blot is shown in Figure 5.

Molecular Weight Markers

The inclusion of molecular weight (MW) standards, or markers, on the gel facilitates the estimation of the sizes of the proteins of interest after resolution by electrophoresis. Two types are available, unstained and pre-stained. Unstained MW markers usually consist of a mixture of purified native or recombinant proteins of defined molecular weights. Visualizing their location on a gel or membrane requires a staining step.

Pre-stained MW markers are shown in Figure 4. There are both advantages and disadvantages to using pre-stained markers. Pre-stained markers allow monitoring of protein separation in the gel during electrophoresis. They also indicate transfer efficiency in the subsequent blotting steps. However, they can be relatively expensive and the addition of dyes may affect protein mobility. Pre-stained markers may be less accurate for molecular weight determination, as dyes attached to the proteins may alter their ability to adsorb to the membrane during blotting.



A Unstained gel and Western transfer

B AP Western blot (S-protein AP conjugate) colorimetric detection

C AP Western blot (S-protein AP conjugate) chemiluminescent detection

Figure 4.

With three prestained markers and eight unstained markers (which have both His tag and S·Tag), Trail Mix™ protein markers (Catalogue No. 70982) enable direct visualization of protein migration during electrophoresis and accurate sizing on any Western blot.

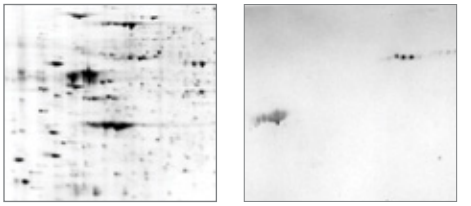


Figure 5.

Chemiluminescent detection of proteins separated by two-dimensional electrophoresis. Silver-stained 2-D gel of rat fibroblast cell line (left) and blot of the same gel (right), probed with a mouse monoclonal antibody and visualized using chemiluminescence on Immobilon®-P membrane.

Polyacrylamide Concentration

The concentration of polyacrylamide in the gel can be homogenous or a gradient. The most common polyacrylamide concentration, 10%, is best suited for the separation of proteins in the range of 10 –150 kDa. If unknown proteins are being analyzed or a broader range of separation is desired, gradient gels are recommended. For example, 4 –12% Tris-glycine gels are suitable for proteins in the range of 30 to 200 kDa, while 10 – 20% gels will successfully separate proteins from 6 to 150 kDa. SDS-PAGE gels are usually 1.0 and 1.5 mm thick; however, for blotting, proteins transfer best out of thinner gels (= 1 mm).

Gel Running Buffers

Most common gel running buffers are composed of Tris-glycine or Tris-tricine. Buffers may contain 0.1% detergent, usually SDS. Tris-glycine buffer systems are useful for separation of proteins over a wide range of molecular weights (6 – 200 kDa) and are compatible with denaturing or non-denaturing conditions. Tris-tricine systems are best for the separation of smaller proteins (<10 kDa) that need to be reduced and denatured prior to loading. Both buffer systems are compatible with protein transfer to PVDF membranes. Tris-acetate buffers are sometimes used for separation of larger proteins.

Transfer of Proteins from Gel to Membrane

The process of transferring proteins from a gel to a membrane while maintaining their relative position and resolution is known as blotting. Blotting can be achieved in three different ways:

Simple diffusion (Kurien and Scofield, 1997) is accomplished by laying a membrane on top of the gel with a stack of dry filter paper on top of the membrane, and placing a weight on top of the filter paper to facilitate the diffusion process (Kurien and Scofield, 2003). This method can be used to transfer proteins from one gel to multiple membranes (Kurien and Scofield, 1997), obtaining several imprints of the same gel. The major disadvantage of the diffusion method is that transfer is not quantitative and only transfers 25 – 50% of the proteins as compared to electroblotting (Chen and Chang, 2001).

Vacuum-assisted solvent flow (Peferoen et al., 1982) uses the suction power of a pump to draw separated proteins from the gel onto the membrane. Both high and low molecular weight proteins can be transferred by this method; however, a smaller pore size membrane (0.2 µm) may be needed for proteins with MW <20 kDa, since they are less readily adsorbed by the 0.45 µm membrane (Kurien, 2003). Vacuum blotting of proteins out of polyacrylamide gels is uncommon and is mostly used for nucleic acid transfer from agarose gels.

Electrophoretic elution, or electrotransfer (Towbin et al., 1979) is by far the most commonly used transfer method. The principal advantages are the speed and completeness of transfer compared to diffusion or vacuum blotting (Kurien et al., 2003).

Electrotransfer Techniques

The two commonly used electrotransfer techniques are tank transfer and semi-dry transfer. Both are based on the same principles and differ only in the mechanical devices used to hold the gel/membrane stack and applications of the electrical field.

Tank transfer (Figure 6) is the traditional technique where the gel/membrane stack is completely immersed in a buffer reservoir and then current is applied. It is an effective but slow technique, using large volumes of buffer. Tank systems are typically run at constant voltage; mixing of the buffer during transfer keeps the current relatively constant.

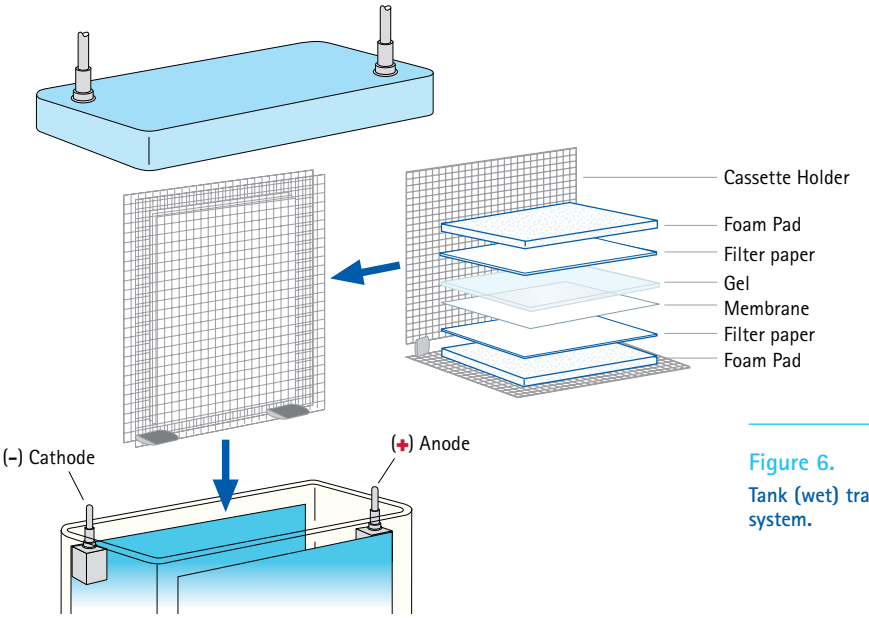


Figure 6.
Tank (wet) transfer system.

Semi-dry transfer (Figure 7) replaces the buffer reservoir with layers of filter paper soaked in buffer. Because the plate electrodes are in direct contact with the filter papers, the field strength across the gel is maximized for fast, efficient transfers. This technique is as effective and far quicker (15 – 45 minutes) than tank transfer. Most semi-dry transfer methods use more than one buffer system to achieve efficient transfer of both large and small proteins. However, semi-dry blotting systems have lower buffering capacity and thus are inappropriate for prolonged transfers. Semi-dry transfer is the preferred method for blotting large 2-D gels. Semi-dry blotters are typically run at constant current; the voltage normally increases during the transfer period.

For semi-dry transfer systems, it is important that the filter papers and membrane are cut to the same size as the gel so that the current is forced to flow through the gel. Otherwise, the current will short-circuit through overlapping filter paper around the edges of the gel. In both types of transfer systems, extra caution should be taken to prevent introduction of air bubbles

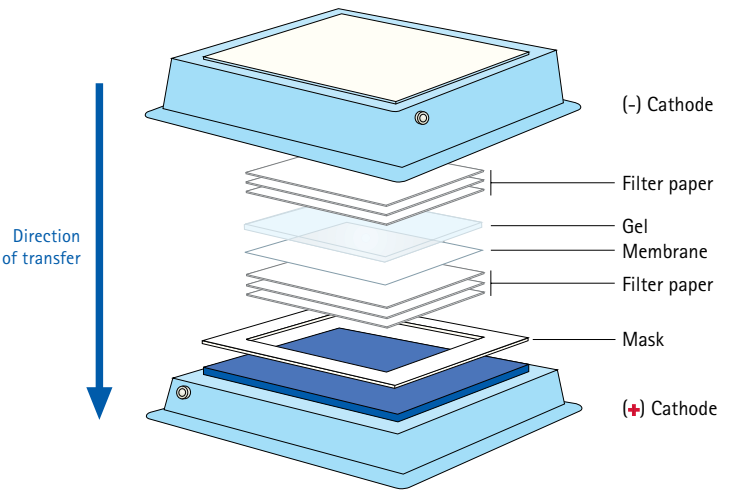


Figure 7.
Semi-dry transfer system.

anywhere between the filter paper, gel and membrane. Bubbles prevent transfer and cause "bald spots" (i.e., areas of non-transfer) on the blot.

Transfer Buffers

The transfer buffer provides electrical continuity between the electrodes and must be conductive. It also provides a chemical environment that maintains the solubility of the proteins without preventing the adsorption of the proteins to the membrane during transfer. Common formulations achieve these functions for the majority of protein samples. Most buffers undergo Joule heating during transfer. For this reason, many tank transfer systems are equipped with built-in cooling coils. The tanks can also be placed in a cold room, and the buffer can be chilled before use. In semi-dry transfer systems, the electrode plates serve as heat sinks. Their heat dissipation capacity is limited, and semi-dry systems are not normally used for prolonged transfers.

Traditional transfer buffers consist of a buffering system and methanol. Towbin buffer (1979), a Tris-glycine buffer, is commonly used in tank systems. The pH of this buffer is 8.3, which is higher than the isoelectric point (pI) of most proteins. The proteins that have been separated on a gel have a net negative charge and migrate toward the anode. Because the buffer is mixed in the tank, the ion distribution remains relatively constant during the transfer.

Semi-dry systems can be run using either a single- or a three-buffer system (defined in Kyhse-Anderson, 1984). Three buffers are used because the transfer is an isotachophoretic process, where the proteins are mobilized between a leading ion and a trailing ion (Schafer-Nielsen, et al., 1980). In some cases, a three-buffer system provides better quantitative transfer. The three buffers are:

- Anode buffer I: 0.3 M Tris at pH 10.4
- Anode buffer II: 25 mM Tris at pH 10.4
- Cathode buffer: 25 mM Tris and 40 mM e-aminocaproic acid at pH 9.4

Anode buffer I neutralizes excess protons generated on the surface of the anode plate. Anode buffer II contains Tris at the same pH as anode buffer I, but at a reduced concentration of 25 mM. The cathode buffer contains ε-aminocaproic acid, which serves as the trailing ion during transfer and is depleted from the cathode buffer as it migrates through the gel toward the anode. Review manufacturer's recommendations for single buffers in a semi-dry system.

Although the buffer systems defined above are suitable for the majority of protein transfers, the literature contains many variations suited to different applications. One of the most significant variations was the recommendation of 10 mM CAPS buffer at pH 11 for protein sequencing applications (Matsudaira, 1987). The glycine used in Towbin buffer and carried over from the gel running buffer caused high backgrounds in automated protein sequencers employing Edman chemistry. By changing the transfer buffer composition, this artifact was significantly reduced. Any modification to the buffer strength and composition should be made with care to ensure that the transfer unit does not experience excessive heating.

Functions of Methanol in Transfer Buffer

The methanol added to transfer buffers has two major functions:

- Stabilizes the dimensions of the gel.
- Strips complexed SDS from the protein molecules.

Polyacrylamide is a hydrogel that has the capacity to absorb water. In pure water, the gel's size increases in all dimensions by a considerable amount. The degree of swelling also depends on the concentration of acrylamide used in the gel. High concentration gels expand more than low concentration gels. Gradient gels highlight this effect quite dramatically with the more concentrated zone at the bottom expanding much more than the top. A gel that starts out rectangular may become trapezoidal. The methanol added to the transfer buffer minimizes gel swelling, and transfer protocols normally include an equilibration step to achieve dimensional stability. At methanol concentrations of 10% to 20%, dimensional stability can be achieved fairly rapidly. At lower methanol concentrations, more time is required for equilibrium to be achieved. If dimensional changes occur during transfer, the resolution of the proteins may be lost. For high MW proteins with limited solubility in methanol, elimination of the methanol can result in a significant increase in protein transfer efficiency, but this may necessitate a longer equilibration time to ensure dimensional stability.

The second function of the methanol is critical for transfer of proteins from gels containing SDS. Methanol helps to strip complexed SDS from the protein molecules (Mozdzanowski and Speicher, 1992). Although the SDS is necessary for resolution of individual proteins on the gel, it can be extremely detrimental to effective blotting. First, by imparting a high negative charge density to a protein molecule, the SDS causes the protein molecule to move very rapidly through the membrane, reducing the residence within the pore structure and minimizing the opportunity for molecular interaction. Second, by coating the protein molecule, the SDS limits the ability of the protein to make molecular contact with the PVDF. These effects increase as the MW of the protein decreases. Methanol reduces both effects by stripping off the SDS and increasing the probability that a protein molecule will bind to the membrane.

Factors Affecting Successful Protein Transfer

Presence of SDS

Some studies have suggested that proteins, in the context of an SDS-PAGE gel, bind to SDS molecules and are therefore unable to bind efficiently to the transfer membrane. Specifically, when the transfer of BSA was monitored over two hours in a standard tank transfer system, the data suggested that within a single protein band there is more than one population of molecules transferring from the gel Figure 8, page 20). About 90% of the BSA eluted from the gel during the first 60 minutes, with an additional 7% eluting in the last 60 minutes. During the first 15 minutes, part of the eluted BSA adsorbed to the Immobilon®-P transfer membrane while the remainder passed through and adsorbed to the Immobilon®-P^{SQ} transfer membrane. BSA that eluted after 15 minutes adsorbed almost exclusively to the sheet of Immobilon®-P transfer membrane.

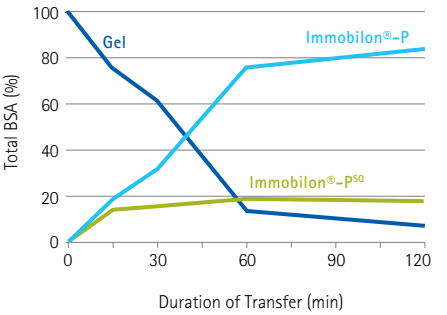


Figure 8.

Electrotransfer of BSA. 25 picomoles of ^{125}I-labeled BSA were resolved by SDS-PAGE on a 10–20% gradient gel. After equilibration for 5 minutes, the BSA was transferred to Immobilon®-P transfer membrane, backed up with Immobilon®-P^{SQ} transfer membrane, in a tank transfer system using 25 mM Tris, 192 mM glycine, and 10% methanol, as the transfer buffer. The system was run at 8 V/cm interelectrode distance. At 15, 30, 60, and 120 minutes, a gel/membrane cassette was removed and stained. The BSA bands were excised and counted.

The simplest interpretation is that the BSA bound to a high level of residual SDS eluted from the gel rapidly and was unable to adsorb to Immobilon®-P transfer membrane. BSA that eluted more slowly was able to adsorb to the Immobilon®-P transfer membrane.

Although removal of SDS from a gel is generally the best approach for routine blotting, there are instances where addition of low amounts of SDS to the transfer buffer is worth considering. One instance is when the proteins to be transferred have low solubility in the absence of SDS. Proteins associated with or integral to cellular membranes may be very hydrophobic and can precipitate within the polyacrylamide as the SDS is removed. High MW proteins also may exhibit solubility problems in the absence of SDS, especially after being exposed to the denaturing conditions of the gel sample buffer and the methanol used in the transfer buffer. Supplementation of the transfer buffer with SDS can help maintain sufficient solubility to permit elution from the gel (e.g., Towbin and Gordon, 1984, Otter et al., 1987; Bolt and Mahoney, 1997). The SDS concentration in the transfer buffer should not exceed 0.05%, and sufficient equilibration time should be allowed to remove all excess SDS from the gel.

Other methods employed to improve the transfer efficiency of high molecular weight proteins include prolonged blotting time, up to 21 hours (Erickson et al., 1982), or the use of a composite agarosepolyacrylamide gel containing SDS and urea (Elkon et al., 1984).

Current and Transfer Time

The appropriate current and transfer time are critical for successful blotting. Insufficient current and/or time will result in incomplete transfer. Conversely, if the current is too high, the protein molecules may migrate through the membrane too fast to be adsorbed. This can be a significant problem for smaller proteins. Usually, blotting systems come with manufacturer's recommendations for current and transfer time that should be used as a guideline.

Optimization may still be required depending on the percentage of acrylamide, the buffer composition and the MW of the protein of interest. Generally, long transfer times are best suited for tank systems, which normally require cooling of the unit and internal recirculation of the transfer buffer. In semi-dry transfer, however, prolonged blotting may result in buffer depletion, overheating and gel drying. If too much drying occurs, the unit can be damaged by electrical arcing between the electrode plates.

Transfer Buffer pH

The pH of the transfer buffer is another important factor. If a protein has an isoelectric point equal to the buffer pH, transfer will not occur. To alleviate this problem, higher pH buffers such as CAPS or lower pH buffer such as acetic acid solutions can be used.

Equilibration Time

In the early days of protein blotting (late 1970s, early 1980s), most protocols called for equilibration of the gel for 30 minutes prior to blotting. Standard gel sizes of 5 inches or more on a side and minimum thicknesses >1 mm required extended equilibration to stabilize the size of the gel. As mini-gels became more common, equilibration times were shortened because there was less volume into which the water and methanol had to equilibrate.

Dimensional equilibrium can be reached in standard mini-gels within 5 to 10 minutes, but the kinetics of SDS stripping are significantly slower, so a minimum equilibration time of 15 minutes is recommended for most mini-gels.

NOTE: For samples containing small peptides, the rapid migration of peptides can occur without electrical force. In this instance, equilibration of the gel in transfer buffer should be limited to less than 10 minutes.

In SDS-PAGE systems, the running buffer is supplemented with SDS. This SDS concentrates from the cathode reservoir and runs into the gel behind the bromophenol blue tracking dye. Since most gels are run until the tracking dye is at the bottom of the gel, all of the excess SDS remains in the gel and is carried over into the blotting procedure. If the SDS isn't allowed to diffuse out of the gel prior to transfer, it will interfere with protein adsorption. Equilibration times can be extended up to 30 minutes, and sufficient buffer should be used to dilute the SDS to a minimal level.

The effect of equilibration time on electrotransfer of BSA is shown in Figure 9. In this study, radioactively labeled BSA was resolved by SDS-PAGE, and the gels were equilibrated in transfer buffer for periods ranging from 0 to 30 minutes. Protein was transferred to Immobilon®-P transfer membrane backed up with a piece of Immobilon®-P^{SQ} transfer membrane to adsorb any BSA not retained by the Immobilon®-P transfer membrane. At the end of the transfer period, the BSA in the gel, on the primary blot (Immobilon®-P transfer membrane) and on the backup blot (Immobilon®-P^{SQ} transfer membrane) was quantified. Retention improved to 90% when the duration of the equilibration period was increased to 30 minutes. Other proteins have been found to behave similarly.

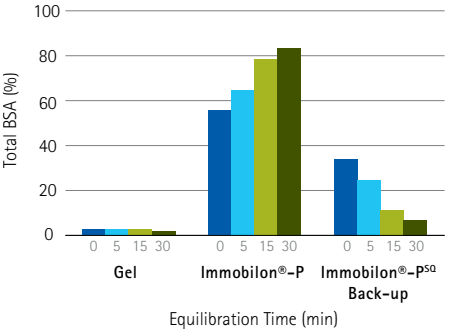


Figure 9.

Effect of equilibration time on electrotransfer of BSA to Immobilon®-P transfer membrane.
^{125}I-labeled BSA was resolved by SDS-PAGE on a 10–20% gradient gel. After equilibration for the times noted, the BSA was transferred to Immobilon®-P transfer membrane, backed up with Immobilon®-P^{SQ} transfer membrane, in a tank transfer system using 25 mM Tris, 192 mM glycine, and 10% methanol, as the transfer buffer. The system was run at 8 V/cm interelectrode distance. At the end of the 2-hour transfer period, the gel and membranes were stained. The BSA bands were excised and counted.

Developing a New Transfer Protocol

Although the previous section suggests that the selection of buffers and transfer conditions can be very complex, the tank transfer system defined by Towbin et al. (1979) and the semi-dry transfer system defined by Kyhse-Anderson (1984) work well for most protein samples. Both represent excellent starting points. If they prove less than optimal for a particular protein, though, transfer conditions can be tailored to fit the biochemical peculiarities of the protein. An interesting optimization strategy for the efficient transfer of proteins over a MW range from 8,000 to >400,000 kDa was demonstrated by Otter et al. (1987). The transfer buffer was supplemented with 0.01% SDS to maintain the solubility of high MW proteins and 20% methanol to enhance adsorption. The electrical field was applied in two phases. The first hour of transfer was at a low current density to slow the migration rate of low MW proteins and increase their residence time in the membrane. This was followed by a prolonged period at high current density to elute the high MW proteins.

When developing a new transfer protocol or working with a new sample type, the gel should be stained to verify that all of the proteins have completely eluted from the gel. It is also highly recommended to have a lane with prestained markers in each gel to monitor the transfer efficiency. Some proteins have limited solubility in typical transfer buffers, requiring modification of the buffer chemistry to prevent precipitation. Other proteins, such as histones and ribosomal proteins, are positively charged in standard transfer buffers and will migrate toward the cathode. These proteins can be successfully transferred by placing a sheet of Immobilon®-P membrane on the cathode side of the gel. Staining the membrane after the transfer can also be helpful to ensure that the target protein is on the blot. See "Protein Visualization," below, for information on stains compatible with subsequent immunodetection.

Another method to monitor protein transfer is to stain SDS-PAGE gels prior to electroblotting (Thompson and Larson, 1992). In this method, the gels are stained with either Coomassie® Brilliant Blue after electrophoresis, or during electrophoresis. The transferred proteins remain stained during immunodetection, providing a set of background markers for protein location and size determination (Thompson and Larson, 1992).

Preparing Membrane for Protein Identification

For Staining and Immunodetection

PVDF membrane should be washed with deionized water to remove any gel debris. The blot can then be incubated with the blocking solution.

For Rapid Immunodetection and Visualization by Transillumination

After the transfer is complete, PVDF membranes should be completely dried before continuing on to staining or immunodetection procedures. Drying enhances the adsorption of the proteins to the PVDF polymer, helping to minimize desorption during subsequent analyses. As the blotted membrane dries, it becomes opaque. This optical change is a surface phenomenon that can mask retention of water within the depth of the pores. The membrane should be dried for the recommended period to ensure that all liquid has evaporated from within the membrane's pore structure (refer to Protocol 1.7. Membrane Drying Methods, page 44).

Storage

PVDF membranes can be stored dry for long periods of time after proteins have been transferred with no ill effects to the membrane or the proteins (up to two weeks at 4 °C; up to two months at –20 °C; for longer periods at –70 °C). Some proteins, however, may be sensitive to chemical changes (e.g. oxidation,

deamidation, hydrolysis) upon prolonged storage in uncontrolled environments. Long term storage at low temperature is recommended. Prior to further analysis, dried membranes must be wetted by soaking in 100% methanol.

Protein Visualization

Transillumination

Transillumination (Figure 10, page 24) is a visualization technique unique to PVDF membranes and was first described for Immobilon®-P transfer membrane (Reig and Klein, 1988). This technique takes advantage of a characteristic unique to PVDF membranes; areas of PVDF coated with transferred protein are capable of wetting out in 20% methanol while the surrounding areas of PVDF are not. In the areas where the PVDF wets, it becomes optically transparent, allowing visualization of protein bands using backlighting and photographic archiving. The process is fully reversible by evaporation. Further denaturation of the proteins is unlikely as the proteins had been previously exposed to methanol during blotting. Even though this technique does not allow for visualization of low abundance proteins, it can be used to assess the overall transfer efficiency and the suitability of the blot for further analysis.

Staining

Staining (Figure 10) is a simple technique to make proteins visible on a blot. Staining can be used to:

- Verify that proteins have transferred onto the membrane.
- Determine if the lanes were loaded equally.
- Evaluate the overall efficiency of the transfer, especially for a new buffer system or protein.
- Identify and excise bands for peptide sequencing.

Many types of stains are available, including organic dyes (Ponceau-S red, amido black, fast green and Coomassie® Blue), fluorescent dyes (fluorescamine, coumarin) and colloidal particles (gold, silver, copper, iron and India ink) (Kurien et al., 2003). Table 5 lists the most common stains for detection of total proteins on Western blots. The dyes are separated into two groups: reversible and irreversible stains.

Reversible Stains

Reversible stains allow assessment of the blot and then can be washed from the membrane. These will not interfere with subsequent immunodetection or other analysis of the proteins on the blot. The most commonly used reversible protein stain is Ponceau-S red.

The major drawnback of reversible stains is that they are less sensitive than irreversible stains. Since the staining pattern of more abundant proteins in a blot is generally a good indicator of how well low abundance proteins transferred, this drawback can be minimized in most cases.

Fluorescent blot stains are highly sensitive and compatible with downstream immunodetection, Edman-based sequencing and mass spectrometry (Berggren et al., 1999). Sypro® Ruby and Sypro® Rose protein blot stains (Life Technologies) can be used prior to chromogenic, fluorogenic or chemiluminescent immunostaining procedures and provide sensitivity of about 1–2 ng/band (Haugland, 2002).

Irreversible Stains

Irreversible stains generally exhibit the best sensitivity but can interfere with or prevent further analysis of the proteins. Examples of irreversible stains are amido black and Coomassie® Brilliant Blue.

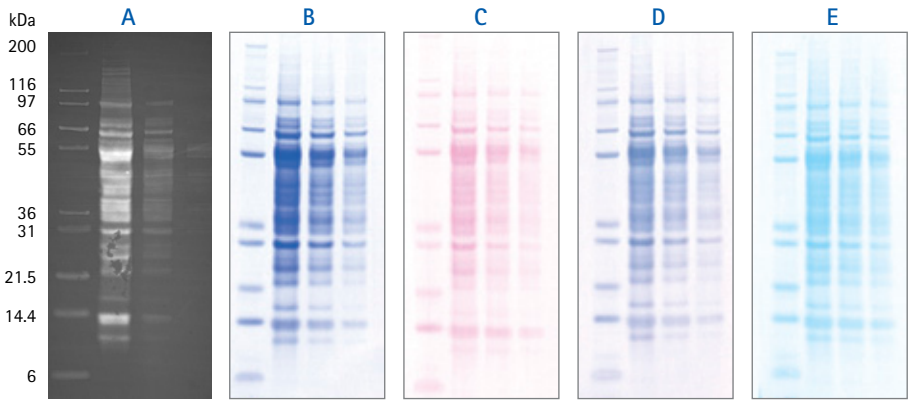


Figure 10.

Calf liver proteins are visualized after electroblotting to Immobilon®-P membranes:
(A) Transillumination, (B) Coomassie® Brilliant Blue, (C) Ponceau-S red, (D) Amido black and (E) CPTS total protein stains. Left to right, molecular weight standards and 12.2 µg, 6.1 µg, 3.1 µg of the lysate per lane.

Table 5. Common stains used in Western blotting and their attributes.

	Detection Reagent (protein per spot)	Approximate Sensitivity	Reference
Reversible	Ponceau-S red	5 µg	Dunn et al., 1999
	Fast green FC	5 µg	Dunn et al., 1999
	CPTS	1 µg	Bickar et al., 1992
	Sypro® Ruby	1–2 ng	Haugland, 2002
	Sypro® Rose	1–2 ng	Haugland, 2002
Irreversible	Amido black 10B	1 µg	Dunn et al., 1999
	Coomassie® Brilliant Blue R-250	500 ng	Dunn et al., 1999
	India ink	100 ng	Dunn et al., 1999
	Colloidal gold	4 ng	Dunn et al., 1999

Protein Identification

4

Immunodetection

Immunodetection uses a specific antibody to detect and localize a protein blotted onto the membrane (Figure 11). The specificity of antigen-antibody binding permits the identification of a single protein in a complex sample.

When developing new immunodetection protocols, all components and their interactions must be considered. Antibody concentrations, buffer compositions, blocking agents and incubation times must be tested empirically to determine the best conditions. Water quality is important 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, and azide, a common preservative in antibody solutions. The quality of the blocking agents must also be considered relative to consistency and contaminants.

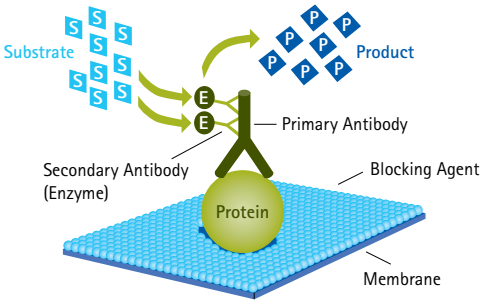


Figure 11.
Membrane-based immunodetection.

Standard vs. Rapid Immunodetection Procedures

There are two types of protocols for immunodetection: standard and rapid.

Standard immunodetection methods include the following steps:

1. Blocking unoccupied membrane sites to prevent nonspecific binding of antibodies.
2. Incubating the membrane with primary antibody, which binds to the protein of interest.
3. Washing to remove any unbound primary antibody.
4. Incubating the membrane with a conjugated secondary antibody, which binds the first antibody.
5. Washing to remove any unbound secondary antibody.
6. Incubating the membrane with a substrate that reacts with the conjugated secondary antibody to reveal the location of the protein.

Rapid immunodetection eliminates the blocking step and reduces the time necessary for the washing and incubation steps. The rapid immunodetection method works well to quickly visualize higher abundance proteins. Standard immunodetection, however, offers higher sensitivity and requires less optimization for new sample types. Procedures for both standard and rapid immunodetection methods are outlined in Protocol 3 (pages 48-54). Table 6 compares the times required to perform the two protocols.

Factors Influencing Immunodetection

Understanding the concepts presented in the following sections on immunodetection will help in optimizing protocols for 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 requirement of the buffer is that it should help preserve the biological activity of the antibodies. Thus, the ionic strength and pH should be fairly close to physiological conditions. PBS formulations with 10 mM total phosphate concentration work well with a wide array of antibodies and detection substrates.

During incubations, the container holding the membrane should be gently agitated. A sufficient volume of buffer should be used to cover the membrane completely so that it is floating freely in the buffer. If more than one blot is placed in a container, insufficient buffer volume will cause the blots to stick together. This will limit the accessibility of the incubation solutions and can cause a variety of artifacts including high backgrounds, weak signals, and uneven sensitivity.

Table 6. Standard vs. rapid immunodetection.

Step	Standard Immunodetection	Rapid Immunodetection
1. Block the membrane	1 hr	None
2. Incubate with primary antibody	1 hr	1 hr
3. Wash the membrane	3 x 10 min	3 x 5 min
4. Incubate with secondary antibody	1 hr	30 min
5. Wash the membrane	3 x 10 min	3 x 5 min
6. Add substrate	5 min	5 min
Total time	**4 hr 5 min**	**2 hr 5 min**

Blocking

For meaningful results, the antibodies must bind only to the protein of interest and not to the membrane. Nonspecific binding (NSB) of antibodies can be reduced by blocking the unoccupied membrane sites with an inert protein, non-ionic detergent, or a protein-free blocker such as Bløk® noise-canceling reagents. The blocking agent should have a greater affinity for the membrane than the antibodies. It should fill all unoccupied binding sites on the membrane without displacing the target protein from the membrane. The most common blocking agents used are bovine serum albumin (BSA, 0.2–5.0%), non-fat milk, casein, gelatin, dilute solutions of Tween®-20 detergent (0.05 –0.1%), and Bløk® reagents. Tween®-20 detergent was also shown to have a renaturing effect on antigens, resulting in improved recognition by specific antibodies (Van Dam et al.,1990; Zampieri et al., 2000). Other detergents, such as Triton® X-100 detergent, SDS, and NP-40, are sometimes used but can be too harsh and disrupt interaction between proteins. The blocking agent is usually dissolved in PBS or TBS buffers.

There are risks associated with blocking; a poorly selected blocking agent or excessive blocking can displace or obscure the protein of interest. Therefore, the correct choice of a blocking agent can be critical to a successful immunodetection. For example, dry milk cannot be used with biotinylated or concanavalin-labeled antibodies, since milk contains both glycoproteins and biotin. The analysis of phosphorylated proteins with phosphospecific antibodies can be compromised if using crude protein preparation as a blocking agent. These preparations may contain phosphatases, and the phosphorylated proteins on the blot could become dephosphorylated by these enzymes. It has been shown that addition of phosphatase inhibitors to the blocking solution increases the signal with phospho-specific antibody (Sharma and Carew, 2002). Finally, a blocking agent that is found to be suitable for one antigen-antibody combination may not be suitable for another.

Compatibility between the blocking agent and the detection reagent can be determined easily using the Spot Blotting Method described in Protocol 1.5 (page 41). The blocking solution gets spotted onto a blank PVDF membrane that has been wet in methanol and equilibrated in TBS. Detection reagents are then added to the blot, the blot is incubated for 5 minutes and then exposed to X-ray film. Appearance of a dark spot indicates that the blocking reagent is incompatible with the detection reagent (Figure 19, page 42). It is important to remember that Immobilon®-P^{SQ} transfer membrane, with its higher surface area and smaller pore size than Immobilon®-P transfer membrane, binds more protein. If Immobilon®-P^{SQ} transfer membrane is substituted directly for Immobilon®-P transfer membrane in a standard Western blotting procedure, there may be insufficient blocking reagent to saturate the membrane surface. Additional washing steps may also be required to reduce the background. Blocking can be conveniently optimized using the Spot Blotting Method (page 40).

Antibodies

After blocking, the blot is incubated with one or more antibodies. The first antibody binds to the target protein, and a secondary antibody binds to the first. The secondary antibody is conjugated to an enzyme or dye that is used to indicate the location of the protein.

Although the primary antibody may be labeled directly, using a secondary antibody has distinct advantages. First, more than one molecule of the secondary antibody may be able to bind to a single molecule of the primary antibody, resulting in signal amplification. Second, a labeled secondary antibody (enzyme-antibody

conjugate) can be used for a large number of primary antibodies of different specificities, thereby eliminating the need to label numerous primary antibodies.

Either polyclonal or monoclonal primary antibodies are used. Polyclonal antibodies usually come in the form of antiserum or affinity-purified antibody. Monoclonal antibodies are expressed in ascites fluid or tissue culture fluid and can be directly used or as an affinity-purified preparation. It is important to remember that a denatured protein may not be recognized by an antibody raised to the native antigen. In some cases, a nondenaturing gel may be required for production of the blot. Antibodies are diluted in buffer and blocking solution to prevent nonspecific binding to the membrane. The antibody diluent also normally contains trace amounts of Tween®-20 or another detergent to prevent nonspecific aggregation of the antibodies. Many published protocols for chemiluminescence require 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 some blotted proteins from the membrane.

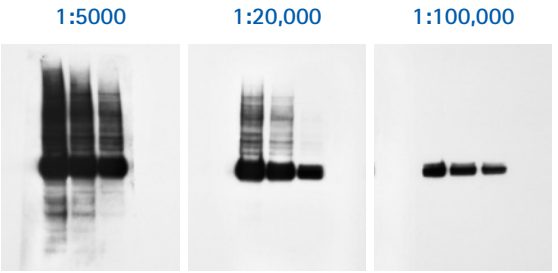


Figure 12.

Optimization of secondary antibody dilution for immunodetection of ERK 1 with Immobilon® Western Chemiluminescent HRP Substrate. Two-fold dilutions of rat liver lysate samples were applied to each gel. Electroblotted proteins were probed with rabbit anti-ERK 1 antibody (1:1,000 dilution) and HRP-conjugated goat anti-rabbit IgG (1:5,000, 1:20,000 and 1:100,000 dilutions, left to right).

Elevating the concentration of Tween®-20 detergent can often reduce the background. Often, a simpler and more cost-effective strategy is to reduce the concentration of the antibodies, especially the secondary antibody (see Figure 12). In addition to being specific for the protein of interest, the antibodies must not cross-react with components of the blocking buffer and should be relatively pure. Impurities in the form of other proteins or aggregates can result in nonspecific binding and increased background.

Immunodetection is an extremely sensitive method. In order to achieve a high signal-to-noise ratio and thereby maximum sensitivity, the concentrations of primary and secondary antibodies should be optimized for each case. Generally, nonspecific signal can be reduced by higher dilution of the primary antibody or decreased protein load on the original gel. High overall background can be minimized by higher dilution of the enzyme-conjugated secondary antibody.

Optimal concentration of both primary and secondary antibodies also depends on the sensitivity of the detection reagents. Up to twenty times less antibody is required for high-sensitivity reagents (detection at femtogram level) as compared to low-sensitivity reagents (detection at the picogram level). An example of optimization of antibody concentration depending on the detection substrate is shown in Figure 13. When using the most sensitive detection reagent (Luminata™ Forte reagent), excessive amounts of antibody led to high nonspecific and increased overall background (top left). In this case, increasing the primary antibody dilution from 1:1,000 to 1:10,000 improved the signal-to-noise ratio. With progressively less sensitive detection reagents (middle and bottom rows, respectively), antibodies need to be more concentrated to yield the same signal.

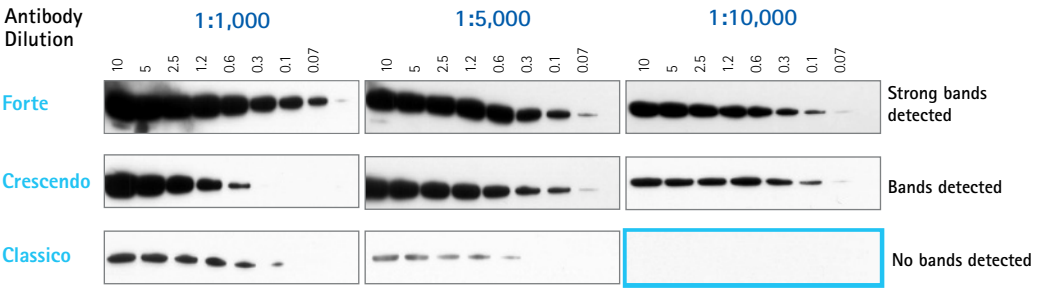


Figure 13.

The more sensitive the detection reagent, the less antibody is needed. Immunoblots containing the indicated amounts of A431 lysate were probed with different concentrations of anti-GAPDH antibody (Catalogue No. MAB374) indicated in the top row, followed by an appropriate secondary antibody. Bands were visualized using the indicated Luminata™ HRP substrate and exposed to x-ray film for 5 minutes. Of the three detection reagents shown, Luminata™ Forte reagent is the most sensitive, followed by Luminata™ Cresendo and then Luminata™ Classico reagents.

Using higher sensitivity HRP substrates was advantageous in three respects:

1. **Better results**: It produced stronger bands for a more quantitative blot (compare the increase in band intensities for Luminata™ Crescendo & Forte substrates at 1:10,000 dilution)
2. **Faster**: It took only 10 minutes to wash blot and add a new substrate relative to the 2.5 hours required to repeat antibody incubations.
3. **Cheaper**: The HRP substrates are much cheaper than the price of antibodies.

Washing

Washing the blot removes any unbound antibodies from the membrane that could cause high background and poor detection. A dilute solution of Tween®-20 (0.05% v/v) in PBS or TBS buffer is commonly used, especially when the antibody preparations are comparatively crude or used at high concentrations. As mentioned previously, concentrated detergent solutions may cause unwanted elution of 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 excessive background, while overwashing may elute the antibodies and reduce the signal. It is recommended that washing be performed a minimum of three times for 5 minutes each.

Persistent background can be reduced by adding up to 0.5 M sodium chloride and up to 0.2% SDS to the TBS wash buffer and extending wash time to 2 hours.

Double Blotting

An innovative method to eliminate nonspecific binding in Western blots was developed by Dr. Françoise Lasne of the Laboratoire National de Dépistage du Dopage (National Anti-Doping Laboratory) in Châtenay-Malabry, France (Lasne, 2001; Lasne, 2003). While studying recombinant human erythropoietin (EPO), Dr. Lasne found that recombinant and naturally occurring EPO have different isoelectric points (pI). Recombinant EPO has a pI of 4.42–5.11, while natural EPO has a more acidic pI of 3.92–4.42. However, when urine samples are blotted, the very high nonspecific binding (NSB)

of the secondary antibodies makes it difficult to distinguish between recombinant and natural EPO. To eliminate the NSB, Dr. Lasne used "double blotting." After the primary antibody is bound to the blotted protein, the antibodies are transferred to a second Immobilon®-P membrane under acidic conditions. The primary antibody desorbs from its corresponding antigen and transfers through an intermediate onto the second (double-blot) membrane. When the double-blot membrane is probed with the secondary antibody, no other proteins are present to bind nonspecifically, thus eliminating the background problem (refer to Protocol 1.6. Double Blotting Procedure, page 43).

"Double blotting" has also been used in the detection of transthyretin and may be a useful method in detecting other low concentration serum or urine proteins.

Detection Substrates

Modern immunodetection methods are based on enzyme-linked detection, utilizing secondary antibodies covalently bound to enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). The conjugated enzyme catalyzes the degradation of specific substrates, resulting in signal generation. Three types of substrates are commonly used: chromogenic, chemiluminescent, and chemifluorescent, as well as detection with fluorophore-labeled secondary antibodies. Immobilon® PVDF membranes have been tested for compatibility with all commercially available chromogenic and chemiluminescent substrates.

Chromogenic Detection

Chromogenic detection (Figure 14) uses the conjugated enzyme to catalyze a reaction resulting in the deposition of an insoluble colored precipitate, for example, the insoluble blue compound obtained through the interaction of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium salt (NBT) (Leary, et al., 1983). This technique is easy to perform and requires no special equipment for analysis. However, the following should be kept in mind:

- Sensitivity of chromogenic detection is typically at least one order of magnitude lower than with chemiluminescent reagents.
- Production of the precipitate can interfere with enzyme activity and limit sensitivity.
- The precipitate is difficult to strip from membrane, limiting reuse of the blot for detection of other proteins.

Figure 14.

Immunodetection of transferrin in human serum with chromogenic substrate BCIP/NBT (KPL). Left to right, 5 µL human serum dilutions 1:1,000, 1:5,000, 1:25,000, 1:125,000. Electroblotted proteins were probed with goat anti-human transferrin (1:10,000 dilution) and AP-conjugated rabbit anti-goat IgG (1:30,000 dilution).

Chemiluminescent Detection

Chemiluminescent detection uses the conjugated enzyme to catalyze a reaction that results in the production of visible light. Some chemiluminescent systems are based on the formation of peroxides by horseradish peroxidase; other systems use 1,2-dioxetane substrates and the enzyme alkaline phosphatase (Cortese, 2002). This technique has the speed and safety of chromogenic detection at sensitivity levels comparable to radioisotopic detection. Detection is achieved by either exposing the blot to X-ray film or acquiring the image directly in a chemiluminescence-compatible digital imaging system, usually equipped with highly-cooled CCD cameras to avoid electronic noise. Reprobing is possible with chemiluminescent substrates.

There are a variety of chemiluminescent substrates offering researchers different levels of sensitivity of detection. Traditional or low-sensitivity substrates allow protein detection at the picogram level. While these substrates may be appropriate for routine applications, they cannot detect low abundance proteins. New, high-sensitivity substrates, such as Luminata™ HRP substrates, allow visualization of proteins at the femtogram level. However, use of these powerful substrates often requires optimization of primary and secondary antibody concentrations (Figure 13, page 29). When switching from a low-sensitivity to a high-sensitivity substrate, like Luminata™ Forte reagent, it is recommended that antibody dilutions be increased to avoid excessive background and appearance of nonspecific bands.

Reagents for ECL immunodetection can be prepared using p-iodophenol (PIP) and luminol (Hengen, 1997). PIP is needed for enhancing the visible light reaction by acting as a co-factor for peroxidase activity toward luminol. When phenolic enhancers are used in combination with HRP, the level of light increases about 100-fold (Van Dyke and Van Dyke, 1990). These homemade reagents are cited to produce excellent results, however, the highest purity of the luminol and PIP is critical (Hengen, 1997).

Fluorescent Detection

Fluorescent detection employs either a fluorophore-conjugated antibody or fluorogenic substrates that fluoresce at the site of enzyme activity (chemifluoresence). One advantage of this method is that the fluorescent signal is stable for long periods of time, and blots can be archived and re-imaged. In addition, the wide variety of fluorophores makes it possible to simultaneously detect multiple protein targets in a single sample (multiplex detection). Until recently, fluorescent detection in Western blotting was limited by the high fluorescent background of most blotting membranes. Immobilon®-FL transfer membrane exhibits low background fluorescence across a wide range of excitation/emission wavelengths compared to other blotting membranes (Figure 15). The membrane is ideal for any application involving fluorescence-based immunodetection, including chemifluorescent substrates and multiplexing (Figure 16). In addition, Immobilon®-FL membrane can be used for standard chemiluminescent or chromogenic detection.

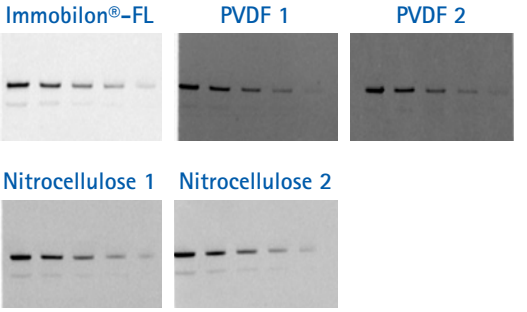


Figure 15.

Reverse image demonstrating fluorescent detection of transferrin in human serum on various blotting membranes. Serum dilutions: 1:4,000, 1:8,000, 1:16,000, 1:32,000 and 1:64,000.

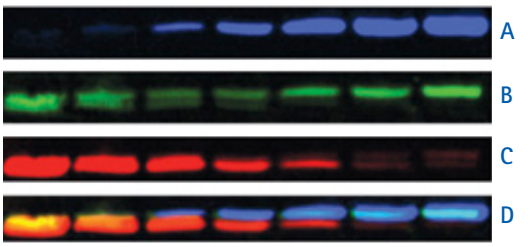


Figure 16.

Western blots of serial dilutions of E. coli cell lysates over-expressing GST protein with either a cMyc or an HA tag. (A) cMyc tag detected with a primary antibody followed by Qdot® 705 (pseudo-colored blue) anti-mouse conjugate; (B) GST detected with Qdot 565 (green) anti-GST conjugate; (C) HA tag detected with a primary antibody followed by Qdot® 605 (red) anti-mouse conjugate; (D) the blot probed with a combination of all three antibodies (multiplexing). The blots were scanned on a Kodak imager. Data provided by Quantum Dot Corporation.

Reprobing Immobilon® PVDF Transfer Membranes

A single blot can be sequentially analyzed with multiple antibodies by stripping the first antibody from the blot and incubating with another. This may be especially useful for co-localization experiments and method optimization or when sample amount is limited (refer to Protocol 5.4 Membrane Stripping Protocols, page 55.)

The stripping process disrupts the antigen antibody bonds and dissolves the antibody in the surrounding buffer. This is usually achieved either by a combination of detergent and heat or by exposure to low pH. Neither method removes 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 an antibody specific for a different target protein.

Mass Spectrometry

Mass Spectrometry with Immobilon® PVDF Transfer Membranes

Mass spectrometry (MS) is an analytical method used to identify proteins on a blot. After staining the proteins on a PVDF membrane with MS-compatible dye (Coomassie® Blue, Amido black or Sypro® stains), the band of interest is cut out of the membrane and subjected to tryptic digestion. The peptide fragments generated are then analyzed by mass spectrometry (Gharahdaghi et al., 1996; Bienvenut, et al., 1999; Bunai et al., 2003). Figure 17 demonstrates a MALDI-TOF spectrum obtained for an on-membrane-digested bovine protein successfully identified as catalase.

Another method to consider is protein mass spectrometry directly from the blotted Immobilon® PVDF transfer membrane. This method usually is applied for 2-D gel separated proteins. Using a parallel process, all proteins on a gel are simultaneously digested proteolytically and electrotransferred onto an Immobilon® PVDF transfer membrane, which is then scanned for the presence of the peptides (Binz et al., 1999; Bienvenut et al., 1999; Bienvenut et al., 2003). Alternatively, in a method called "chemical printing" (Wallace et al., 2001; Gooley et al., 1998), two-dimensionally separated proteins are first transferred to PVDF membrane and visualized, and then digested by dispensing miniscule amounts of trypsin directly onto the spots (Sloane et al., 2002). In both methods, the membrane is sprayed with matrix and directly scanned by MALDI-TOF MS. Protein identification is obtained by peptide mass fingerprinting. Figure 18 shows 2-D-separated human plasma proteins transferred to Immobilon®-P^{SQ} transfer membrane and two MALDI-TOF spectra obtained for identification of membrane-immobilized proteins.

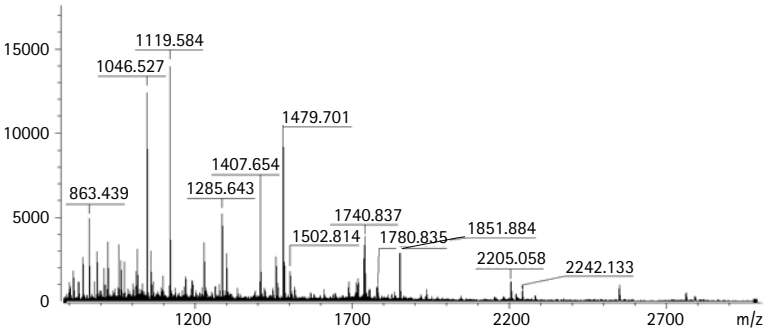


Figure 17.

MALDI-TOF spectrum of a band from Coomassie® Blue-stained blot of liver proteins, using Protocol 5.1. On-membrane Protein Digestion, page 57 (Bienvenut et al., 1999). The protein was identified as bovine catalase, with 6.4e+06 MOWSE score and 34% coverage. Data were obtained on a Bruker® Autoflex™ mass spectrometer. The search was done using Protein Prospector.

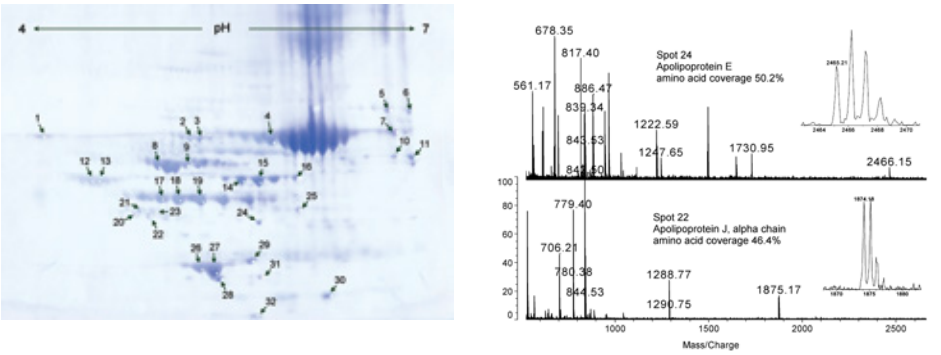


Figure 18.

Human plasma separated by 2-D electrophoresis and transferred to (left) Immobilon®-P^{SQ} transfer membrane and (right) MALDI-TOF mass spectra collected directly from the membrane surface of tryptic digested proteins. Human plasma was separated by 2D electrophoresis, electro-blotted onto Immobilon®-P^{SQ} transfer membrane, and adhered to a MALDI target. Digestion of proteins with the endoproteinase trypsin was performed directly on the membrane surface. Nanoliters of enzyme were required for digestion and were microdispensed with drop-on-demand piezoelectric ink-jet devices onto the membrane surface using a ChIP™ (Proteome Systems and Shimadzu Corp.) instrument. The resultant peptides were analyzed with an AXIMA™-CFR (Shimadzu Corp.) MALDI-TOF MS and identified with peptide mass fingerprinting. The peptides were analyzed directly from the membrane surface, where MALDI matrix was microdispensed on top of the digested protein prior to analysis. Data courtesy of Drs. J.L. Duff, F.G. Hopwood, C.J. Hill, A.A. Gooley (Proteome Systems, Ltd., Sydney, Australia).

5 Protocols

This section of the handbook is a compendium of the most frequently used protein blotting protocols. The methods are general enough that they can be used with all commercially available detection reagents. They can be optimized using the numerous tips that are also included in this section. These optimization tips are a product of Merck Millipore's collective years of experience with transfer membranes and blotting, as well as referenced literature and feedback from our customers.

- **TIP**: Ethanol or isopropanol can be substituted for methanol in the transfer buffer.
- **TIP**: SDS in the transfer buffer (up to 0.05%) can improve transfer efficiency but may also reduce the membrane's protein retention.
- **TIP**: Immobilon® transfer membrane precut sheets fit all standard mini-gel sizes and common electrophoresis systems. (Tables page 8 and 9.)

  Recommendation - Chill transfer buffers prior to tank transfer.
- **TIP**: Thicker gels or larger proteins may require longer transfer times or increased field strength. The actual transfer conditions should be optimized for each system.

- **TIP**: Methanol in the transfer buffer (8 -20%) deceases efficiency of protein elution from the gel but improves absorption to PVDF transfer membrane.
- **TIP**: Because of the hydrophobic nature of PVDF the membrane requires wetting with alcohol.
- **TIP**: Tris-glycine buffer will produce higher background in N-terminal sequencing. Use CAPS or TBE buffer for transfer if protein bonds are to be sequenced by Edman degradation.
- **TIP**: Replace 0.45 µm Immobilon®-P transfer membrane with 0.2 µm Immobilon®-P^{SQ} transfer membrane when analyzing lower molecular weight proteins.
- **TIP**: Use 0.45 µm Immobilon®-FL transfer membrane for fluorescent detection methods.

1. Protein Transfer

Protocol 1.1
Electrotransfers: Tank (Wet) Transfer

The following protocol describes the standard procedure for transferring proteins from a polyacrylamide gel (SDS-PAGE) onto an Immobilon® PVDF transfer 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® PVDF transfer 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).
- Tank transfer system large enough to accommodate gel.
- Methanol, 100%.
- Milli-Q® water.
- Tris/glycine transfer buffer: 25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3; or CAPS buffer: 10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, pH 11 (adjust with NaOH).

  NOTE: Both buffers can be prepared as 10X stock solutions and mixed with methanol prior to use.

Setup

1. Prepare sufficient transfer buffer to fill the transfer tank, plus an additional 200 mL to equilibrate the gel and membrane, and wet the filter paper.
2. Remove the gel from its cassette; trim away any stacking gel and wells.
3. Immerse the gel in transfer buffer for 10 to 30 minutes.
4. Soak filter papers in transfer buffer for at least 30 seconds.
5. Prepare the membrane:
   a. Wet the membrane in methanol for 15 seconds. Membrane should uniformly change from opaque to semi-transparent.
   b. Carefully place the membrane in Milli-Q® water and soak for 2 minutes.
   c. Carefully place the membrane in transfer buffer and let equilibrate for at least 5 minutes.

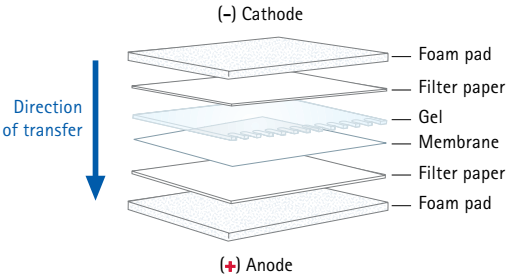
Transfer Stack Assembly

1. Open the cassette holder.

   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 to prevent damaging the membrane and gel.
2. Place a foam (fiber) 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 the 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:



Protein Transfer

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 into 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 on the transfer unit.
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 inter-electrode distance. Follow the tank manufacturer's guidelines, and refer to Transfer of Proteins from Gel to Membrane, page 16, for optimization details.
7. After the transfer is complete, remove the cassette holder from the tank.
8. Using forceps, carefully disassemble the transfer stack.

For protein visualization protocols, see page 45; for immunodetection protocols, see page 48.

Protocol 1.2 Electrotransfers: Semi-dry Transfer

The following protocol describes the standard procedure for transferring proteins from a polyacrylamide gel (SDS-PAGE) onto an Immobilon® PVDF transfer 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. This protocol describes a three-buffer system. Single-buffer systems can also be used. Please consult manufacturer's recommendations.

▶ **TIP:** For semi-dry transfer systems, it is important that the filter papers and membrane are cut to the same size as the gel so that the current is forced to flow through the gel.

▶ **TIP**: In both types of transfer systems (tank and semi-dry), extra caution should be taken to prevent introduction of air bubbles anywhere between the filter paper, gel or membrane.

▶ **Recommended** - Transfer proteins at constant current. If transferring at constant voltage, monitor current to make sure it doesn't exceed 0.4 amp. Start from 100 V and reduce voltage if current is too high.

▶ **TIP**: Both sides of Immobilon® transfer membranes work equally well. The appearance of either side (shiny or dull) has no effect on the transfer and detection efficiency of the membrane.

▶ **TIP**: For samples containing small peptides, equilibration of the gel in transfer buffer should be limited to less than 10 minutes.

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

Required Equipment and Solutions

For single transfers:

- Polyacrylamide gel containing the resolved proteins.
- Immobilon® PVDF transfer 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.
- Semi-dry transfer system large enough to accommodate gel.
- Anode buffer I: 0.3 M Tris, pH 10.4, 10% (v/v) methanol.
- Anode buffer II: 25 mM Tris, pH 10.4, 10% (v/v) methanol.
- Cathode buffer: 25 mM Tris, 40 mM 6-amino-n-caproic acid (glycine may be substituted), 10% (v/v) methanol, pH 9.4.
- Methanol, 100%.
- Milli-Q® water.

For multiple transfers, all of the above plus the following:

- Dialysis membrane, cut to the same dimensions as the gel and wet with Milli-Q® water. (The membrane should have a molecular weight exclusion small enough to retain the lowest molecular weight protein in the gel).
- Additional pieces of filter paper .

Setup

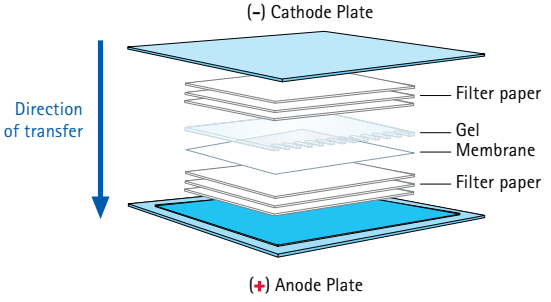
1. Prepare 200 mL of each anode buffer and 400 mL of cathode buffer.
2. Remove the gel from its cassette; trim away any stacking gel.
3. Immerse the gel in 200 mL of cathode buffer for 15 minutes.
4. Soak two pieces of filter paper in anode buffer I for at least 30 seconds.
5. Soak one piece of filter 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 the 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 Milli-Q® water and soak for 2 minutes.
   c. Carefully place the membrane in anode buffer II and let equilibrate for at least 5 minutes.

Transfer Stack Assembly

Refer to the manufacturer's specific operating instructions for the semi-dry transfer system being used. 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 to prevent damaging the membrane and gel.

For single transfers:

1. Place the anode electrode plate on a level bench top.
2. Place 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 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.



For multiple transfers:

1. Place the anode electrode plate on a level bench top.
2. Place 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 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.

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

Protein Transfer

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/cm²*	1–2 hours
1.2 mA/cm²	1 hour
2.5 mA/cm²	30–45 minutes
4.0 mA/cm²	10–30 minutes

*The surface area (cm²) is calculated 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.

6. Turn off the power supply when the transfer is complete.
7. Disconnect the system leads.
8. Remove the cover.
9. Remove and discard the filter papers.
   NOTE: When using graphite plates, graphite particles from the anode electrode plate occasionally appear on the filter paper. These particles do not affect operation.
10. Remove the gel.
11. Remove the blotted membrane with a pair of forceps.

For protein visualization protocols, see page 45; for immunodetection protocols, see page 48.

Protocol 1.3
Dot Blotting/Slot Blotting: Vacuum Filtration Method

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

▶ **TIP**: Detergents may inhibit the binding of proteins to the Immobilon® PVDF transfer membrane.

▶ **Recommended** - Viscous samples must be diluted with buffer to reduce viscosity.

Required Equipment and Solutions

- Two sheets of Immobilon® PVDF transfer membrane, cut to size for blotting unit.
- Filter paper, cut to size for blotting unit.
- Methanol, 100%.
- Milli-Q® water.
- Buffer, for sample loading and washes.
- Blotting unit, dot blot or slot blot format.

Set Up

1. Prepare the 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 Milli-Q® 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. If the sample solution is cloudy, centrifuge to remove particles. If the sample is viscous, dilute with additional buffer.

Blotting Unit Assembly

See manufacturer's instructions for detailed assembly instructions.

1. Place one sheet of moistened filter paper on unit. Some units may require more than one sheet.
2. Place two sheets of the membrane on top of the filter paper*.
3. Close the unit.
4. Connect to vacuum line.

Protein Transfer

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. Apply the vacuum to filter through the wash buffer.
6. When all of the wash buffer has filtered through the membrane, turn off the vacuum.
7. To remove the blot, open the blotting unit.
8. Using forceps, carefully remove the membrane.

For protein visualization protocols, see page 45; for immunodetection protocols, see page 48.

* 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.

Protocol 1.4
Spot Blotting: Manual Spotting Method

Required Equipment and Solutions

- Immobilon® PVDF transfer membrane.
- Filter paper, cut to size of transfer membrane.
- Paper towels.
- Methanol, 100%.
- Buffer.

Setup

1. Prepare the 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 Milli-Q® water and soak for 2 minutes.
   c. Carefully place the membrane in buffer and let equilibrate for at least 5 minutes.
2. If the sample solution is cloudy, centrifuge to remove particles. If the sample is viscous, dilute with additional buffer.

Transfer Stack Assembly

Assemble stack as follows (from the bottom up):

1. Place paper towels on work surface.
   NOTE: Bottom towels should remain dry throughout blotting procedure.
2. Place dry filter paper (i.e., Whatman® 3MM paper) on paper towels.
3. Place filter paper (pre-wet with buffer) on the dry filter paper.
4. Place the pre-wet membrane on wet filter paper.

Protein Transfer

1. Spot 1 – 5 µL of sample onto the membrane. Sample should wick into membrane.
   NOTE: Membrane should be wet enough to absorb sample, but not so wet that sample spreads across membrane.
2. After sample is absorbed, place membrane on clean filter paper to dry.

Protocol 1.5
Optimization of Blocking Reagents

Required Equipment and Solutions

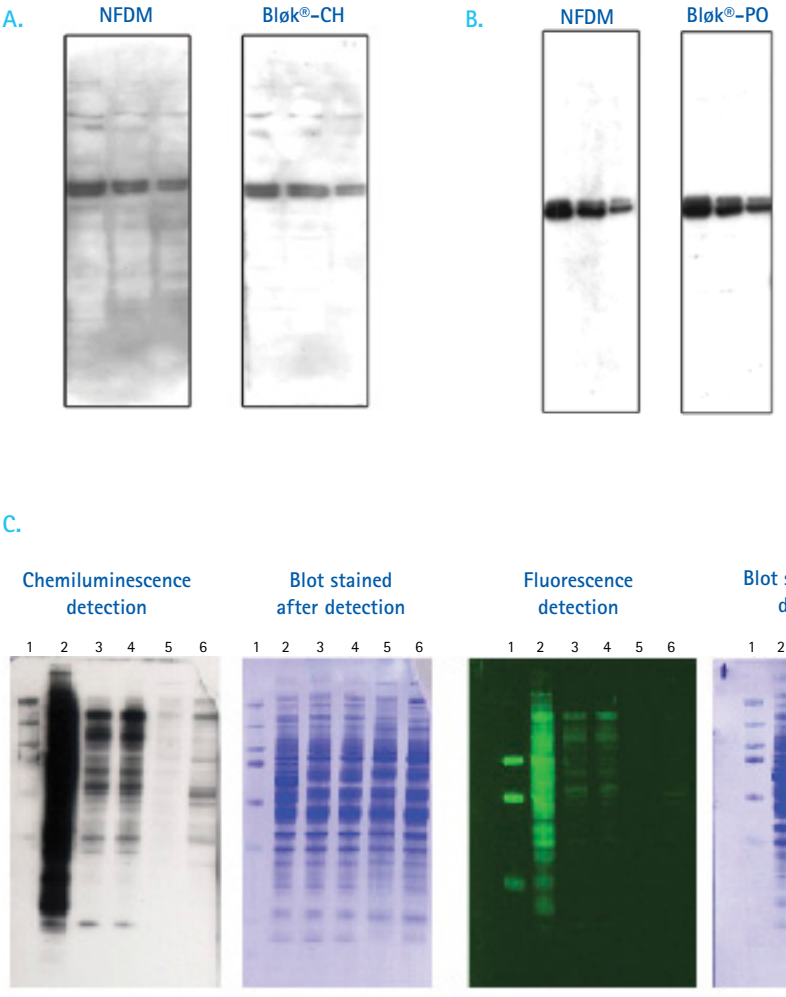
- Small piece of Immobilon® PVDF transfer membrane.
- Piece of filter paper cut to the dimension of the membrane.
- Methanol, 100%.
- Wash buffer: Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) containing 0.05 –0.1% Tween®-20 surfactant.
- Blocking solution(s) to be tested.
- Detection reagents to be tested.

Set Up

1. Prepare the membrane:
   a. Wet PVDF membrane in methanol for 15 seconds. The membrane should uniformly change from opaque to semi-transparent.
   b. Carefully place the membrane in Milli-Q® water and soak for 2 minutes.
   c. Carefully place the membrane in buffer and let equilibrate 5 minutes with gentle shaking.
2. Wet filter paper in wash buffer.

Procedure

1. Place the membrane on wet filter paper.
2. Spot 5 µL of blocking solution onto the membrane and allow it to absorb completely.
3. Add detection reagents to the blot and incubate for 5 minutes.
4. Drain the excess reagents.
5. Cover the blot with a clean plastic wrap or sheet protector and remove any air bubbles.
6. Expose the blot to a suitable X-ray film for a few minutes. Appearance of a black spot on the film indicates cross-reactivity between the blocking reagent and the detection substrate.



Lane 1: Molecular weight marker; Lane 2: pervanadate-stimulated A431; Lane 3:, non-stimulated A431 control; Lane 4: EGF-stimulated A431; Lane 5: non-stimulated A431 control; Lane 6: EGF-stimulated A431

Figure 19.

Bløk® noise-canceling reagents, featuring optimized formulations for chemiluminescence, fluorescence and phosphoprotein detection, can provide superior signal-to-noise ratios and also permit staining of the membrane after detection. (A) Chemiluminescence detection of p53 in EGF-stimulated A431 lysate (10 - 2.5 µg/lane, Catalogue No. 12-110). Blots were blocked with NFDM or Bløk®-CH reagent, then probed with anti-p53 antibody (1:1,000, Catalogue No. AB565) diluted in Bløk®-CH reagent. Bands were detected using Luminata™ Forte Western HRP substrate (Catalogue No. WBLUF0500). NFDM = Non-fat dry milk. (B) Chemiluminescence detection of pERK in EGF-stimulated A431 lysate (10 – 2.5 µg/lane, Catalogue No. 12-110). Blots were blocked with NFDM or Bløk®-PO reagent, then probed with anti-pERK antibody (1:10,000, Catalogue No. 05-797R) diluted in Bløk®-PO reagent. Bands were detected using Luminata™ Forte Western HRP substrate (Catalogue No. WBLUF0500). (C.) Two blots containing different samples of A431 cell lysate, some freshly prepared (lanes 2 - 4) and some old samples (5 - 6), were normalized to 10 µg of total protein per lane. The blots were probed with anti-phosphotyrosine, clone 4G10®, and detected by chemiluminescence (left two blots) and by fluorescence (right two blots). Lanes 5 and 6 showed significantly lower signal than lanes 3 and 4 in both detection methods. Staining with Coomassie® blue right after immunodetection ruled out the possibilities of loading and transfer errors.

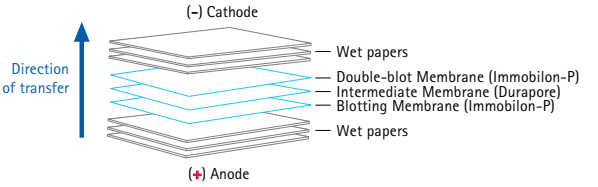
Protocol 1.6
Double Blotting: An Innovative Procedure for Eliminating the Nonspecific Binding of Secondary Antibodies

Required Equipment and Solutions

- Semi-dry transfer apparatus.
- Two sheets Immobilon®-P membrane (blot and double-blot membranes).
- Durapore® hydrophilic PVDF membrane, 0.65 µm pore size (Catalogue No. DVPP 000 10).
- Filter paper.
- 0.7% (v/v) acetic acid solution.
- Phosphate-buffered saline (PBS), pH 7.4.

Procedure

1. Blot resolved proteins onto Immobilon®-P membrane; block; apply primary antibody; and wash as described in Protocol 3.1. Standard Immunodetection Method, page 48.
2. Cut two stacks of filter paper and a sheet of Durapore® membrane to the dimension of the membranes.
3. Immerse the Durapore® membrane in the acid solution for at least 10 minutes. Prewet the double-blot (unblotted) Immobilon®-P membrane in methanol for 3 seconds; rinse in water for 2 minutes; and equilibrate in acid solution for 10 minutes. Wet the stacks of filter paper in the acetic acid solution by capillary wicking.
4. Place the blotted membrane onto a stack of filter paper and cover it with the Durapore® membrane and the double-blot membrane. Quickly put a second stack of filter paper onto the double-blot membrane to prevent drying. Position this sandwich in the blotting instrument with the blotted membrane facing the anode and the double-blot membrane facing the cathode.



5. Apply a constant intensity of 0.8 mA/cm^2 for 10 minutes.
6. Disassemble the sandwich.
7. Rinse the double-blot membrane twice in PBS using fresh buffer each time.
8. Block the double-blot membrane.
9. If the original blotting membrane (on which the blotted proteins are retained) will be reprobed with the same or another primary antibody, it can be kept in PBS after the acidic transfer.
10. Proceed with probing of the double-blot membrane with the appropriate secondary antibody and protein detection as described in Protocol 3.1. Standard Immunodetection Method, page 48.

For an explanation of double blotting, see pages 29-30.

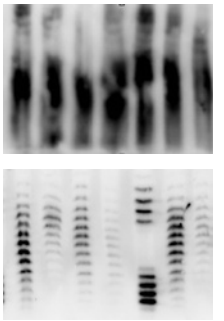


Figure 20.
Isoelectric patterns of urinary endogenous erythropoetin (EPO) without (top) and with (bottom) double blotting. Data courtesy of Dr. Françoise Lasne, Laboratoire National de Dépistage du Dopage.

Protocol 1.7
Membrane Drying Methods

The membrane must be dried before continuing on to transillumination or rapid immunodetection procedures. After proteins have been blotted onto the membrane, rinse the membrane with Milli-Q® water. 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.

▶ **TIP**: Allow the membrane to dry completely before continuing on to transillumination or rapid immunodetection.

Drying Method	Required Time*
Soak in 100% methanol for 10 seconds. Remove from 15 minutes methanol and place on piece of filter paper until dry.	1.5 minutes
Secure between two sheets of filter paper and place 30 minutes in a vacuum chamber.	30 minutes
Incubate at 37 °C for 1 hour	1 hours
Place on lab bench and let dry at room temperature.	2 hours

*Longer times required in higher humidity environments.

2. Protein Visualization

The following protocols describe typical procedures for staining proteins immobilized on an Immobilon® PVDF transfer 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.

▶ **TIP**: Only reversible stains or transillumination should be used prior to immunodetection.

▶ **TIP**: To visualize fainter bands, increase the methanol concentration to 40%.

Protocol 2.1
Visualization by Transillumination

Transillumination is a nondestructive, reversible technique (Reig and Klein, 1988). This method is based on the change of PVDF from opaque to semi-transparent when wet in methanol. 20% methanol will wet out regions of the membrane coated with protein, but not areas without protein.

▶ **TIP**: When developing a new transfer protocol or working with a new sample type, stain the gel to verify that all of the proteins have eluted from the gel.

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.
- Black paper.

Procedure

1. Dry the blot completely using one of the drying methods in Protocol 1.7. Membrane Drying Methods, page 44.
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 (Figure 10, blot A, page 24.)
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.

Protocol 2.2
Visualization by Reversible Staining

Ponceau-S Red

This stain produces pink bands on a light background.

Required Equipment and Solutions

- Stain: 0.2% Ponceau-S red, 1% acetic acid. Prepare by diluting stock solution (2% dye in 30% (w/v) trichloroacetic acid and 30% (w/v) sulfosalicylic acid) 1:10 in 1% (v/v) acetic acid.
- Methanol, 100%.
- 0.1 N NaOH.
- Milli-Q® water.
- Shallow tray, large enough to hold membrane.

Procedure

1. If the blot is dry, 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 Milli-Q® 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 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%.
- Milli-Q® water.
- Protein destain solution: 0.5 M $NaHCO_3$.
- Shallow tray, large enough to hold membrane.

Procedure

1. If the blot is dry, 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.

Protocol 2.3
Visualization by Irreversible Staining

Coomassie® Brilliant Blue R Dye

This 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%.
- Milli-Q® water.
- Shallow tray, large enough to hold membrane.

Procedure

1. If the blot is dry, 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 Milli-Q® water.
5. Place the blot in destain solution I and agitate for 10 minutes to remove excess stain.
6. Place the blot in destain solution II and agitate for 10 minutes to completely destain the background.

Amido Black

This stain produces dark bands on a light background.

IMPORTANT: This stain will interfere with immunodetection.

Required Equipment and 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%.
- Milli-Q® water.
- Shallow tray, large enough to hold membrane.

Procedure

1. If the blot is dry, 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 Milli-Q® water.
5. Place the blot in destain solution and agitate for 5 to 10 minutes to remove excess stain.

3. Immunodetection

Protocol 3.1
Standard Immunodetection Method

Standard immunodetection is performed on blotted proteins directly after electrotransfer. (If the membrane was dried after transfer, thoroughly wet the blot in methanol for 5 minutes before proceeding to immunodetection.) The unoccupied membrane binding sites on the wet blot are blocked with optimized reagents (see Protocol 1.5. Optimization of Blocking Reagents, page 41). 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 optimization for new sample types.

- **TIP**: Immobilon®-P^{SQ} transfer membrane has a smaller pore size (0.2 µm) and higher surface area than Immobilon®-P ransfer membrane (0.45 µm). Increased background can be expected on Immobilon®-P^{SQ} and the blocking and wash steps will need to be adjusted accordingly.
- **TIP**: Phosphatases in the blocking solution may dephosphorylate blotted proteins
- **TIP**: Do not use sodium azide in the buffers as it inhibits HRP activity.
- **Recommended**- Do not let the blot dry out at any time during and after blocking.
- **TIP**: If more than one blot is placed in a container, insufficient buffer volume will cause the blots to stick together.
- **TIP**: Dry milk powder cannot be used with biotin-avidin systems.
- **TIP**: Sensitivity of chromogenic detection is typically at least an order of magnitude lower than of chemiluminescent detection.
- **TIP**: High nonspecific signal can be alleviated by higher dilution of the primary antibody or reduced protein load on the gel.
- **TIP**: Immobilon®-FL membrane can be scanned dry or wet.
- **TIP**: High overall background can be minimized by higher dilution of the enzyme-conjugated secondary antibody.

Required 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 (PBST): 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl, up to 0.1% Tween®-20 detergent.
- TBST: 10 mM Tris, pH 7.4, 0.9% (w/v) NaCl, up to 0.1% Tween®-20.
- Blocking solution: 1% (w/v) BSA (bovine serum albumin), 0.05% Tween®-20.
- Milli-Q® water.

Required Equipment

- Shallow trays, large enough to hold blot.
- Glass plates.
- Plastic wrap (e.g., Saran™ film), freezer bag, or sheet protector.
- Autoradiography film and cassette.
- Dark room.
- Autoradiography film processing equipment.

Set Up

1. Dilute the primary antibody in the blocking solution to the desired working concentration.
2. Dilute the secondary antibody in the blocking solution to the desired working concentration. **NOTE**: Enough solution should be prepared to allow for 0.1 mL of antibody solution (primary and secondary) per cm^2 of membrane.

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 at RT or 37 °C.
5. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
6. Proceed with either chromogenic, chemiluminescent or fluorescent detection.

Chromogenic Detection

1. Prepare the substrate according to manufacturer'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 with mild agitation or until signal reaches desired contrast.
3. Rinse the blot with Milli-Q® water to stop the reaction.
4. Store the blot out of direct light to minimize fading. Blot may be stored dry.

Chemiluminescent Detection

Follow manufacturer's instructions.

1. Prepare the substrate according to manufacturer's instructions.
2. Place the blot in a container and add substrate to completely cover the membrane. Incubate for 1 minute.
3. Drain excess substrate.
4. Place the blot on a clean piece of glass and wrap in plastic wrap.

   NOTE: A cut-to-size sheet protector or a freezer bag can also be used.
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 done to determine the optimum exposure time; 1 to 5 minutes is common.

Fluorescent Detection

Required Equipment

- Proteins blotted onto Immobilon®-FL transfer membrane and probed with antibodies.
- Mylar® wrap.
- Fluorescent imaging equipment.

The following is a general protocol for fluorescent immunodetection. For optimal results, refer to manufacturer's protocol provided with the reagents. **NOTE**: If using chemifluorescent reagents, follow reagent manufacturer's directions.

1. Place the blot in diluted fluorescent dye-labeled secondary antibody solution and incubate for 1 hour with gentle agitation.
2. Wash the blot with wash buffer 3–5 times for 5 minutes each.
3. Place the blot onto a piece of clean filter paper to dry.
4. If using a wrap, use Mylar®. Do not use Saran™ wrap because it permits light to shine through and quench fluorescence.
5. Image the blot using an appropriate fluorescence scanner.

Protocol 3.2
Rapid Immunodetection Method

Rapid immunodetection takes advantage of the fact that antibodies cannot bind to the hydrophobic (non-wetted) surface of the Immobilon®-P transfer membrane, but will bind to a protein immobilized on the membrane. Rapid immunodetection is compatible with both chromogenic and chemiluminescent substrates.

▶ **TIP**: Use the Rapid Immunodetection Method to quickly visualize higher abundance proteins. For higher sensitivity, use the Standard Immunodetection Method, page 48.

The major advantage of rapid immunodetection is that blocking is not required, saving time and eliminating the risks involved (Mansfield, 1994). 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.

IMPORTANT: The blot must be thoroughly dry before beginning rapid immunodetection. Refer to Protocol 1.7. Membrane Drying Methods, page 44.

Required 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.
- Antibody dilution buffer: 1% (w/v) BSA (bovine serum albumin), 0.05% Tween®-20 detergent.
- Methanol, 100%.
- Milli-Q® water.

Required Equipment

- Shallow trays, large enough to hold blot.
- Plastic wrap (e.g., Saran™), freezer bag, or sheet protector.
- Autoradiography film and cassette.
- Dark room and autoradiography film processing equipment.

Set Up

1. Dry the blot completely using one of the drying methods in Protocol 1.7. Membrane Drying Methods, page 44. Do not re-wet the blot in methanol.
2. Dilute the primary antibody in dilution buffer to the desired working concentration.
3. Dilute the secondary antibody in dilution buffer to the desired working concentration. **NOTE**: Enough solution should be prepared to allow for 0.1 mL of antibody solution (primary and secondary) per cm^2 of membrane.

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 5 minutes. Repeat twice with fresh buffer.
3. Place the blot in the secondary antibody solution and incubate with agitation for 30 minutes.
4. Place the blot in PBS and wash for 5 minutes. Repeat twice with fresh buffer.
5. Proceed with chromogenic, chemiluminescent or fluorescent protein detection as described in Protocol 3.1, Standard Immunodetection Method, page 48.

Protocol 3.3
Rapid Immunodetection Using the SNAP i.d.® 2.0 System

The SNAP i.d.® 2.0 Protein Detection System is the second generation of the SNAP i.d.® method for detecting immunoreactive proteins on Western blots. With this unique, vacuum-driven system, reagents are pulled through the membrane, increasing contact between the reagents and the interior of the membrane, without depending on slow diffusion and agitation. This improvement in three-dimensional reagent distribution decreases the length of time required for immunodetection. While traditional Western blotting required 4 to 24 hours for the blocking, antibody incubation and washing steps,the SNAP i.d.® 2.0 protocol takes only 30 minutes with no loss of signal intensity or reduction in blot quality. All immunodetection steps after protein transfer to a membrane (i.e., blocking, washing, and primary and secondary antibody incubations) can be performed with the SNAP i.d.® 2.0 System.

Required Solutions

- Blocking reagent such as non-fat/low fat dry milk (0.5% or less), casein, bovine serum albumin (BSA) or other commercially available blocking agents such as Bløk®-CH reagent.
- Antibodies (monoclonal and/or polyclonal).
- Detection reagents.
- Wash buffer: Tris- or phosphate-buffered saline solution, pH 7.4, supplemented with Tween® 20 surfactant (TBST or PBST).

Required Equipment

- Vacuum source: Pump or other uniform vacuum source that can deliver a sustained minimum pressure of 410 millibar (12 in. Hg) and 34 L/min.

  NOTE: Merck Millipore WP61 series Chemical Duty Pumps can be used, but may require longer processing times.

- One liter or larger vacuum flask with stopper (for waste collection). A Millex®-FA50 filter (or equivalent) is recommended between the vacuum flask and the vacuum source to protect the vacuum source from contamination.
- Vacuum tubing to connect vacuum flask to vacuum source.
- Forceps.
- Blot with transferred proteins.

Setup

1. Place the SNAP i.d.® 2.0 base on a level bench top.
2. Attach the vacuum tubing to the back of the system by pushing the coupling insert on the end of the tubing into the quick disconnect fitting at the back of the system base until it clicks.
3. Connect the other end of the tubing to a vacuum source. Use a one-liter vacuum flask as a trap and a Millex®-FA50 filter (Catalogue No. SLFA05010) to protect the vacuum source from contamination.

   NOTE: Any vacuum source that can deliver 410 millibar (12 in. Hg) and 34 L/min is sufficient. If the vacuum source operates at higher than 410 millibar, the SNAP i.d.® 2.0 system will automatically regulate the vacuum pressure.

Blot Assembly & General Protocol

1. Hold the blot holder by the support layer (blue edges) and wet the membrane layer (white) with distilled water in the wetting tray provided. Place the wetted blot holder on the rolling pad.
2. If required, pre-wet the blot in methanol and water, then place it in the center of the blot holder with the protein side down.
3. Roll the blot gently to remove air bubbles, then close the blot holder and roll one more time.
4. Open the blot holding frame, flip the blot holder so that it is protein side up, then place it inside the frame. A notch in the blot holder ensures correct placement in the frame.
5. Close and lock the frame. Add 30 mL of blocking solution. Press the frame down and turn the system knob to apply vacuum. When frame is completely empty, TURN VACUUM OFF.
6. Apply 5 mL (for Mini blot) or 10 mL (for Midi blot) of primary antibody across the surface of the blot holder.
7. Incubate for 10 minutes at room temperature. Solution will be absorbed into the blot holder and surface may appear dry.
8. Press the frame down and apply vacuum. Wait 5–8 seconds until the frame is completely empty.
9. With vacuum running continuously, add 30 mL of wash buffer. Repeat the washing step 3 more times (total of 4 washes). When frame is completely empty, TURN VACUUM OFF.
10. Apply 5 mL (for Mini blot) or 10 mL (for Midi blot) of secondary antibody evenly across the blot holder surface. Incubate for 10 minutes at room temperature with vacuum off. Again, solution will be absorbed into the blot holder and surface may appear dry.

11. Press frame down and apply vacuum. Wait 5–8 seconds until frame is completely empty. With vacuum running continuously, add 30 mL of wash buffer. Repeat the washing step 3 more times (total of 4 washes).
12. Turn vacuum off and remove blot holder from frame. Remove blot from blot holder and incubate with the appropriate detection reagent.

Extended Primary Antibody

Incubation: One Hour to Overnight

1. Perform steps 1 through 5 of the General Protocol.
2. Add 5 mL (for Mini blot) or 10 mL (for Midi blot) of 1X primary antibody (concentration normally used during standard immunodetection).
3. Cover the blot holding frame with the lid. If desired, remove it from the base and incubate with constant shaking. If overnight incubation is required, refrigeration is recommended. Although the surface of the blot holder may look partially dry, the blot will not dry out, since the solution is contained in the frame.

   NOTE: If processing more than one blot for extended period of time, the blot holding frames can be stacked to reduce the working space.
4. **OPTIONAL**: If recovering primary antibody, place antibody recovery trays into base before proceeding to step 4.
5. After the extended incubation period, place the frame back on the base, remove the lid, and follow steps 8 through 12 of the General Protocol.

   NOTE: Extended incubation periods for the secondary antibody are not required for the SNAP i.d.® 2.0 System. A 5X concentration of secondary antibody for 10 minutes is recommended.

Antibody Recovery

1. Perform steps 1 through 5 of the General Protocol, but increase the vacuum time to 2 minutes to ensure that all the blocking solution has been removed from the grooves and channels of the frame.
2. Remove the blot holding frame from the base and wipe any residual liquid from the bottom of the frame with a paper towel.
3. Place the antibody collection tray into the base, making sure that the positioning holes in the antibody collection tray line up with the positioning pins in the base.
4. Place the blot holding frame back into position on the base.
5. Apply primary antibody and incubate as indicated in steps 6 and 7 of the General Protocol.
6. After the 10-minute incubation, turn the vacuum on and wait one minute to ensure that all of the antibody has been collected.

   NOTE: When processing two frames at the same time, apply vacuum first to one side, then to the other. This ensures full vacuum force on each frame and improves volume recovery.
7. Turn the vacuum off and remove the frame. Remove the antibody collection tray.
8. Transfer the antibody to a suitable container for storage or analysis.
9. Place the blot holding frame back into position and continue with step 9 of the General Protocol.

Protocol 3.4
High Salt Wash to Remove Persistent Background

Required Solutions

- High salt buffer: PBS or TBS buffer supplemented with 0.5M NaCl and 0.2% SDS

Procedure

1. Follow Protocol 3.1. Standard Immunodetection Method on page 48, through primary and secondary antibody incubations.
2. After the secondary antibody incubation, place the blot in the high salt buffer and incubate for 30 minutes with gentle shaking.
3. Rinse blot with Milli-Q® water and proceed with chromogenic, chemiluminescent or fluorescent protein detection as described in the Standard Immunodetection Method section on page 48.

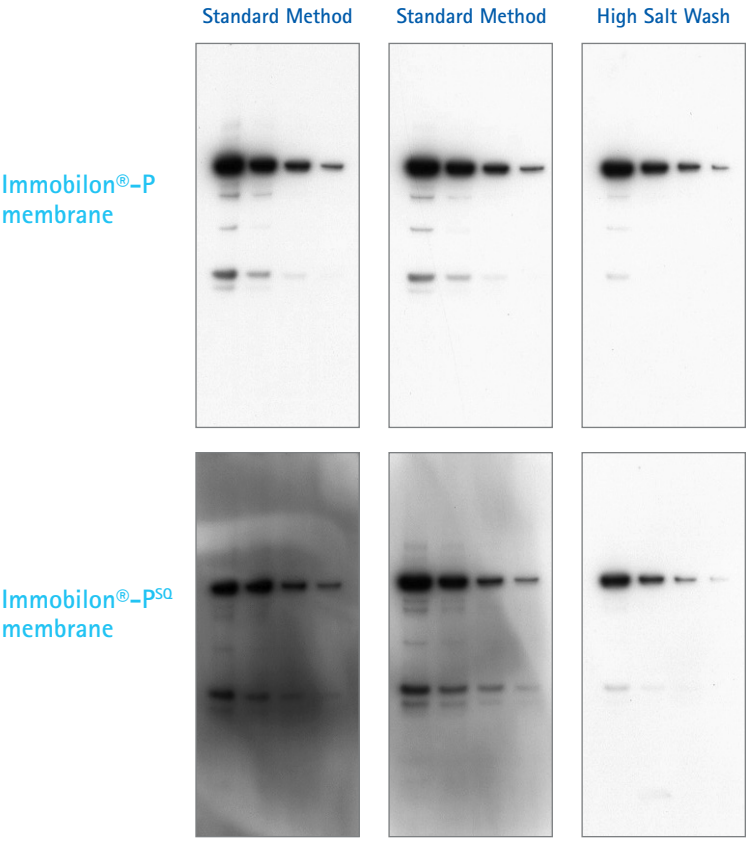


Figure 21.
Adding 0.5 M NaCl and 0.2% SDS to the wash buffer reduced the background considerably on the Immobilon®-P^SQ membrane, which has a high protein binding capacity.

4. Membrane Stripping

Two protocols for removing antibodies from blots 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 specific to a different target protein.

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.

Protocol 4.1
Stripping by Heat and Detergent

Required Equipment and Solutions

- Stripping solution: 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7.
- Phosphate buffered saline (PBS): 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl.
- Shallow tray, large enough to hold the membrane.

Procedure

1. In a fume hood, place the blot in stripping solution and incubate with agitation 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 immunodetection.

Protocol 4.2
Stripping by Low pH

Required Equipment and Solutions

- Stripping solution: 25 mM glycine-HCl, pH 2, 1% (w/v) SDS.
- Phosphate buffered saline (PBS): 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl.
- Shallow tray, large enough to hold the membrane.

Procedure

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 immunodetection.

Protocol 4.3
Stripping with the ReBlot™ Plus Western Blot Recycling Kit

Required Equipment and Solutions

- Standard blot or blot strips, on nitrocellulose or PVDF/nylon membrane.
- Blocking solutions.
- Plastic wrap for storage of blots that will not be reprobed immediately.
- Distilled water, for reagent dilution.
- Plastic trays for incubation of blots or blot strips in stripping, washing and blocking solutions.
- Positive and negative stripping controls.

Procedure

NOTE: The blots or individual strips that are to be reused should be prepared for stripping immediately after their first usage. If stripping cannot be performed right away, membranes can be wrapped in plastic wrap and stored moist in PBS at 4°C. DO NOT STORE BLOTS IN DRY FORM.

1. Fill a plastic tray with appropriate amount of 1X Antibody Stripping Solution (supplied in kit).
2. Using tweezers or forceps, submerge blot or blot strips in stripping solution. Incubate with gentle mixing for 15 minutes at room temperature.
3. Fill a clean plastic tray with an equal amount of blocking buffer. Conventional blocking buffers such as 20 mM Tris HCl, pH 8.0; 150 mM NaCl; 0.1% Tween 20; 5% dry milk or similar are appropriate.
4. Wash blots two times 5 minutes each with blocking buffer.
5. The blot is now ready for reprobing with antibodies.

5. Protein Digestion

Protocol 5.1
On-Membrane Protein Digestion for Mass Spectrometry*

This method describes the preparation of proteins immobilized on Immobilon® PVDF transfer membrane for analysis by mass spectrometry.

> **TIP**: Mass spectrometry-compatible membrane stains include Coomassie® Blue, amido black, and Sypro blot stains.

Required Equipment and Solutions

- 50% methanol in Milli-Q® water.
- 30% acetonitrile, 50 mM ammonium bicarbonate.
- Trypsin, dissolved in Milli-Q® water at 0.1 mg/mL.

  80% acetonitrile in Milli-Q® water.
- Vacuum centrifuge.
- Microcentrifuge tubes.
- MALDI-TOF matrix.

Procedure

1. Transfer protein from the gel to the membrane (Protein Transfer Protocols, pages 35-38).
2. Stain the blotted membrane with Coomassie® Brilliant Blue or amido black (Protocol 2.3. Visualization by Irreversible Staining, page 47).
3. Wash the stained membrane with water and dry.
4. Excise pieces of membrane containing proteins of interest with a clean scalpel and place in separate microcentrifuge tubes.
5. Destain membrane pieces with 500 µL of 50% methanol for 2 hours at room temperature.
6. Remove supernatant and dry the membrane.
7. Add 10 µL of 30% acetonitrile, 50 mM ammonium bicarbonate, and 4 µL of 0.1 mg/mL trypsin.
8. Incubate overnight at room temperature.
9. Transfer supernatants to clean microcentrifuge tubes.
10. Extract peptides with 20 µL of 80% acetonitrile for 15 minutes with sonication.
11. Pool the extracts with the previous supernatants.
12. Dry the digests in the vacuum centrifuge.
    NOTE: Alternatively, Merck Millipore ZipTip®SCX pipette tips can be used to purify and concentrate the peptide digest instead of drying. To do this, acidify the digest with 0.5% TFA (make sure the pH is <3) and follow the instructions in the ZipTip®SCX Pipette Tip User Guide (Merck Millipore Lit. No. P36444).
13. Resuspend each peptide extract in a small volume of 30% acetonitrile, 0.1% TFA.
14. Load 1 –2 µL of the digest onto a MALDI plate, and mix with 1 µL of matrix.

*Based on Bienvenut, et al. (1999)

6. Blot Storage

Protocol 6.1
Preparation of Protein Blots for Long-term Storage

PVDF is a chemically resistant polymer with excellent long term stability. For blots that need to be stored for use at a later date, storage conditions are determined by the stability of the proteins bound to the membrane. We recommend storage at low temperature, room temperature may be adequate for some proteins.

Required Materials

- Dry blotted Immobilon® PVDF transfer membrane.
- Two sheets of Whatman® 3MM paper.
- Two sheets of card stock or thin cardboard.
- Paper clips.
- Plastic bag.

Procedure

1. Place the dry blot between two clean sheets of Whatman® 3MM paper.
2. Place the blot-filter paper sandwich between two sheets of card stock.
3. Clip the stack together along the edges. The clips should not overlap the blot.
4. Place the stack into a sealable plastic bag.
5. Close or seal the bag.
6. Store the blot at the desired temperature:

4 °C	For up to 2 weeks
20 °C	For up to 2 months
70 °C	For longer term storage

NOTE: Blots stored in a freezer should not be subjected to mechanical shock, which can cause breakage of the membrane. The blot should be allowed to come to room temperature before removal from the plastic bag. Blots may also be stored wet at 4 °C in a plastic bag, but a bacteriocide such as sodium azide should be added to prevent bacterial growth. The azide must be thoroughly washed out of the blot prior to use as it inhibits HRP activity.

Appendices

Troubleshooting Blotting Problems

1. Dot/Slot (Filtration) Blotting

Symptom	Possible Cause	Remedy
Slow or no filtration of the sample through the membrane	Inadequate vacuum	Make sure the blotting unit is closed properly and the seal is intact. Make sure the vacuum source (e.g. pump) is operating properly. Seal off any open wells with a high quality laboratory tape. Increase vacuum level.
	Membrane pores clogged	Centrifuge or filter samples to remove particulates. Dilute viscous samples with buffer.
Little or no protein observed on the blot	Not enough protein applied to the membrane	Minimize sample dilution and filter more sample through the membrane.
	Detergents (e.g., SDS) may inhibit lower molecular weight proteins from binding to the membrane	Eliminate detergents if possible.
	Stain not sensitive enough.	Use a more sensitive stain.
Stained blot is not uniform	Membrane structure was compressed by filter paper	Place a second membrane in the blotting unit to protect the membrane receiving the samples.
	Air bubbles trapped in the interior of the membrane	Pre-wet membrane by laying it on the surface of the methanol. Immersing the membrane can entrap air.
	Membrane not pre-wet in methanol	Membrane must be pre-wet with methanol; entire membrane should change uniformly from opaque to semi-transparent.
	Air bubbles in the sample.	Carefully pipette samples into well to avoid the formation of air bubbles.
	Not enough sample volume loaded	Sample must cover the entire exposed membrane area.
Protein smeared across the top of the membrane	Sample leaked across the wells	Make sure the blotting unit is properly assembled, closed and sealed prior to filtration.
Protein smeared across the back of the membrane	Membrane capacity was exceeded	Reduce the amount of protein loaded into the well.

2. Semi-dry or Tank Electrotransfer

Symptom	Possible Cause	Remedy
Band smeared/ distorted	Membrane not uniformly wetted with methanol	The entire membrane must be pre-wet with methanol; the entire membrane should change uniformly from opaque to semi-transparent.
	Air bubbles under membrane and between other layers in the stack	Using a pipette or stirring rod, gently roll out any trapped air bubbles while assembling the stack.
	Uneven contact between gel and membrane	Make sure entire gel and membrane surfaces are in good contact.
	Too much heat generated during the transfer	The temperature of the run should not exceed 20 °C. For a tank transfer, pre-chill the buffer or carry out the transfer in a cold room. For a semi-dry transfer, either shorten the run time, increase the number of filter papers, or reduce the current.
	Filter paper dried out during semi-dry transfer	Make sure filter paper is thoroughly drenched prior to transfer or use additional sheets. Be sure the stack is assembled in less than 15 minutes.
	Proteins transferred too rapidly; protein buildup on the membrane surface	Reduce the strength of the electrical field.
Weak signal	Proteins passing through the membrane	Increase the time the proteins have to interact with membrane by reducing the voltage by as much as 50%. Highly negatively charged proteins (due to high aspartic acid and glutamic acid content) tend to move very fast in an electric field. Decrease the voltage to slow down migration of these proteins. Presence of SDS in the gel may inhibit protein binding. Equilibrate the gel in the transfer buffer for at least 15 minutes. Methanol concentration in transfer buffer is too low to facilitate removal of SDS. Increase the methanol to 15 – 20%, especially for smaller molecular weight proteins. The membrane must be pre-wet with methanol; the entire membrane should change uniformly from opaque to semi-transparent. Switch to Immobilon®-P^{SQ} transfer membrane.
	Proteins retained in the gel	If the methanol concentration in the transfer buffer is too high, it can remove SDS from proteins and lead to protein precipitation in the gel. This would reduce the transfer of large molecular weight proteins out of the gel. If protein precipitation is an issue, the transfer buffer can be supplemented with SDS (0.01% – 0.05%) to aid in solubility. In addition, excess methanol can tend to shrink or tighten a gel, thus inhibiting transfer of large molecular weight proteins.

2. Semi-dry or Tank Electrotransfer (continued)

Symptom	Possible Cause	Remedy
Weak signal (continued)	Isoelectric point of the protein is at or close to the pH of the transfer buffer	A protein that has the same isoelectric point as the pH of of the transfer buffer will have no net charge and thus will not migrate in an electric field. To facilitate transfer, try a higher pH buffer such as 10 mM CAPS buffer at pH 11, including 10% methanol or a lower pH buffer such as an acetic acid buffer.
	Poor detection when urea is used in the gel and/or transfer buffer	Reduce the temperature by using a circulating buffer setup or run your transfer in a cold room. Urea in the presence of heat can cause carbamylation of proteins, which can change the charge of amino acids in a protein. This could affect the epitopes essential for antibody recognition and binding.
	Incomplete transfer of proteins	Stain the gel to check for residual proteins. If transfer was not complete, review your transfer technique.
	Poor protein retention	Once transfer is complete, be sure to dry the membrane completely to obtain optimal binding and fixation of the proteins. This should be done prior to any downstream detection method.
No signal	No transfer of proteins	Check for the gel and membrane orientation during the transfer process. Use pre-stained molecular weight standards to monitor transfer.
Poor transfer of small molecular weight proteins	Insufficient protein retention SDS interferes with binding of small molecular weight proteins	Switch to Immobilon®-P^{SQ} transfer membrane. Remove SDS from the transfer solution.
	Low methanol concentration in the transfer buffer	Use higher percentage of methanol (15% – 20%) in the transfer buffer.
	Insufficient protein binding time	A lower voltage may optimize binding of small proteins to the membrane.
	Current doesn't pass through the membrane	Cut membrane and blotting paper exactly to the gel size; do not allow overhangs.
Poor transfer of large molecular weight proteins (~ >80 kDa)	Methanol concentration is too high	Reducing the methanol concentration to 10% (v/v) or less should help in the transfer of large molecular weight proteins by allowing the gel to swell. Moreover, a lower methanol percentage would also reduce SDS loss from the proteins and reduce protein precipitation in the gel. Proteins >200 kDa are not as sensitive to interference from the SDS in binding to membrane as are proteins <100 kDa.
Poor transfer of positively charged proteins	Protein net charge in the transfer buffer is positive; proteins move toward cathode.	Reverse the transfer stack such that the Immobilon® transfer membrane is on the cathode side of the gel.
Poor semi-dry transfer	Current bypasses the gel stack	Make sure the membrane and blotting paper are cut exactly to the gel size and there are no overhangs.
Poor transfer of a wide range of protein sizes	Different conditions required to transfer large and small proteins	Refer to "Transfer of a broad MW range of proteins may require a multi-step transfer" (T. Otter et al., Anal. Biochem. 162:370-377 (1987). Use three-buffer system for semi-dry transfer (Protocol 1.2, page 36.)

3. Protein Visualization

Symptom	Possible Cause	Remedy
Poor detection by transillumination	Inappropriate membrane	Transillumination works best with Immobilon®-P transfer membrane. It is not recommended for nitrocellulose or Immobilon®-P^{SQ} transfer membrane.
	Membrane wasn't completely dried prior to wetting with methanol	Be sure that the membrane was dried completely after the transfer prior to immersing it in the 20% methanol solution. Make sure to use a 20% methanol solution.
	Blot saturated with water only	Saturate the blot with 20% methanol.
	Insufficiently saturated blot	Increase concentration of methanol up to 40%.
Weak or uneven stain	Membrane wasn't wetted in methanol prior to staining	The membrane must be pre-wet with methanol; the entire membrane should change uniformly from opaque to semitransparent.
Uneven/splotchy results	Use sufficient volume of incubation solutions and ensure that the membrane is completely covered with these solutions during incubation.	The container used should be large enough to allow solution to move freely across the blot. Do not incubate more than one blot at a time in that same container. In addition, the protein side of the blot should be facing up so as not to be interacting with the bottom surface of the container.
	Air bubbles	The blot should not have any air bubbles on the surface. Gently pull the membrane across the edge of the container to remove bubbles.
	Poor reagent quality	All of the buffers and reagents should be fresh and free of particulates and contaminants. Filtration of buffers with Millex® syringe filters or Stericup®, Steritop® or Steriflip® filter units and centrifugation of antibody stocks may be required.
High background staining	Nonspecific protein binding to the membrane	Make sure to use clean electrotransfer equipment and components and high quality reagents and Milli-Q® water.

4. Immunodetection

Symptom	Possible Cause	Remedy
Weak signal	Improper blocking reagent	The blocking agent may have an affinity for the protein of interest and thus obscure the protein from detection. Try a different blocking agent and/or reduce both the amount or exposure time of the blocking agent.
	Insufficient antibody reaction time	Increase the incubation time.
	Antibody concentration is too low or antibody is inactive	Multiple freeze-thaw or bacterial contamination of antibody solution can change antibody titer or activity. Increase antibody concentration or prepare it fresh.
	Outdated detection reagents	Use fresh substrate and store properly. Outdated substrate can reduce sensitivity.
	Protein transfer problems	Optimize protein transfer (see above).
	Dried blot in chromogenic detection	If there is poor contrast using a chromogenic detection system, the blot may have dried. Try rewetting the blot in water to maximize the contrast.
	Tap water inactivates chromogenic detection reagents	Use Milli-Q® water for reagent preparation.
	Azide inhibits HRP	Do not use azide in the blotting solutions.
	Antigen concentration is too low	Load more antigen on the gel prior to the blotting.
No signal	Antibody concentration too low	Increase concentration of primary and secondary antibodies.
	HRP inhibition	HRP-labeled antibodies should not be used in solutions containing sodium azide.
	Primary antibody was raised against native protein	Separate proteins in non-denaturing gel or use antibody raised against denatured antigen.
Uneven blot	Fingerprints, fold marks or forceps imprints on the blot	Avoid touching or folding membrane; use gloves and blunt end forceps
Speckled background	Aggregates in the blocking reagent	Filter blocking reagent solution through 0.2 µm or 0.45 µm Millex® syringe filter unit.
	Aggregates in HRP-conjugated secondary antibody	Filter secondary antibody solution through 0.2 µm or 0.45 µm Millex® syringe filter unit.
High background	Insufficient washes	Increase washing volumes and times. Pre-filter all of your solutions including the transfer buffer using Millex® syringe filter units or Steriflip® filter units.
	Secondary (enzyme conjugated) antibody concentration is too high	Increase antibody dilution.
	Protein-protein interactions	Use Tween®-20 (0.05%) in the wash and detection solutions to minimize protein-protein interactions and increase the signal to noise ratio.
	Immunodetection on Immobilon®-P^{SQ} transfer membrane	Increase the concentration or volume of the blocking agent used to compensate for the greater surface area of the membrane. Persistent background can be reduced by adding up to 0.5M NaCl and up to 0.2% SDS to the wash buffer and extending the wash time to 2 hours.
	Poor quality reagents	Use high quality reagents and Milli-Q® water.
	Crossreactivity between blocking reagent and antibody	Use different blocking agent or use Tween®-20 detergent in the washing buffer.
	Film overexposure	Shorten exposure time.
	Membrane drying during incubation process	Use volumes sufficient to cover the membrane during incubation.
	Poor quality antibodies	Use high quality affinity purified antibodies.
	Excess detection reagents	Drain blots completely before exposure.
Persistent background	Nonspecific binding	Use High Salt Wash. (Protocol 3.4, page 54.)

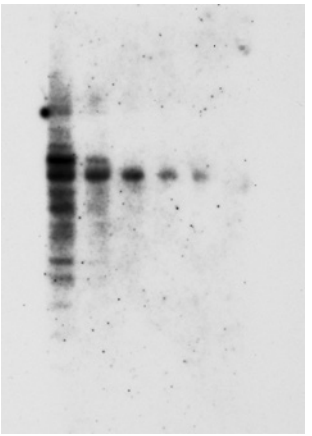
4. Immunodetection (continued)

Symptom	Possible Cause	Remedy
High background (rapid immuno-detection)	Membrane wets out during rapid immunodetection	Reduce the Tween®-20 (<0.04%) detergent in the antibody diluent. Use gentler agitation during incubations. Rinse the blot in Milli-Q® water after electrotransfer to remove any residual SDS carried over from the gel. Be sure to dry the blot completely prior to starting any detection protocol.
	Membrane was wet in methanol prior to the immunodetection	Do not pre-wet the membrane.
	Membrane wasn't completely dry prior to the immunodetection	Make sure the membrane is completely dry prior to starting the procedure.
Nonspecific binding	Primary antibody concentration too high	Increase primary antibody dilution.
	Secondary antibody concentration too high	Increase secondary antibody dilution.
	Antigen concentration too high	Decrease amount of protein loaded on the gel.
Reverse images on film (white bands on dark background)	Too much HRP-conjugated secondary antibody	Reduce concentration of secondary HRP-conjugated antibody.
Poor detection of small proteins	Small proteins are masked by large blocking molecules such as BSA	Consider casein or a low molecular weight polyvinylpyrrolidone (PVP). Surfactants such as Tween® and Triton® X-100 may have to be minimized. Avoid excessive incubation times with antibody and wash solution.

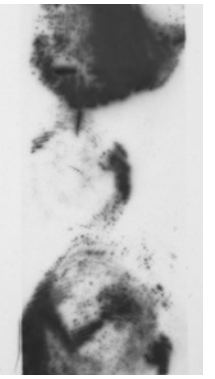
5. Fluorescent Detection

Symptom	Possible Cause	Remedy
High overall background	High background fluorescence from the blotting membrane	Use Immobilon®-FL PVDF blotting membrane.
	Blocking reagents not optimized for fluorescence detection.	Use Bløk®-FL Noise-canceling Reagents, optimized for fluorescent Western blots.
Multiplexing problems	Experimental design	The two antibodies must be derived from different host species so that they can be differentiated by secondary antibodies of different specificities. Before combining the two primary antibodies, test the banding pattern on separate blots to determine where bands will appear. Use cross-adsorbed secondary antibodies in two-color detection.
Speckled background	Dust/powder particles on the Handle blots	Handle blots with powder-free gloves and clean surface of the scanner.
Low signal	Wet blot	Drying the blot may enhance signal strength. The blot can be scanned after re-wetting. Do not wrap the blot in plastic/Saran™ wrap while scanning.
	Blot photo-bleached	While fluorescent dyes usually provide long-lasting stable signal, some fluorescent dyes can be easily photo-bleached. To prevent photo-bleaching, protect the membrane from light during secondary antibody incubations and washes, and until the membrane is ready to be scanned. Store developed blots in the dark for subsequent imaging.
	Wrong excitation wavelength	Follow dye manufacturers' instructions for blot imaging or use an emission filter

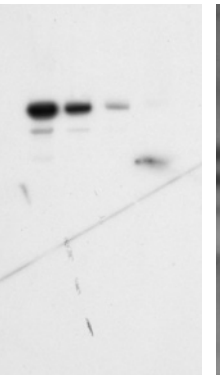
Examples and Causes of Blot Failure



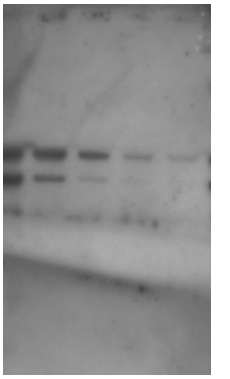
Splotchy background caused by insufficient washes and/or unfiltered blocking solution. Can be improved by filtering the blocking solution through 0.2 µm Millex®-GP syringe filter and adding extra washes.



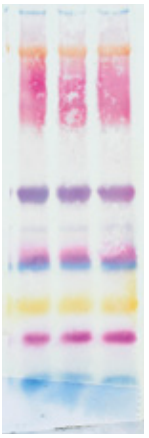
Fingerprints



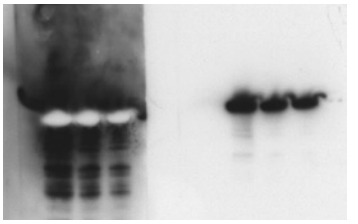
Scratches and forceps imprints



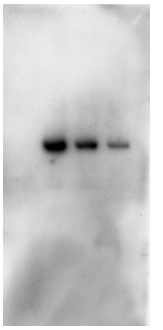
Folded membrane



Bubbles between the membrane and SDS-PAGE gel introduced during the transfer.



Negative (reverse) image on film caused by excess secondary HRP-conjugated antibody. The detection was improved by increasing secondary antibody dilution 10-fold. Blot on left was incubated with a 1:5,000 dilution of secondary antibody, and blot on right was incubated with a 1:50,000 dilution.



High background and dark edges caused by insufficient washing of the blot. Can be alleviated by additional or/and longer washes or a high salt wash (Protocol 3.4, page 54.)

Glossary

1-D
One-dimensional

2-D
Two-dimensional

Adsorption
The process whereby molecules or particles (e.g., protein) bind to a surface (e.g., membrane).

Anode
Positively charged electrode in an electrophoresis system.

Blocking
Technique used to reduce nonspecific binding of antibodies during immunodetection; unoccupied membrane sites are blocked with an inert protein, nonionic detergent, or protein-free blocking reagent.

Blot
A microporous membrane with biomolecules adsorbed onto the polymer.

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

Cathode
Negatively charged electrode in an electrophoresis system.

Chemiluminescent detection
Immunodetection technique that results in the production of visible light at the site of the target protein.

Chromogenic detection
Immunodetection technique that results in the deposition of a colored precipitate at the site of the target protein.

Dot blot
A blot prepared by filtration of liquid samples through a membrane using a dot blot manifold (see "Filtration").

Edman Degradation
A process that uses the Edman reagent, phenyl isothiocyanate (PITC), to sequentially remove one amino acid from the N-terminus of a protein. The chemically derivatized amino acid is then analyzed after it is cleaved from the protein. Sequential processing of the protein provides the amino acid sequence of the protein.

Electrotransfer
Common method for the transfer of proteins from a gel to a membrane. Proteins elute from the gel and get absorbed onto the membrane when an electrical field is applied perpendicular to the plane of the gel.

ELISA
Enzyme-Linked Immunosorbent Assay; a rapid method to determine the presence and quantity of a specific substance. It is based on an antigen-antibody interaction where the antibody or antigen is linked to an enzyme as a means of detecting its presence.

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.

Fluorescent detection
Immunodetection of a protein using a primary or secondary antibody conjugated to a fluorescent dye.

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

Immunoblot
A Western blot that has been analyzed for a target protein using a specific antibody.

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

Isoelectric focusing
Method of protein separation on the basis of isoelectric points. Usually achieved by electrophoresis of proteins in a stabilized pH gradient where proteins migrate to the pH corresponding to their isoelectric points.

Isoelectric point (pI)
The pH value at which the net electric charge of a molecule, such as a protein or amino acid, is zero. It is usually the first dimension of a 2-D gel.

Polyacrylamide
A branched polymer of acrylamide that is used in gel electrophoresis.

Primary antibody
The first antibody used in an immunodetection protocol. The primary antibody is specific for the target antigen.

PVDF
Polyvinylidene fluoride; the polymer used to make Immobilon®-P, Immobilon®-P^{SQ} and Immobilon®-FL transfer membranes. (Occasionally this acronym is erroneously defined in the blotting literature as polyvinylidene difluoride.)

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

Reprobing
The process of sequentially cycling a blot through multiple rounds of immunodetection.

Retention
In the context of stripping and reprobing immunoblots, retention refers to the ability of a protein to remain adsorbed to a membrane surface under conditions that disrupt immunocomplexes.

SDS
Sodium dodecyl sulfate. SDS is a detergent that binds to proteins, giving them a net negative charge. It is used in denaturing protein gel electrophoresis. It is also widely used to disrupt cell walls and dissociate protein complexes. Lithium dodecyl sulfate (LDS) can be substituted for SDS.

SDS–PAGE
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Secondary antibody
The second antibody used in an immunodetection protocol. The secondary antibody is specific for the primary antibody and is typically conjugated to an enzyme used for signal amplification.

Semi–dry transfer
Electrotransfer technique where the traditional buffer reservoir is replaced by layers of filter paper soaked in buffer; an equally effective, but faster technique than tank transfer. Multiple buffer systems can be used during the transfer.

Slot blot
A blot prepared by the filtration of liquid samples through a membrane using a slot blot manifold.

Spot blotting
A blot prepared by manually applying sample to the membrane.

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

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

Substrate
The compound that interacts with the enzyme conjugated to the secondary antibody to yield a detectable signal in immunodetection. The signal is typically in the form of a colored precipitate (chromogenic substrates) or electromagnetic radiation (light from chemiluminescent or fluorescent substrates).

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

Transfer buffer(s)
The buffer(s) used as the chemical environment to facilitate the transfer of biomolecules from a gel onto a membrane.

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

Vacuum blotting
The process of transferring biomolecules from a gel to a membrane using vacuum as the driving force; not typically used for protein gels.

Western blot
A blot prepared by transferring protein from a polyacrylamide gel to a membrane.

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Patents

U.S. Pat. No. 6,632,339, issued to W.V. Bienvenut et al. on October 14, 2003.

U.S. Pat. App. Pub. No. 2002/0136668 (published September 26, 2002), filed by D. Wallace et al. on December 18, 2001.

International Patent Application No. PCT/AU98/00265 of A. Gooley et al. (published October 22, 1998 as International Publication No. WO 98/47006).

International Patent Application No. PCT/FR01/01331 of Laboratoire National de Dépistage du Dopage with Hospices Civils de Lyon which also holds the patent (2 786 273).

7 Ordering Information

2X Sample Buffer (2105)

Component	Catalogue No.
130 mM Tris HCl pH 8.0	9310 ■
20% (v/v) Glycerol	4750 ■
4.6% (w/v) SDS	7910 ■
0.02% Bromophenol blue	2830 ■
2% DTT	3860 ■

8X Resolving Gel Buffer: 100 mL

Component	Catalogue No.
0.8 g SDS (add last)	7910 ■
36.3 g Tris base (=3 M)	9210 ■

Adjust pH to 8.8 with concentrated HCl

4X Stacking Gel Buffer: 100 mL

Component	Catalogue No.
0.4 g SDS (add last)	7910 ■
6.05 g Tris base (=0.5 M)	9210 ■

Adjust pH to 6.8

10X Running Buffer: 1 L

Component	Catalogue No.
30.3 g Tris base (=0.25 M)	9210 ■
144 g Glycine(=1.92 M)	4810 ■
10 g SDS (=1%, add last)	7910 ■

Do not adjust pH!

10X Transfer Buffer: 1 L (Catalogue No. 9000, ready to use)

Component	Catalogue No.
30.3 g Tris base (=0.25 M)	9210 ■
144 g Glycine(=1.92 M)	4810 ■

pH should be 8.3, do not adjust

Wash Buffer

Component	Catalogue No.
OmniPur® 10X PBS, Premixed Powder	6508 ■

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	26.5 cm × 3.75 m	1 roll	IPVH00010
Immobilon®-FL: PVDF 0.45 µm	7 × 8.4 cm	10/pk	IPFL07810
	26.5 cm × 3.75 m	1 roll	IPFL00010
Immobilon®-P^{SQ}: PVDF 0.2 µm	7 × 8.4 cm	50/pk	ISEQ07850
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SNAP i.d.® 2.0 systems

Description	Catalogue No.
SNAP i.d.® 2.0 System-Mini (7.5 x 8.4 cm)	SNAP2MINI
SNAP i.d.® 2.0 System-Midi (8.5 x 13.5 cm)	SNAP2MIDI
SNAP i.d.® 2.0 System-Mini and Midi (7.5 x 8.4 cm and 8.5 x 13.5 cm)	SNAP2MM

SNAP i.d.® 2.0 consumables

Description	Qty	Catalogue No.
SNAP i.d.® 2.0 Mini Blot Holders (7.5 x 8.4 cm)	100/pk	SNAP2BHMN0100
SNAP i.d.® 2.0 Midi Blot Holders (8.5 x 13.5 cm)	100/pk	SNAP2BHMD0100

SNAP i.d.® 2.0 accessories

Description	Qty	Catalogue No.
SNAP i.d.® 2.0 Antibody Collection Tray	20/pk	SNAPABTR
SNAP i.d.® 2.0 Blot Roller	1/pk	SNAP2RL
SNAP i.d.® 2.0 Mini Blot Holding Frames (double pack)	2/pk	SNAP2FRMN02
SNAP i.d.® 2.0 Midi Blot Holding Frames (double pack)	2/pk	SNAP2FRMD02
SNAP i.d.® 2.0 Mini Blot Holding Frame (single pack)	1/pk	SNAP2FRMN01
SNAP i.d.® 2.0 Midi Blot Holding Frame (single pack)	1/pk	SNAP2FRMD01

Bløk® noise cancelling reagents

Description	Detection Method	Qty	Catalogue No.
Bløk®-CH Reagent	Chemiluminescence Detection	500 mL	WBAVDCH01
Bløk®-FL Reagent	Fluorescence Detection	500 mL	WBAVDFL01
Bløk®-PO Reagent	Phosphorylated Protein Detection	500 mL	WBAVDP001

Luminata™ Western HRP substrates

Description	Qty	Catalogue No.
Luminata™ Classico Western HRP Substrates	500 mL	WBLUC0500
Luminata™ Crescendo Western HRP Substrates	500 mL	WBLUR0500
Luminata™ Forte Western HRP Substrates	500 mL	WBLUF0500

Western blotting enhancing reagents

Description	Qty	Catalogue No.
SignalBoost™ Immunoreaction Enhancer Kit	400 mL	407207 ■

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