

MILLIPORE

protein
blotting
handbook



About the Third Edition

Millipore is pleased to publish the third edition of the Protein Blotting Handbook. Much work has been done to substantially enhance this current edition, with the inclusion of more information on substrates, more protocols, more tips, and a new troubleshooting section.

The publication represents the collective experience of Millipore's 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.

About Millipore Corporation

Millipore has been one of the leading suppliers of transfer membranes for nearly three decades. E.M. Southern used Millipore membrane to develop the first nucleic acid transfer from an agarose gel in 1975.¹ The first 0.45 μm PVDF membrane for Western blotting, Immobilon-P, was introduced by Millipore in 1985, and the first 0.2 μm PVDF membrane for protein blotting and sequencing, Immobilon-P^{SQ}, was introduced by Millipore in 1988.

In addition to Immobilon transfer membranes, Millipore provides a wide selection of other tools for protein research, including Amicon® centrifugal devices, Montage® antibody purification kits, ZipTip® pipette tips for MS sample prep, and a full line of devices for sterilizing tissue culture media.

Where to Get Additional Information

If you have questions or need assistance, please contact a Millipore technical service specialist, or pose your question on-line at www.millipore.com/techservice.

You'll also find answers to frequently asked questions (FAQs) concerning Western blotting on the Millipore web site, as well as FAQs for other methods related to protein blotting.

¹The Scientist, Nov. 3, 2003, pp. 14.

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I. 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 uses vacuum filtration to transfer protein to a micro-porous membrane, known as dot blot or slot blot. While this method may provide quantitative information about 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). A specific protein can be identified through its reaction with a labeled antibody or antigen. Through spatial resolution this method provides molecular weight information on individual proteins and separates isoforms from processing products. After proteins have been transferred to a PVDF membrane, they can be stained and directly identified by N-terminal sequencing, mass spectrometry or immunodetection.

In the clinical laboratory, immunoblotting has emerged for applications in fields such as infectious and autoimmune diseases, allergy, and others (Towbin *et al.*, 1989; Stahl *et al.*, 2000). Western blotting is considered as a reliable confirmatory diagnostic test following a repeatedly reactive ELISA over the course of viral infection, and is reported to be the most sensitive, unequivocal and simple test system available, with the highest complexity of information obtained (Bauer, 2001; Mylonakis *et al.*, 2000; Heermann *et al.*, 1988). Examples of western blotting applications include global 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).

This guide contains background information and protocols for every step of the protein blotting procedure.

II. Membrane Selection

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

PVDF as a substrate was first introduced by Millipore Corporation in 1985. There are many advantages to electroblotting onto PVDF membranes rather than onto nitrocellulose membranes. PVDF membranes offer better protein retention, physical strength and 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 purposes, such as staining with Coomassie™ Blue followed

by band excision and N-terminal sequencing, proteolysis/peptide separation/internal sequencing, and immunodetection (Kurien, *et al.*, 2003).

Typical binding capacity for commercially available nitrocellulose membranes is 80 – 100 µg/cm² while PVDF membranes offer a binding capacity of 100 – 200 µg/cm².

In direct comparison of PVDF and nitrocellulose membranes in a serological assay for human immunodeficiency virus (HIV), 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 – 200 µg/cm ²
Solvent resistance	No	Yes
Western transfer	Yes	Yes
Total protein stain	Colloidal gold Ponceau-S red Amido black India ink	Colloidal gold Ponceau-S red Amido black India ink Coomassie Blue
Detection	Chromogenic Chemiluminescent Fluorescent Radioactive	Chromogenic Chemiluminescent Fluorescent Radioactive Chemifluorescent (ECF™)
Rapid immunodetection	No	Yes
Western reprobing	Yes	Yes
Edman sequencing	No	Yes
Amino acid analysis	Yes	Yes
Glycoprotein detection	No	Yes
Binding in the presence of SDS	Poor	Good
On-membrane digestion for mass spectrometry	No	Yes
Direct MALDI-TOF MS analysis	No	Yes

Immobilon™ PVDF Transfer Membranes

Millipore offers PVDF membranes with 0.45 µm and 0.2 µm pore sizes. The larger pore size Immobilon-P transfer membrane is commonly used for immunoblotting applications, while the 0.2 µm pore size Immobilon-PS^Q transfer membrane is used for optimal immunoblotting of low molecular weight proteins and sequencing applications due to its higher binding capacity and improved protein retention.

Immobilon transfer membrane is provided in cut sheets of different sizes and rolls. Millipore's cuts are compatible with all the pre-cast gels and most commercially available gel running systems. See Table 2 (page 6) for properties of Immobilon-P and Immobilon-PS^Q transfer membranes. See Table 3 (page 7) to match Immobilon membrane cuts with the most commonly used electrophoresis systems. See Table 4 (page 7) to match Immobilon membrane cuts with available pre-cast gels.

Table 2: Properties and applications of Immobilon-P and Immobilon-P^{SQ} transfer membranes.

	Immobilon-P transfer membrane	Immobilon-P^{SQ} 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
Composition	PVDF	PVDF
Pore size	0.45 µm	0.2 µm
Phobicity	Hydrophobic	Hydrophobic
Applications	Western blotting Binding assays Amino acid analysis N-terminal protein sequencing Dot/slot blotting Glycoprotein visualization Lipopolysaccharide analysis Mass spectrometry	Low molecular weight western blotting Amino acid analysis Mass spectrometry N-terminal protein sequencing
Detection methods	Chromogenic Radioactive Fluorescent Chemifluorescent Chemiluminescent	Chromogenic Radioactive Fluorescent Chemifluorescent Chemiluminescent
Protein binding capacity	Insulin: 85 µg/cm ² BSA: 131 µg/cm ² Goat IgG: 294 µg/cm ²	Insulin: 262 µg/cm ² BSA: 340 µg/cm ² Goat IgG: 448 µg/cm ²
Compatible stains	Coomassie Brilliant Blue Amido black India ink Ponceau-S red Colloidal gold CPTS Toluidine blue Transillumination	Coomassie Brilliant Blue Amido black India ink Ponceau-S red Colloidal gold CPTS Toluidine blue Transillumination

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 ^{5Q} 0.2 µm
Amersham	SE 250 Mighty Small™	8 × 7	8.4 × 7	IPVH07850	ISEQ07850
	SE 260 Mighty Small	8 × 9.5	8 × 10	IPVH08100	ISEQ08100
	miniVE	8 × 9.5	8 × 10	IPVH08100	ISEQ08100
	miniVE	10 × 10	10 × 10	IPVH10100	ISEQ10100
	SE 400	14 × 16	15 × 15	IPVH15150	ISEQ15150
	SE 600	14 × 16	15 × 15	IPVH15150	ISEQ15150
	SE 600	14 × 8	13.5 × 8.5	IPVH08130	ISEQ08130
Bio-Rad	Mini-PROTEAN® 3, Mini-PROTEAN 3 Dodeca	8.3 × 7.3	8.4 × 7	IPVH07850	ISEQ07850
	Criterion™, Criterion Dodeca	13.3 × 8.7	13.5 × 8.5	IPVH08130	ISEQ08130
	PROTEAN II xi	16 × 16	15 × 15	IPVH15150	ISEQ15150
	PROTEAN II xi	16 × 20		IPVH00010	ISEQ00010
	PROTEAN II XL	19.3 × 18.3	20 × 20	IPVH20200	ISEQ20200
	PROTEAN Plus Dodeca	20 × 20.5	26 × 26	IPVH304F0	ISEQ304F0
	Mini-PROTEAN II	8.3 × 7.3	8.4 × 7	IPVH07850	ISEQ07850
Invitrogen	XCell SureLock™ Mini-Cell, XCell6™ MutiGel	8 × 8	7 × 8.4	IPVH07850	ISEQ07850
Owl	P81 Puffin™, P82 Wolverine™, P8DS Emperor Penguin™	10 × 10	10 × 10	IPVH10100	ISEQ10100
	P8DS Emperor Penguin	8 × 10	8 × 10	IPVH08100	ISEQ08100
	P9DS Emperor Penguin	16 × 16	15 × 15	IPVH15150	ISEQ15150
	P10DS Emperor Penguin	20 × 20	20 × 20	IPVH20200	ISEQ20200
Thermo Electron	EC120	7 × 8	7 × 8.4	IPVH07850	ISEQ07850

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 ^{5Q} 0.2 µm
Bio-Rad	Ready Gel®	8.3 × 7.3	8.4 × 7	IPVH07850	ISEQ07850
	Criterion	13.3 × 8.7	13.5 × 8.5	IPVH08130	ISEQ08130
	PROTEAN Ready Gel	16 × 16	15 × 15	IPVH15150	ISEQ15150
	PROTEAN Ready Gel	19.3 × 18.3	20 × 20	IPVH20200	ISEQ20200
	PROTEAN Ready Gel	20 × 20.5	26 × 26	IPVH304F0	ISEQ304F0
Cambrex	PAGEr®	9 × 10	8 × 10	IPVH08100	ISEQ08100
	PAGEr	10 × 10	10 × 10	IPVH10100	ISEQ10100
Gradipore	MicroGel®	8 × 2.5	8 × 10 (cut in ¼)	IPVH08100	ISEQ08100
	igels™	8 × 5.8	8 × 10 (cut in ½)	IPVH08100	ISEQ08100
	LongLife Gels	8 × 5.8	8 × 10 (cut in ½)	IPVH08100	ISEQ08100
Invitrogen	NuPAGE®	8 × 8	7 × 8.4	IPVH07850	ISEQ07850
	Novex®	8 × 8	7 × 8.4	IPVH07850	ISEQ07850
	Zoom®	8 × 8	7 × 8.4	IPVH07850	ISEQ07850
Pierce	Precise Protein Gels	8 × 5.8	8 × 10 (cut in ½)	IPVH08100	ISEQ08100

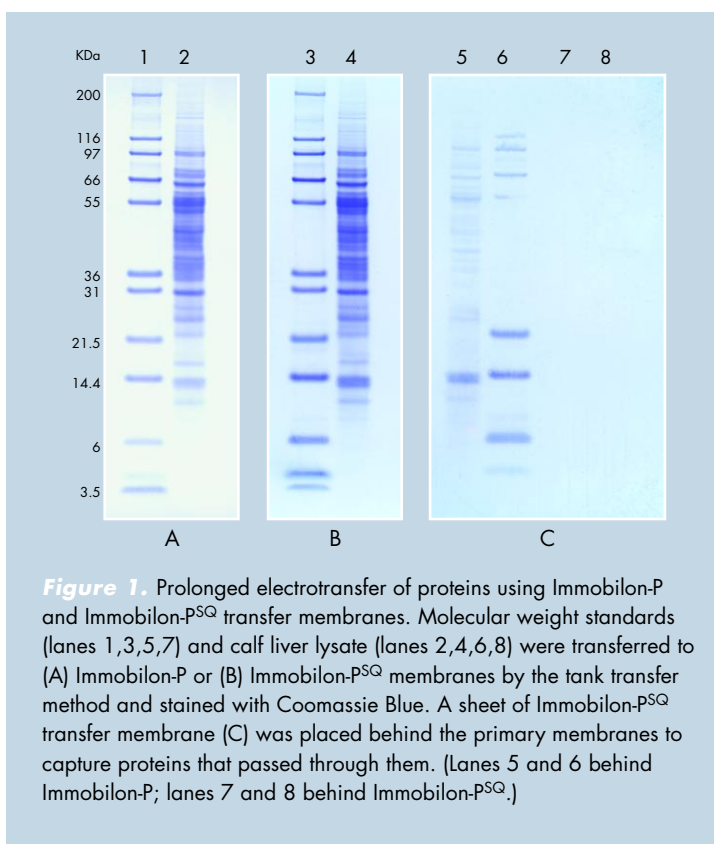
III. Protein Binding

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

Binding Differences between Immobilon-P and Immobilon-PSQ 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-PSQ transfer membrane has approximately three times the internal surface area of Immobilon-P transfer membrane, resulting in higher adsorptive capacity (see Table 2, page 6). 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-PSQ 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-PSQ 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 back-up membrane. In contrast, all of the proteins were bound to the Immobilon-PSQ coupon.



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 will result in a higher background. Thus, the choice of the 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.

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.

Sample blot



Immunodetection of human transferrin on Immobilon-P transfer membrane with Perkin Elmer Western Lightning[®] Western Blot Chemiluminescence Reagent. Left to right, 5 μ L of human serum dilutions 1:5,000, 1:25,000, and 1:125,000. Electroblotted proteins were probed with goat anti-human transferrin (1:10,000 dilution) and AP-conjugated rabbit anti-goat IgG (1:20,000 dilution).

IV. 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 a vacuum. Proteins adsorb to 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 are then available for analysis.

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 can not be performed by conventional techniques such as ELISA. Grid immunoblotting can be 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.

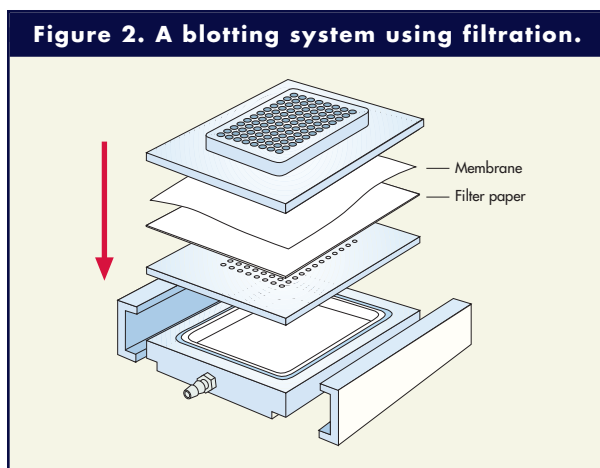
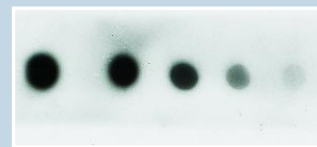


Figure 3.

Rapid Immuno-detection of dot blotted human serum on Immobilon-P



membrane (see Protocol 1.3, Dot Blotting, page 31; and Protocol 3.2, Rapid Immunodetection Method, page 38). The proteins were probed with goat anti-human transferrin (1:10,000 dilution) and HRP-conjugated rabbit anti-goat IgG (1:10,000 dilution) and detected with Amersham ECL reagents according to the manufacturer's instructions.

- 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, with only the supernatant applied to the membrane. Viscous samples should be diluted in buffer.

Western Blotting

Western blotting comprises a series of steps involving:

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

In order for the western blotting process to be successful, 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—a protein must remain adsorbed to the blot during post-transfer processing.
- Accessibility during processing—the adsorbed protein must be available to the chemistries being used to detect it. If the protein is masked, it can not 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 (shown in Figure 4). In some cases, non-denaturing electrophoresis is 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 modern 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 allows 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 proteins, native or recombinant. 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

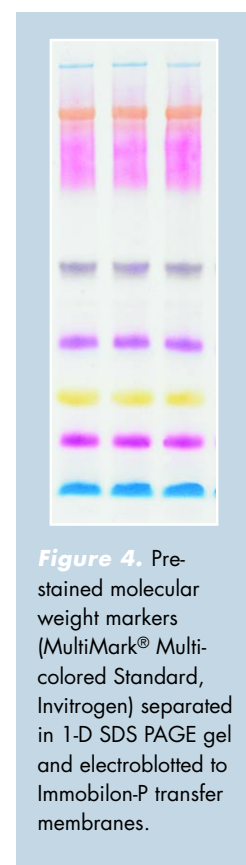


Figure 4. Pre-stained molecular weight markers (MultiMark® Multi-colored Standard, Invitrogen) separated in 1-D SDS PAGE gel and electroblotted to Immobilon-P transfer membranes.

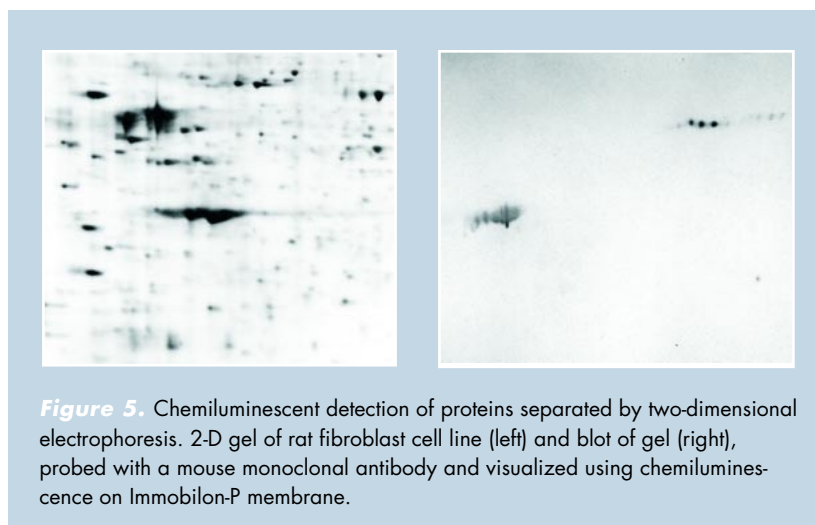


Figure 5. Chemiluminescent detection of proteins separated by two-dimensional electrophoresis. 2-D gel of rat fibroblast cell line (left) and blot of gel (right), probed with a mouse monoclonal antibody and visualized using chemiluminescence on Immobilon-P membrane.

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, and the dyes attached to the proteins may alter their ability to adsorb to the membrane during blotting.

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 gels 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 <14 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 apply the electrical field.

Sample blot



Immunodetection of serine/threonine protein phosphatase 2A/A in calf liver lysate with Amersham ECL Advance reagents, after 1 minute (left) and 10 minutes (right) exposure to X-ray film. Each panel, left to right, 3, 0.6, 0.12 and 0.024 μg of total liver protein per lane. Rabbit primary antibody (1:1000 dilution) and HRP-conjugated goat anti-rabbit IgG (1:50,000 dilution) were used to visualize the antigen.

Tank transfer (Figure 6), is the traditional technique where the gel/membrane stack is immersed in a buffer reservoir and then current is applied. It is an effective but slow technique, uses large volumes of buffer and can only use one buffer. Also, it can be difficult to set up a tank and buffer to accommodate large gels typically used in 2-D electrophoresis. Tank systems are typically run at constant voltage; mixing of the buffer during transfer keeps the current relatively constant.

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 capacity for the buffers 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 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 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.

Figure 6. Tank transfer system.

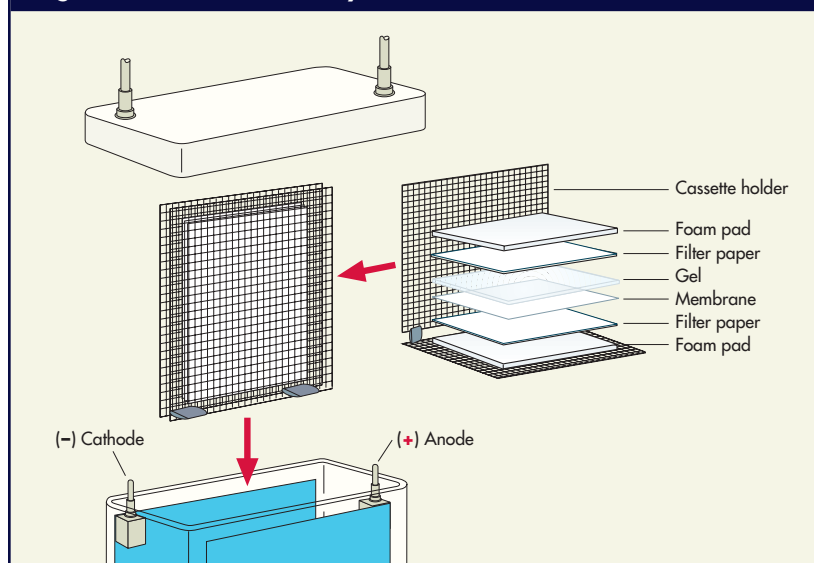
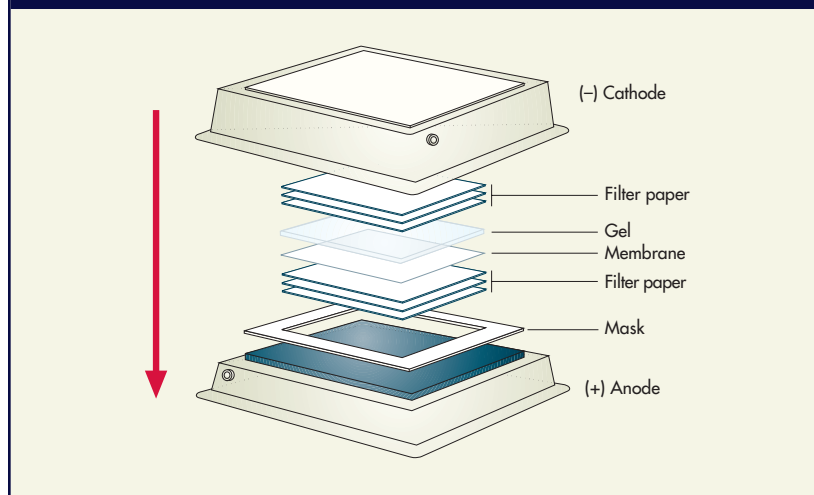


Figure 7. Semi-dry transfer system.



Semi-dry systems use 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). 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 ϵ -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.

In general, Millipore does not recommend substitution of a single system for the three buffer system. With a single buffer system, transfer tends to be inconsistent across the gel and often incomplete.

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, meaning that it 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. Higher 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 swelling and transfer protocols normally include an equilibration step to achieve dimensional stability. At concentrations of 10% to 20%, dimensional stability can be achieved fairly rapidly. At lower 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 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

Sample blot



Immunodetection of serine/threonine protein phosphatase 2A/A in calf liver lysate with Pierce SuperSignal West Femto Maximum Sensitivity Substrate reagents, after 1 minute (left) and 10 minutes (right) exposure to X-ray film. Each panel, left to right, 3, 0.6, 0.12 and 0.024 μ g of total liver protein per lane. Rabbit primary antibody (1:1000 dilution) and HRP-conjugated goat anti-rabbit IgG (1:50,000 dilution) were used to visualize the antigen.

the PVDF. These effects increase as the MW of the protein decreases. Methanol reduces both effects by stripping off the SDS and improving the probability that a protein molecule will bind to the membrane.

Factors Affecting Successful Protein Transfer

Presence of SDS

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). 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-PSQ transfer membrane. BSA that eluted after 15 minutes adsorbed almost exclusively to the sheet of Immobilon-P transfer membrane. 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 when the proteins to be transferred have low solubility in the absence of SDS. Proteins from 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 be used to 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 were prolonged blotting time, up to 21 hours (Erickson *et al.*, 1982), or the use of a composite agarose-polyacrylamide 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 gel percentage, 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.

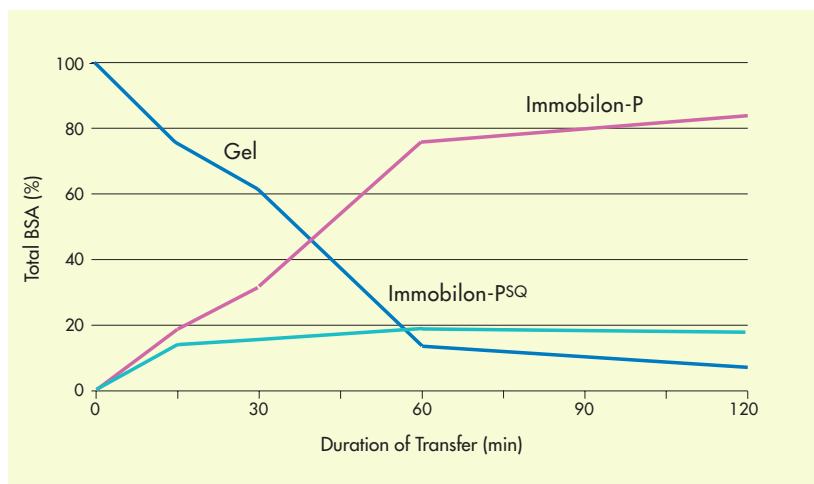


Figure 8. Electrotransfer of BSA. 25 picomoles of ^{125}I -labeled BSA were resolved by SDS-PAGE on a 10–20% gradient gel. After equilibration for 5 minutes, the BSA was transferred to Immobilon-P transfer membrane, backed up with Immobilon-PSQ transfer membrane, in a tank transfer system using 25 mM 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.

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 be promoted. To alleviate this problem, the higher pH buffers such as CAPS or the 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. Unfortunately, the kinetics of SDS stripping are significantly slower; and protein transfer through the membrane is significant. Therefore, 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 it 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 reduce the SDS to a minimal level.

The effect of equilibration time on electrotransfer of BSA is shown in Figure 9. In this study, radioactive 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-PS^Q 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 back-up blot (Immobilon-PS^Q 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.

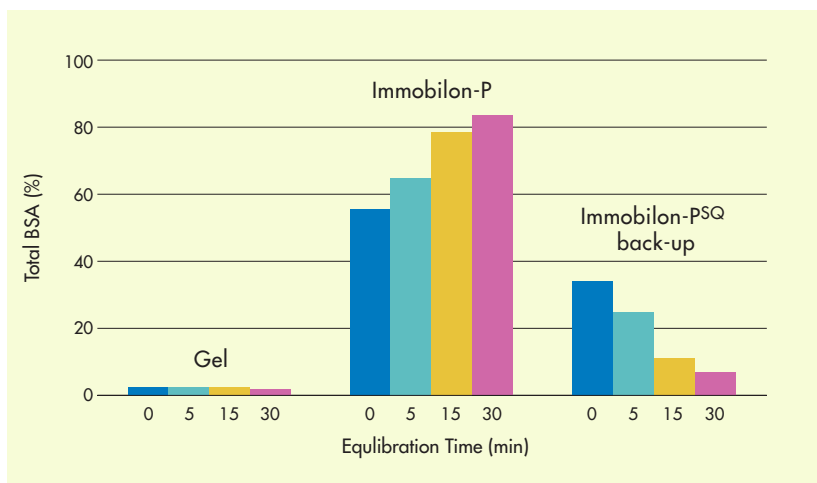
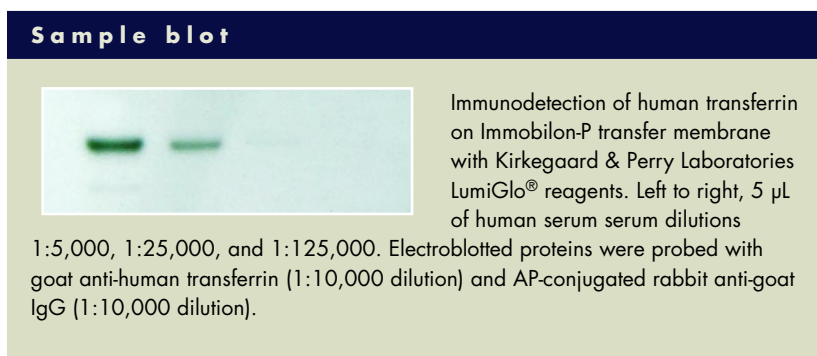


Figure 9. Effect of equilibration time on electrotransfer of BSA to Immobilon-P transfer membrane. ¹²⁵I-labeled BSA was resolved by SDS-PAGE on a 10–20% gradient gel. After equilibration for the times noted, the BSA was transferred to Immobilon-P transfer membrane, backed up with Immobilon-PS^Q transfer 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. They 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 reduce 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 actually eluted from the gel. It is also highly recommended to have a lane with stained 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. Staining the membrane after the transfer can also be helpful to ensure that the target protein is on the blot. See Protein Visualization, on the following page, for information on stains compatible with immunodetection.

Another method to monitor protein transfer is to stain SDS-PAGE gels prior to the electroblotting (Thompson and Larson, 1992). In this method, the gels are stained with either Coomassie Brilliant Blue after electrophoresis, or during electrophoresis using

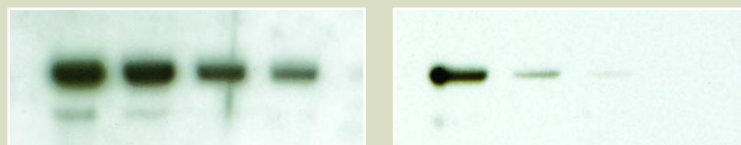
the ChromaPhor™ visualization system (Promega). The transferred proteins remain stained during immunodetection, providing a set of background markers for protein location and size determination (Thompson and Larson, 1992).

Preparing Membrane for Protein Identification

Drying

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 Membrane Drying Methods, page 33). PVDF membranes can be stored dry for long periods of time after proteins have been transferred with no ill effects to the membrane (up to 2 weeks at 4 °C; up to 2 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. Once dried, a membrane should be wet prior to any further analysis by immersion in 100% methanol.

Sample blot



Immunodetection of human transferrin on Immobilon-P transfer membrane with Amersham ECL Advance (left) and ECL (right) reagents. Left to right, 5 μ L of human serum dilutions 1:1000, 1:5,000, 1:25,000, 1:125,000, and 1:625,000. Electroblotted proteins were probed with anti-human transferrin (1:50,000 dilution for ECL Advance and 1:10,000 for ECL) and HRP-conjugated mouse anti-goat IgG (1:50,000 dilution for ECL Advance and 1:10,000 for ECL).

Protein Visualization

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 sample

Many types of stains are available, such as organic dyes (Ponceau-S red, amido black, fast green, Coomassie Blue), fluorescent dyes (fluorescamine, coumarin) and colloidal particles (gold, silver, copper, iron or India ink) (Kurien *et al.*, 2003). Table 5 lists the most common stains for detection of total proteins on western blots.

The dyes fall into two general categories: reversible and non-reversible. Non-reversible stains generally exhibit the best sensitivity but can interfere with or prevent further analysis of the proteins. Examples of non-reversible stains are amido black and Coomassie Brilliant Blue. Although less sensitive, reversible stains allow assessment of the blot and then can be washed from the membrane.

The most commonly used reversible protein stain is Ponceau-S red. Even if the target protein is too limited in abundance to be detected by staining, the staining pattern of more abundant proteins is generally indicative of how well minor proteins transferred.

New 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 (Molecular Probes) can be used prior to chromogenic, fluorogenic or chemiluminescent immunostaining procedures and provide sensitivity of about 1–2 ng/band. (Haugland, 2002).

Transillumination

Transillumination (Figure 11) is a visualization technique unique to PVDF membranes and was first described for Immobilon-P transfer membrane (Reig and Klein, 1988). The technique is based on the premise that areas of PVDF coated with transferred protein are capable of wetting out in 20% methanol while the surrounding areas of exposed PVDF are not. In areas where the PVDF wets, it becomes optically transparent, allowing

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

Detection Reagent	Approximate Sensitivity (protein per spot)	Reversible (compatible with immunodetection)	Reference
Ponceau-S red	5 µg	Yes	Dunn <i>et al.</i> , 1999
Fast green FC	5 µg	Yes	Dunn <i>et al.</i> , 1999
CPTS	1 µg	Yes	Bickar <i>et al.</i> , 1992
Sypro Ruby	1–2 ng	Yes	Haugland, 2002
Sypro Rose	1–2 ng	Yes	Haugland, 2002
Amido black 10B	1 µg	No	Dunn <i>et al.</i> , 1999
Coomassie Brilliant Blue R-250	500 ng	No	Dunn <i>et al.</i> , 1999
India ink	100 ng	No	Dunn <i>et al.</i> , 1999
Colloidal gold	4 ng	No	Dunn <i>et al.</i> , 1999

Figure 10. Western blots of calf liver proteins on Immobilon-P membrane were detected with (A) Ponceau-S red, (B) CPTS, and (C) Coomassie Brilliant Blue total protein blot stains. Left to right, molecular weight standards, 20, 5 and 1.25 μ g of liver proteins per lane loaded.

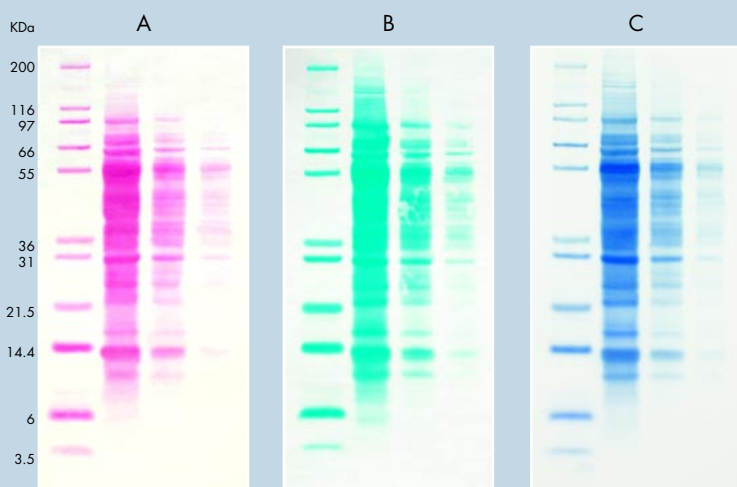
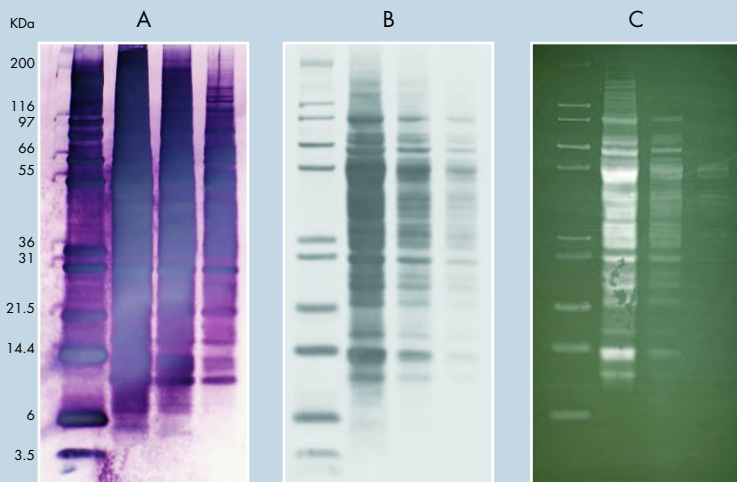


Figure 11. Western blots of calf liver proteins on Immobilon-P membrane were detected with (A) AuroDye™ colloidal gold (Amersham) and (B) Sypro Ruby (Molecular Probes) total protein blot stains, and (C) by transillumination. Left to right, molecular weight standards, 20, 5 and 1.25 μ g of liver proteins per lane loaded.



for 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 probably were exposed to methanol during blotting. Even though this technique does not allow for visualization of minor proteins, it can be used to assess the overall transfer efficiency and the suitability of the blot for further analysis.

Sample blot



Immunodetection of human transferrin on Immobilon-P transfer membrane with Applied Biosystems Western-Star™ Immunodetection System. Left to right, 5 μ L of human serum dilutions 1:1,000, 1:5,000, 1:25,000, and 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).

V. Protein Identification

Immunodetection

Immunodetection uses a specific antibody to detect and localize a protein blotted to the membrane (Figure 12). The specificity of antibody-antigen binding permits the identification of a single protein in a complex sample.

When developing procedures for one's own samples, 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.

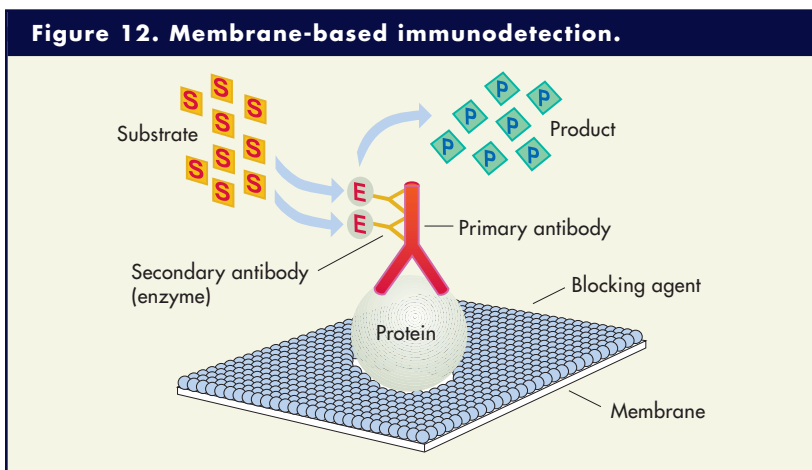
Standard vs. Rapid Immunodetection Procedures

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 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 in the Protocols section. Table 6 compares the times required to perform the steps of two protocols.



The following sections provide important information regarding immunodetection. Understanding these basic concepts will help to optimize 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 feature is that the buffer must preserve the biological activity of the antibodies. Thus, the ionic strength and pH should be fairly close to physiological conditions. PBS formulations with 10 mM total phosphate work well with a wide array of antibodies and detection substrates.

While incubating, the container holding the membrane should be gently agitated. A sufficient volume of buffer should be used to cover the membrane 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.

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 or non-ionic detergent. The blocking agent should have a greater affinity for the membrane than the antibodies. It should fill all unoccupied binding sites without displacing the target protein from the membrane, binding to epitopes on the target protein, or cross-reacting with the antibodies or detection chemistry.

The most common blocking agents are bovine serum albumin (BSA, 0.2–0.5%), non-fat milk, casein, gelatin, and dilute solutions of Tween®-20 (0.05–0.1%). Tween-20 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, SDS, and NP-40, are sometimes used but can be too harsh

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	10 min	10 min
Total time	4 hr 10 min	2 hr 10 min

and disrupt interaction between proteins. The blocking agent is usually dissolved in an appropriate buffer, such as PBS or TBS. There are risks associated with blocking; a poorly selected blocking agent or excessive blocking can displace or obscure the protein of interest. It is also important not to let the blot to dry out at any time during and after blocking.

The correct choice of a blocking reagent can be critical. For example, dry milk can not be used with biotinylated or concanavalin-labeled antibodies since it contains both glycoproteins and biotin. The analysis of phosphorylated proteins with phospho-specific antibodies can be compromised if the blocking agents contain phosphatases, which, upon contact with the phosphorylated protein on the blot, can dephosphorylate it. It was shown that addition of the phosphatase inhibitors in 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.

It is important to remember that Immobilon-PSQ transfer membrane, with its smaller pore size and higher surface area than Immobilon-P transfer membrane, binds more protein. If Immobilon-PSQ transfer membrane is substituted directly for Immobilon-P transfer membrane in a standard western blotting procedure, there may be insufficient blocking agent to saturate the membrane surface. Additional washing steps may also be required to reduce the background.

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 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. One 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. Also, because more than one molecule of the secondary antibody may be able to bind to the primary antibody, a secondary antibody can enhance the signal.

Either polyclonal or monoclonal antibodies are used. Polyclonal antibodies usually come in a form of antiserum or affinity-purified antibody. Monoclonal antibodies are expressed in ascites fluid or tissue culture fluid. 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 call for 0.1% (v/v) Tween-20 in the blocking solution and antibody diluent. It is important to recognize that concentrations above 0.05% (v/v) have the potential to wash some blotted proteins from the membrane. Elevating the concentration of Tween-20 is often used to reduce the background. Often, a simpler and more cost-effective strategy is to reduce the concentration of the antibodies, notably the secondary antibody.

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 concentration of primary and secondary antibodies should be optimized for each case. Generally, nonspecific signal can be alleviated 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 secondary, enzyme-conjugated antibody.

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, higher concentration detergent solutions could elute the protein of interest from the membrane. For highly purified antibodies, buffer alone is often sufficient for washing.

The amount of washing required is best determined experimentally. Too little washing will yield 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 time.

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

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 fluorescent.

Chromogenic Detection

Chromogenic detection (Figure 13) uses the enzyme to catalyze a reaction resulting in the deposit of an insoluble colored precipitate, for example 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 facts should be kept in mind:

- Sensitivity of chromogenic detection is 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.

Chemiluminescent Detection

Chemiluminescent detection uses the 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 alkaline phosphatase (Cortese, 2002). This technique has the speed and safety of chromogenic detection at sensitivity levels comparable to radioisotopic detection. The blots then are either exposed to X-ray films, or are directly scanned in chemiluminescence-compatible imaging systems, usually equipped with highly cooled CCD cameras to avoid electronic noise. Reprobing is possible with chemiluminescent substrates.

Fluorescent Detection

Fluorescent detection employs either a fluorophore-conjugated antibody or fluorogenic substrates (known as chemifluorescence) that fluoresce at the site of enzyme activity. One advantage of this method is that the fluorescent signal is stable indefinitely, and blots can be archived and re-imaged. In addition, the wide variety of fluorophores makes it possible to detect multiple protein targets in a single sample simultaneously (multiplex detection).

Table 7, on the following page, lists commercially available kits for chromogenic, chemiluminescent and fluorescent immunodetection that have been

tested and proven with Millipore's Immobilon transfer membranes.

It is possible to make reagents for ECL immunodetection using p-iodophenol (PIP) and the 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).

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 (Figure 14). This may be especially useful for method optimization or when sample amount is limited. Refer to Membrane Stripping, page 40.



Figure 13. Immunodetection of transferrin in human serum with chromogenic substrate BCIP/NBT (KPL). Left to right, 5 μ L human serum serum dilutions 1:1,000, 1:5,000, 1:25,000, 1:125,000,

1:625,000. Electroblotted proteins were probed with goat anti-human transferrin (1:10,000 dilution) and AP-conjugated rabbit anti-goat IgG (1:30,000 dilution).

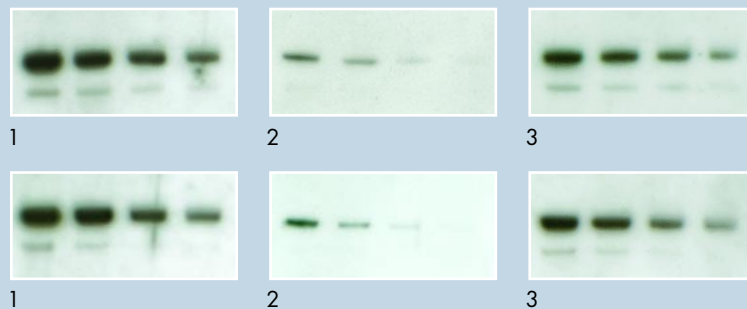


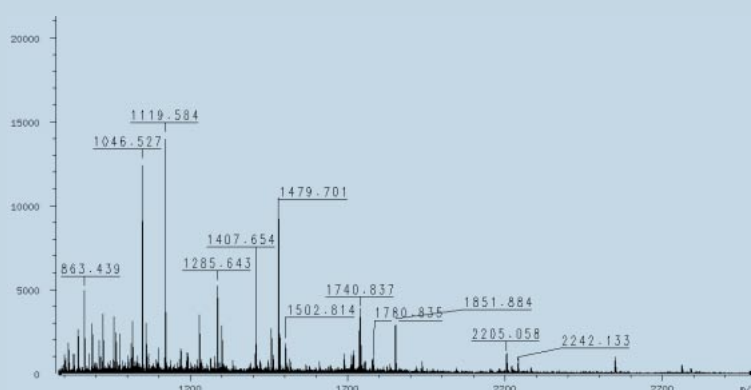
Figure 14. Reprobing Immobilon-P membrane with anti-human transferrin by (top row) detergent and (bottom row) low pH methods. (1) First cycle of detection; (2) stripped membrane detected with the secondary antibody only; (3) second cycle — stripped membrane detected with primary and secondary antibody. ECL (Amersham) detection reagents were used. Left to right, 5 μ L of human serum serum dilutions 1:12,500, 1:25,000, 1:50,000, and 1:100,000. Electroblotted proteins were probed with anti-human transferrin (1:10,000 dilution) and HRP-conjugated rabbit anti-goat IgG (1:20,000).

Table 7. Chromogenic, chemiluminescent, and fluorescent immunodetection kits.

Detection Kit	Manufacturer	Detection Level*	Type
SuperSignal® West Femto Maximum Sensitivity Substrate (See sample blot on page 14)	Pierce	Low femtogram	Chemiluminescent
SuperSignal West Dura Extended Duration Substrate	Pierce	Mid femtogram	Chemiluminescent
ECL™ (See sample blot on page 17)	Amersham	Picogram	Chemiluminescent
ECL PLus	Amersham	50 femtograms	Chemiluminescent
ECL Advance (See sample blots on pages 12 and 17)	Amersham	Low femtogram	Chemiluminescent
ECF Western Blotting Kit	Amersham	Picogram	Chemifluorescent
WesternBreeze® Chemiluminescent Kit (See sample blot on page 29)	Invitrogen	Femtograms	Chemiluminescent
WesternBreeze Chromogenic Kit	Invitrogen	low picogram	Chromogenic
Immun-Blot® BCIP/NBT Kit	Bio-Rad	100 µg	Chromogenic
Amplified Alkaline Phosphatase ImmunBlot Kit	Bio-Rad	10 µg	Chromogenic
Immun-Star®-AP Kit	Bio-Rad	10 µg	Chemiluminescent
Immun-Star-HRP Kit	Bio-Rad	Low picogram	Chemiluminescent
Phototope® HRP Western Detection System	Cell Signaling	Subpicogram	Chemiluminescent
Western-Light™ and Western-Star Immunodetection System (See sample blot on page 19)	Applied Biosystems (ABI)	Low picogram	Chemiluminescent
DyeChrome™ Western Blot Stain Kits	Molecular Probes	1–8 ng	Fluorescent
Ampex Gold Western Blot Stain Kits	Molecular Probes	1–3 ng	Fluorescent
Protein Detector™ Western Blotting Kit (See sample blot on page 16)	Kirkegaard & Perry Laboratories, Inc. (KPL)	low picogram	Chemiluminescent
Western Lightning® Western Blot Chemiluminescence Reagent (See sample blot on page 9)	Perkin Elmer	1–10 µg	Chemiluminescent
Western blot detection kit	Upstate	Low picogram	Chemiluminescent

*Based on manufacturer claims

Figure 15. MALDI-TOF spectrum of a band from Coomassie Blue-stained blot of liver proteins, using on-membrane digestion, Protocol 5.1 on page 41 (Bienvenu *et al.*, 1999). The protein was identified as bovine catalase, with 6.4×10^6 MOWSE score and 34% coverage. Data were obtained on a Bruker® Autoflex™ mass spectrometer. The search was done using Protein Prospector.



The stripping process disrupts the antigen-binding capacity of the antibody and dissolves it into 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 with Immobilon PVDF Transfer Membranes

Mass spectrometry (MS) is a relatively new method to identify proteins on blots. It involves first staining the PVDF membrane with MS-compatible dye (Coomassie Blue, amido black or Sypro stains are effective). Then, the band of interest is cut out of the membrane, and proteolysis on the membrane, peptide extraction and MS analysis are performed (Gharahdaghi *et al.*, 1996; Bienvenut, *et al.*, 1999; Bunai *et al.*, 2003). Figure 15 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 off 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 16 shows 2-D-separated human plasma proteins transferred to Immobilon-PSQ transfer membrane and two MALDI-TOF spectra obtained for identification of membrane-immobilized proteins.

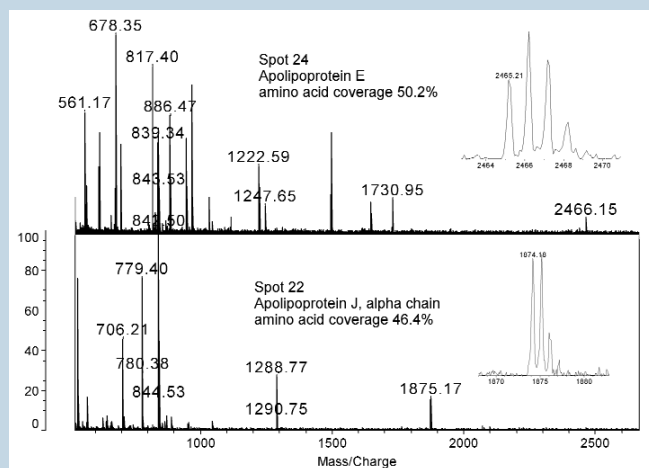
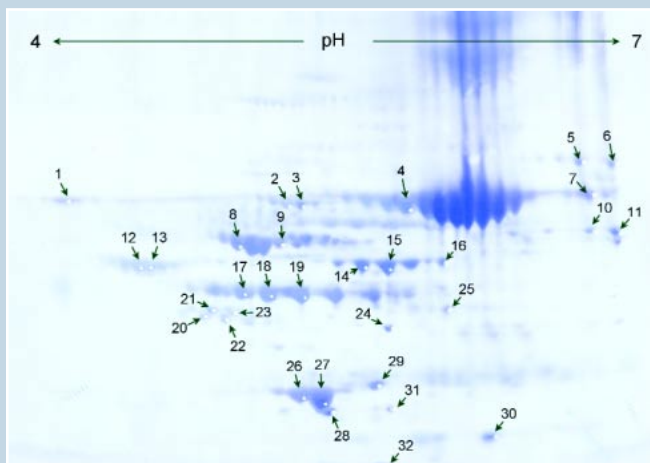


Figure 16. Human plasma separated by 2-D electrophoresis and transferred to (left) Immobilon-PSQ transfer membrane and (right) MALDI-TOF mass spectra collected directly from the membrane surface of tryptic digested proteins.

Human plasma was separated by 2D electrophoresis, electroblotted onto Immobilon-PSQ 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).

VI. 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 Millipore's collective years of experience with transfer membranes, as well as referenced literature and feedback from our customers.

► **Tip**

Immobilon transfer membrane pre-cut sheets fit all standard mini-gel sizes and common electrophoresis systems. See tables on page 7.

► **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.

► **Recommendation**

Chill transfer buffers prior to tank transfer.

1. Protein Transfer

Protocol 1.1. Electrotransfers: Tank 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 glass cassette; trim away any stacking gel.
3. Immerse the gel in transfer buffer for 15 to 30 minutes.
4. Soak filter paper in transfer buffer for at least 30 seconds.

Notes

For protein visualization protocols, see page 34; for immunodetection protocols, see page 37.

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.

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

Set Up

1. Prepare 200 mL of each anode buffer and 400 mL of cathode buffer.
2. Remove the gel from its glass 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.

▶ 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 and 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.

Notes

For protein visualization protocols, see page 34; for immunodetection protocols, see page 37.

Protocol 1.4. Dot Blotting/Slot Blotting: Manual Spotting Method

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.

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 dry filter paper.
4. Place the pre-wet membrane on wet filter paper.

Protein Transfer

Load protein either by spotting or intrusion.

Transfer by Spotting

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.

Transfer by Intrusion (based on Oprandy et al., 1988)

1. Place a 1 mL tuberculin syringe directly against the dry membrane and inject up to 50 μL of protein sample into membrane.
2. Place membrane on clean filter paper to dry.

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**

If more than one blot is placed in a container, insufficient buffer volume will cause the blots to stick together.

▶ **Tip**

Dry milk powder can not be used with biotin-avidin systems.

▶ **Tip**

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

▶ **Tip**

Sensitivity of chromogenic detection is at least an order of magnitude lower than of chemiluminescent detection.

▶ **Tip**

High overall background can be minimized by higher dilution of the enzyme-conjugated secondary antibody.

▶ **Tip**

High non-specific signal can be alleviated by higher dilution of the primary antibody or decreased protein load on the gel.

Chromogenic Protein 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 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.

Chemiluminescent Protein 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: 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 run to determine the optimum exposure time; 2 to 5 minutes is common.

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.

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 Membrane Drying Methods, page 33.*

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
- Blocking solution for diluting antibodies: 1% (w/v) BSA (bovine serum albumin), 0.05% Tween-20
- Methanol, 100%
- Milli-Q water

Notes

4. Membrane Stripping

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

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

Applicable to any chemiluminescent substrate system.

Required Equipment and Solutions

- Stripping solution: 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7
- 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 agitate for 30 minutes at 50 °C.
2. Place the blot in buffer and agitate for 10 minutes. Repeat with fresh buffer.
3. *(Optional)* Repeat the initial detection protocol (omitting the primary antibody step) to make sure that the antibodies have been inactivated or stripped from the membrane.
4. Place the blot in buffer and agitate for 10 minutes.
5. Proceed to the blocking step for the next round of detection.

Protocol 4.2. Stripping by Acidic pH

Applicable to any chemiluminescent substrate system.

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 detection.

Notes

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 instability of the proteins bound to the membrane. While Millipore recommends cold storage, room temperature may be adequate for some proteins.

Required Materials

- Blotted Immobilon PVDF transfer membrane (dry)
- Two sheets of Whatman 3MM paper
- Two sheets of card stock or thin cardboard
- Paper clips
- Plastic bag

Procedure

1. Place the 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 bactericide 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.

VI. 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.
	Membrane pores clogged	Centrifuge or filter samples to remove particulates. Dilute viscous samples. Increase vacuum level.
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 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 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 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.
	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.
	Proteins transferred too rapidly; protein buildup on the membrane surface	Reduce the strength of the electrical field.
	Uneven contact between gel and membrane	Make sure entire gel and membrane surfaces are in good contact.
Weak signal	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 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 from opaque to semi-transparent.
Switch to Immobilon-PSQ 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.	
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 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.	

Symptom	Possible Cause	Remedy
Weak signal (continued)	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	SDS interferes with binding of small molecular weight proteins	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.
	Membrane pore size is too large	Switch to Immobilon-PSQ transfer 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 aid 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 (e.g., histones)	Protein net charge in the transfer buffer is positive; proteins move to the cathode	Reorient or 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 <i>et al.</i> , <i>Anal. Biochem.</i> 162:370-377 (1987).
		Use three-buffer system for semi-dry transfer (see Protocol 1.2, page 28.)

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-PSQ 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.
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 from opaque to semi-transparent.

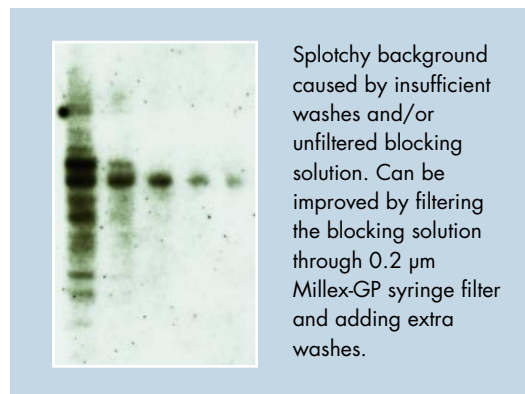
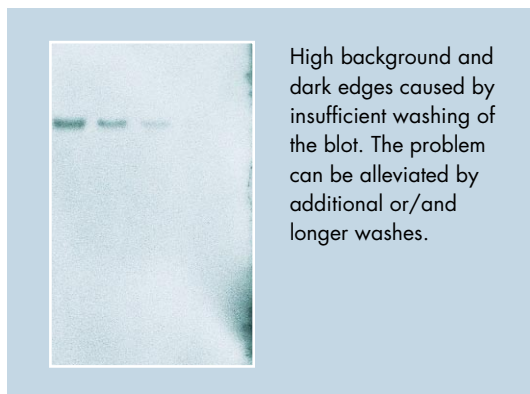
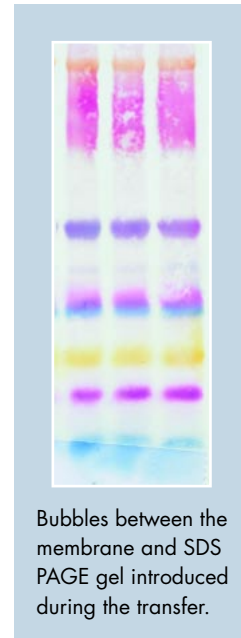
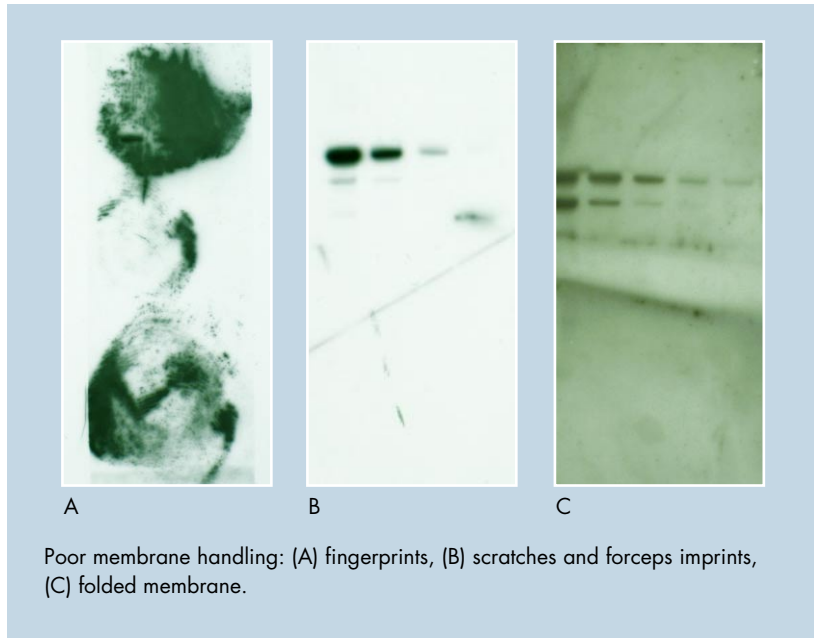
Symptom	Possible Cause	Remedy
Uneven/splotchy results	Insufficient volume of staining solutions	Use sufficient volume of incubation solutions and ensure that all of the membrane is exposed to these solutions during incubation. The container used should be large enough to allow solution to move freely 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 filter units 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 high quality reagents and Milli-Q water.

4. Immunodetection

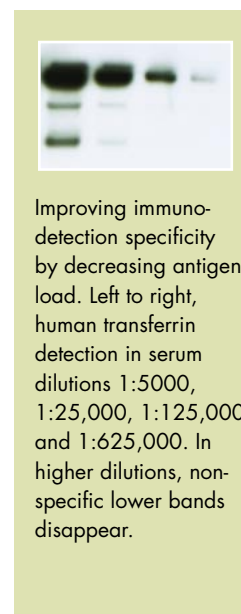
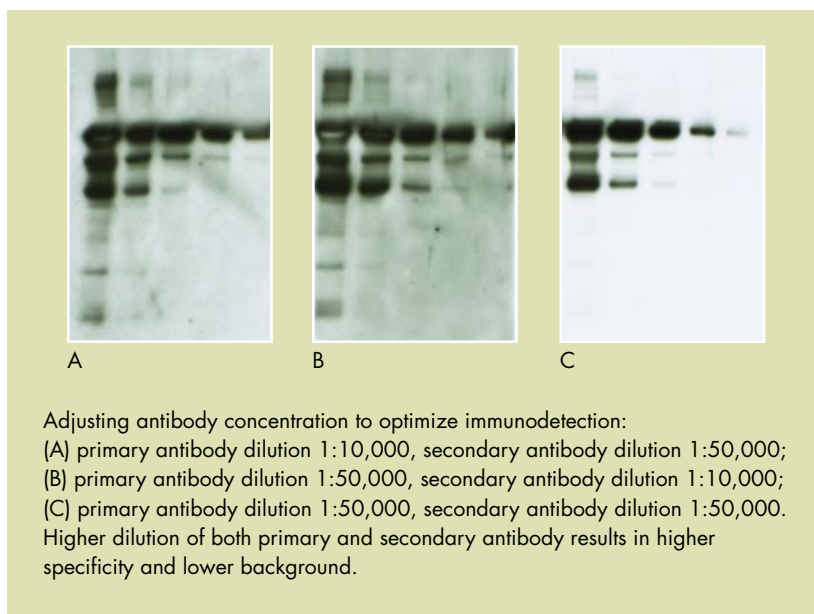
Symptom	Possible Cause	Remedy
Weak signal	Improper blocking reagent	The blocking agent may have an affinity for the protein of interest and thus obscure the protein from detection. Try a different blocking agent and/or reduce both the amount or exposure time of the blocking agent.
	Insufficient antibody 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.
No signal	Antigen concentration is too low	Load more antigen on the gel prior to the blotting.
	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.
Uneven blot	Primary antibody was raised against native protein	Separate proteins in non-denaturing gel or use antibody to denatured antigen.
	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 dry milk powder or other 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.

Symptom	Possible Cause	Remedy
<i>High background</i>	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	Decrease the antibody concentration.
	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-PS ^Q transfer membrane	Increase the concentration or volume of the blocking agent used to compensate for the greater surface area of the membrane. In addition, incubation times for both the wash and blocking steps may need to be extended.
	Poor quality reagents	Use high quality reagents and Milli-Q water.
	Crossreactivity between blocking reagent and antibody	Use Tween-20 in the washing buffer or use different blocking agent.
	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.
<i>High background (rapid immunodetection)</i>	Excess detection reagents	Drain blots completely before exposure.
	Membrane wets out during rapid immunodetection	Reduce the Tween-20 (<0.04%) 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.
<i>Non-specific binding</i>	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.
<i>Reverse images on film (white bands on dark background)</i>	Too much HRP	Reduce concentration of secondary, HRP-conjugated antibody.
<i>Poor detection of small proteins</i>	Small proteins are masked by large blocking molecules such as BSA	Consider casein, gelatin 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.

Examples and Causes of Blot Failure



Impact of Antibody Concentration and Antigen Load on Blot Quality



Glossary, References, Patents and Ordering Information

Glossary

1-D

One-dimensional

2-D

Two-dimensional

Adsorption

The process whereby a soluble molecule (e.g., protein) binds to a solid 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 or non-ionic detergent.

Blot

A microporous membrane with biomolecules adsorbed to the polymer.

Blotting

Process of transferring proteins or nucleic acids 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 deposit of a colored substance 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.

Edman Degradation

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

Electrotransfer

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

ELISA

Enzyme-linked immunosorbent assay; a rapid test to determine the presence and quantity of a specific substance. It is based on an antibody-antigen interaction where the antibody or antigen is linked to a measurable enzyme as a means of detecting its presence. Western blotting is often used to verify ELISA results.

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 method that results in the deposition of a fluorophore at the site of the target protein.

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 membrane-bound protein. The specificity of antibody-antigen binding permits the identification of a single protein in a complex sample.

Isoelectric focusing

Method of protein separation on the basis of isoelectric points. Usually achieved by electro-phoresis 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.

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 protein.

PVDF

Polyvinylidene fluoride; the polymer used to make Immobilon-P and Immobilon-PSQ 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 more than one round of detection.

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.

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.

Slot blot

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

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

Relative to blotting, the compound that interacts with the enzyme on the secondary antibody to yield a detectable signal.

Tank transfer

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

Transfer buffer(s)

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

Transillumination

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

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).

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Type	Size	Qty/Pk	Catalogue No.
Cut Sheet	7 x 8.4 cm	50	IPVH 078 50
	8 x 10 cm	10	IPVH 081 00
	8.5 x 13.5 cm	10	IPVH 081 30
	9 x 12 cm	10	IPVH 091 20
	10 x 10 cm	10	IPVH 101 00
	15 x 15 cm	10	IPVH 151 50
	20 x 20 cm	10	IPVH 202 00
	26 x 26 cm	10	IPVH 304 F0
Roll	26.5 x 375 cm	1	IPVH 000 10

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	8.5 x 13.5 cm	10	ISEQ 081 30
	9 x 12 cm	10	ISEQ 091 20
	10 x 10 cm	10	ISEQ 101 00
	15 x 15 cm	10	ISEQ 151 50
	20 x 20 cm	10	ISEQ 202 00
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