Millipore®

User Guide

# Immobilon®-P Transfer Membrane

## For High Sensitivity Immunodetection

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# Introduction

Immobilon®-P transfer membrane is a polyvinylidene fluoride (PVDF) microporous membrane used for transfer of proteins from a variety of gel matrices. When compared to a nitrocellulose membrane, it has improved handling characteristics and staining capabilities, increased solvent resistance, and a higher signal-to-noise ratio for enhanced sensitivities.

This hydrophobic membrane has a nominal pore size of 0.45 micron (µm) and is capable of binding a wide range of molecular weight proteins. While Immobilon®-P membrane is capable of binding proteins smaller than 20,000 in molecular weight, Immobilon®-P$^{SQ}$ 0.2 micron (µm) membrane could be considered for proteins smaller than 20,000, because it has more surface area and higher binding capacity for small proteins.

Immobilon®-P membrane has excellent protein retention, high physical strength, and broad chemical compatibility, making it ideal for a variety of staining applications and reprobing in immunodetection methods. This user guide describes some of the most common techniques for Western blotting. For more detailed information please refer to TP001EN, the "Protein Blotting Handbook" (available at www.millipore.com).

**Table 1. Immobilon-P® Membrane Properties and Applications**

| | | |
|---|---|---|
| Composition | PVDF | |
| Pore size | 0.45 µm | |
| Phobicity | Hydrophobic | |
| Protein binding capacity | Insulin: 160 µg/cm$^2$<br>Bovine serum albumin (BSA): 215 µg/cm$^2$<br>Goat IgG: 294 µg/cm$^2$ | |
| Applications | Binding assays<br>Dot/slot blotting<br>Glycoprotein visualization<br>Lipopolysaccharide analysis<br>Mass spectrometry<br>Amino acid analysis<br>N- terminal protein sequencing | |
| Detection methods* | Chemiluminescent (Immobilon® HRP substrates)<br>Chromogenic (TMB, Insoluble)<br>Radioactive | |
| Protein visualization methods | Transillumination | |
| | Reversible stains | Ponceau-S<br>Copper phthalocyanine tetrasulfonic acid, tetrasodium salt (CPTS)<br>Toluidine blue<br>Sypro® blot stains |
| | Irreversible stains | Coomassie™ Brilliant Blue dye<br>Amido black<br>India ink<br>Colloidal gold |
| * For fluorescence detection methods, low-autofluorescent Immobilon®-FL membrane is recommended. | | |

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## Guidelines for Working with Immobilon®-P membrane

- Always wear gloves when handling the membrane, in order to avoid fingerprints.
- Use blunt forceps to prevent membrane damage.
- Keep the patapar (blue paper) with the membrane during cutting or handling, but discard when wetting the membrane.
- Handle with care to avoid scratches on the membrane surface. Do not fold the membrane.
- Hydrophobic Immobilon®-P membrane must be wet in an alcohol solution (> 50% v/v methanol, ethanol, or isopropanol) before use. Once the membrane is wet, it changes from opaque to semi-transparent.
- After protein transfer, wash the blot with Milli-Q® water to eliminate any gel residues.
- Blots can be air dried and stored at 4° C for several months (for later use) or they can be used immediately.

## Materials Recommended for Western Blotting

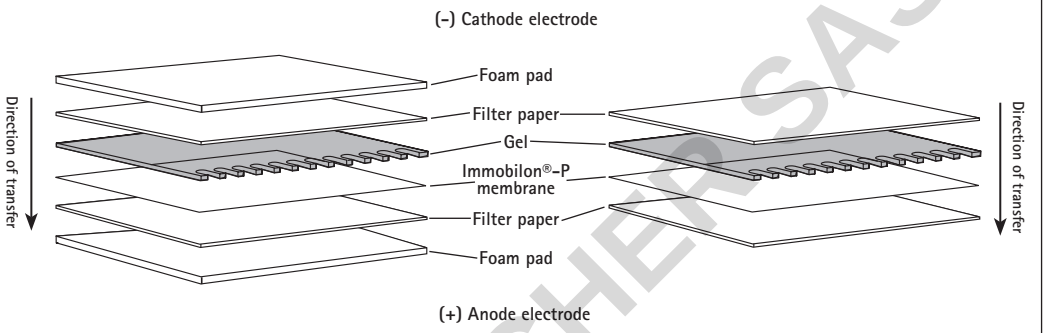
- Immobilon®-P membrane cut to the dimensions of the gel
- Alcohol (> 50% methanol, ethanol, or isopropanol) for wetting dry membrane
- Milli-Q® water
- Transfer buffer: 25 mM Tris-base, 192 mM glycine, pH 8.3, 10% alcohol for tank transfer or 48 mM Tris, 39 mM glycine, pH 9.2, 10% alcohol for semi-dry transfer)
- Sheets of filter paper, cut to the dimensions of the gel and soaked in transfer buffer for at least 30 seconds
- Blocking buffer: Immobilon® Block-CH buffer (cat. no. WBAVDCH01) or 0.5–5% (w/v) blocking agent (bovine serum albumin, casein, nonfat dry milk) in wash buffer
- Wash buffer: Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) containing 0.5-0.1% Tween®-20 surfactant (PBST or TBST)
  PBS: 10 mM sodium phosphate, pH 7.2, 0.9% NaCl
  TBS: 10 mM Tris, pH 7.4, 0.9% NaCl
- Primary antibody (specific for the protein of interest), diluted in blocking buffer or wash buffer
- Secondary antibody (specific for the primary antibody), labeled with a detection enzyme (e.g., horseradish peroxidase [HRP] or alkaline phosphatase [AP]), diluted in blocking buffer or wash buffer

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# Protein Transfer

Proteins can be transferred to Immobilon®-P membrane by two common electro-transfer methods: tank and semi-dry transfer. Table 2 describes the general conditions and major differences for the two methods.

**Table 2. Transfer Methods**




| | Tank Transfer | Semi-dry Transfer |
|---|---|---|
| Conditions | Membrane gel/ stack immersed in buffer | Filter paper soaked in buffer |
| Buffer volume | 0.5 L or higher depending on system | ~ 0.05 L per mini-gel |
| Transfer time | Slow (1 or more hours) | Fast (7–45 minutes) |
| Typical run | Constant voltage | Constant current |
| Gel equilibration time | Not necessary but recommended | Minimum of 15 minutes equilibration per mini-gel |
| Required buffer system | Continuous (single buffer) | Continuous (single buffer) or Discontinuous (3 buffers) |
| Typical continuous buffer name/ composition | **Towbin buffer, pH 8.3**<br>25 mM Tris, 192 mM glycine | **Bjerrum-Shafer-Nielsen buffer, pH 9.2**<br>48 mM Tris, 39 mM glycine |
| Typical discontinuous buffer name/composition | N/A | Anode buffer I: 300 mM Tris, pH 10.4<br>Anode buffer II: 25 mM Tris, pH 10.4<br>Cathode buffer: 25 mM Tris, 40 mM amino-caproic acid, pH 9.4 |
| % Alcohol* | 10–20% alcohol | 10–20% alcohol |
| % SDS* | 0.02–0.05% SDS | 0.02–0.05% SDS |

* Alcohol (methanol, ethanol, or isopropanol) in the transfer buffer has two important functions; it stabilizes the gel dimensions and strips complexed sodium dodecyl sulfate (SDS) from protein molecules, improving protein binding to the membrane. However, for large proteins, or proteins that exhibit solubility problems, it is recommended that the alcohol concentration be decreased and that a small amount of SDS be added to the transfer buffer. This improves protein elution from the gel while maintaining protein solubility during the transfer process.

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# Immunodetection

Immunodetection is an antibody-based method that allows the detection, identification, and quantitation of a protein or antigen in the blotting membrane. The typical protocol follows these six general steps:

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

Standard immunodetection takes at least 4 hours and is widely used, but the SNAP i.d.® 2.0 Protein Detection System (www.millipore.com/snapwb) can perform the same process with significant time savings (Table 3).

**Table 3. Comparison of Standard vs. SNAP i.d.® 2.0 Immunodetection**

| | Standard **Immunodetection** | SNAP i.d.® 2.0 Immunodetection |
|---|---|---|
| 1. Block membrane | 1 hour | 10 seconds |
| 2. Incubate with primary antibody | 1 hour | 10 minutes |
| 3. Wash membrane | 3 times, 5 minutes each | 4 times, ~10 seconds each |
| 4. Incubate with secondary antibody | 1 hour | 10 minutes |
| 5. Wash membrane | 3 times, 5 minutes each | 4 times, ~10 seconds each |
| Total time | 4 hours | 22 minutes |

# Common Protocols Used in Western Blotting and Immunodetection

## Membrane Wetting

1. Wet the dry membrane in alcohol (>50% methanol, ethanol, or isopropanol) for 10–20 seconds, or until it changes from a opaque white to uniform, translucent gray.
2. Immerse the membrane in Milli-Q® water for 1–2 minutes to displace the alcohol.
3. Equilibrate the membrane in transfer buffer for 2–3 minutes or until ready to use.

**CAUTION:** Once the membrane has been wet out, do not allow it to dry out. It can be kept in buffer until protein transfer. If the membrane dries out (turns opaque white) even partially, it must be wet out again (steps 1–3).

## Semi-dry Transfer

1. Resolve the protein mixture on a 1D or 2D polyacrylamide gel.
2. Immerse the gel in the transfer buffer and allow it to equilibrate for 10–15 minutes.
3. Assemble the transfer stack according to manufacturer's instructions for the transfer

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**Semi-dry Transfer,** continued

apparatus used.

**CAUTION:** To ensure an even transfer, remove air bubbles by carefully rolling a clean pipette or blot roller over the surface of each layer in the stack. Do not apply excessive pressure, as this may damage the gel and membrane.

4. Transfer proteins according to transfer apparatus manufacturer's instructions.
5. Remove the blot from the transfer system and rinse the membrane briefly in Milli-Q® water to remove gel debris. The blot may be air dried for storage, or it can be use immediately for the immunodetection step.

**NOTE:** Drying the blot before immunodetection may enhance the binding of some proteins and reduce background noise.

## Protein Visualization (Optional)

To visualize the protein transfer efficiency, Immobilon®-P membrane may be stained with any reversible blot stain compatible with immunodetection (e.g., Ponceau-S, toluidine blue, CPTS, Sypro® blot stains) or viewed by transillumination using a light box. For a list of reversible and nonreversible compatible stains and protocols refer to TP001EN, the "Protein Blotting Handbook" at www.millipore.com.

## Immunodetection

The following is a general protocol for immunodetection with Immobilon®-P membrane. Some of the critical factors for obtaining a "perfect" Western blot such as protein concentration, blocking solution, and antibody concentration may require optimization.

## Standard Immunodetection

1. If blot has been dried, rewet it in alcohol (> 50% methanol, ethanol, or isopropanol) for 15 seconds or until it changes from opaque white to translucent gray.
2. Rinse the blot in Milli-Q® water for 1 minute.
3. Place the blot in blocking buffer and incubate for 1 hour with gentle agitation. Dilute the primary antibody in wash or blocking buffer.
4. Place the blot in diluted primary antibody solution and incubate for 1 hour with gentle agitation.
5. Wash the blot with wash buffer (tris- or phosphate-buffered saline solution, supplemented with Tween®-20 surfactant (TBST or PBST)) 3–5 times for 5 minutes each. Prepare secondary antibody in wash or blocking buffer.
6. Place the blot in diluted enzyme-labeled secondary antibody solution and incubate for 1 hour with gentle agitation.
7. Wash the blot with wash buffer 3–5 times for 5 minutes each.
8. Place the blot into a clean container and add the appropriate detection reagent (HRP, AP, or chromogenic).
9. Incubate 1–5 minutes, according to the detection reagent manufacturer's instructions.
10. For HRP or AP chemiluminescent reagents, expose blot to x-ray film or acquire the image using a digital imaging system. For chromogenic detection, add the reagent and wait until signal appears.

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### SNAP i.d.® 2.0 Immunodetection Using Vacuum Filtration

(refer to the SNAP i.d.® 2.0 Protein Detection System User Guide for full protocol details)

1. If blot has been dried, rewet it in > 50% alcohol (methanol, ethanol, or isopropanol) for 15 seconds, or until it changes from opaque white to translucent gray. Prepare all the required solutions and antibodies ahead of time.

   **NOTE:** Antibodies should be 3 to 5 times more concentrated than in standard immunodetection, but in volumes of 2.5 to 10 mL depending of the blot size/blot holder.
2. Wet the SNAP i.d.® 2.0 blot holder in Milli-Q® water and assemble the blot with the protein side down.
3. Using the blot roller, remove all air bubbles and excess water, and insert the blot holder inside the SNAP i.d.® 2.0 frame.
4. Block by adding 15–30 mL of blocking solution and immediately turn the vacuum on.
5. Depending of the blot holder size used, add 2.5 to 10 mL of diluted primary antibody and incubate for 10 minutes.
6. Turn vacuum on to flush the antibody, then with the vacuum still on, wash 4 times with 15–30 mL of wash buffer.
7. Turn vacuum off, add 2.5–10 mL (depending of the blot holder size used) of diluted secondary antibody, and incubate for 10 minutes.
8. Turn vacuum on to flush the antibody, then wash 4 times with 15–30 mL of wash buffer.
9. Remove the blot from the blot holder and continue with the detection method of choice (chemiluminescence or chromogenic).

## Guidelines for Choosing an Immobilon® PVDF Membrane

The following table provides general guidelines for choosing the appropriate membrane for a specific post-Western blot application. Due to variations in protein properties such as charge density, conformation, and hydrophobicity, not all proteins behave the same way on a given membrane surface. Experiments with a variety of Immobilon® membranes may be necessary to optimize results for your specific application.

| Application after Western blotting | Membrane of choice for most proteins |
|---|---|
| General immunodetection | Immobilon®-P or Immobilon®-E |
| Amino acid analysis | Immobilon®-P |
| Immunodetection of low molecular weight or low-abundance proteins | Immobilon®-P$^{SQ}$ |
| Sequencing of low molecular weight or low-abundance proteins | Immobilon®-P$^{SQ}$ |
| Fluorescence immunodetection and chemifluorescence methods | Immobilon®-FL |

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# Ordering Information

See the Technical Assistance section for contact information. You can purchase these products on-line at www.SigmaAldrich.com/westernblot.

**Immobilon®-P Membrane (0.45 µm pore size) for general Western blotting applications**

| Size | Qty/Pk | Catalogue Number |
|---|---|---|
| 8.5 × 1000 cm roll | 1 | IPVH85R |
| 26.5 × 375 cm roll | 1 | IPVH00010 |
| 26.5 × 187.5 cm roll | 1 | IPVH00005 |
| 26 × 26 cm sheet | 10 | IPVH304F0 |
| 20 × 20 cm sheet | 10 | IPVH20200 |
| 15 × 15 cm sheet | 10 | IPVH15150 |
| 10 × 10 cm sheet | 10 | IPVH10100 |
| 9 × 12 cm sheet | 10 | IPVH09120 |
| 8.5 × 13.5 cm sheet | 10 | IPVH08130 |
| 8 × 10 cm sheet | 10 | IPVH08100 |
| 7 × 8.4 cm sheet | 50 | IPVH07850 |

**Immobilon®-P$^{SQ}$ Membrane (0.2 µm pore size) for blotting applications of proteins with molecular weights less than 20,000**

| Size | Qty/Pk | Catalogue Number |
|---|---|---|
| 8.5 × 1000 cm roll | 1 | ISEQ85R |
| 26.5 × 375 cm roll | 1 | ISEQ00010 |
| 26.5 × 187.5 cm roll | 1 | ISEQ00005 |
| 26 x 26 cm sheet | 10 | ISEQ26260 |
| 20 x 20 cm sheet | 10 | ISEQ20200 |
| 15 x 15 cm sheet | 10 | ISEQ15150 |
| 10 × 10 cm sheet | 10 | ISEQ10100 |
| 9 × 12 cm sheet | 10 | ISEQ09120 |
| 8.5 × 13.5 cm sheet | 10 | ISEQ08130 |
| 8 × 10 cm sheet | 10 | ISEQ08100 |
| 7 × 8.4 cm sheet | 50 | ISEQ07850 |

**Immobilon®-FL Membrane (0.45 µm pore size) for fluorescence detection applications**

| Size | Qty/Pk | Catalogue Number |
|---|---|---|
| 8.5 × 1000 cm roll | 1 | IPFL85R |
| 26.5 × 375 cm roll | 1 | IPFL00010 |
| 26.5 × 187.5 cm roll | 1 | IPFL00005 |
| 20 × 20 cm sheet | 10 | IPFL20200 |
| 10 × 10 cm sheet | 10 | IPFL10100 |
| 7 × 8.4 cm sheet | 10 | IPFL07810 |

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## Related products for general Western blotting applications

| Description | Catalogue Number |
|---|---|
| Immobilon® NOW Dispenser for 8.5 x 1000 cm rolls | IMDISP |
| Immobilon® Block Noise Cancelling Reagents for chemiluminescence detection, 500 mL | WBAVDCH01 |
| Immobilon® blotting filter paper, 7 × 8.4 cm sheet, 100/pk | IBFP0785C |
| Immobilon® blotting filter paper, 8.5 × 13.5 cm sheet, 100/pk | IBFP0813C |
| Immobilon® ECL Ultra Western HRP substrate, 100 mL | WBULS0100 |
| Immobilon® Signal Enhancer for immunodetection, 500 mL | WBSH0500 |
| Immobilon® Western HRP substrate, 100 mL | WBKLS0100 |
| Immunoblot Blocking Reagent, 20 g | 20-200 |
| Immobilon® Forte Western HRP substrate, 100 mL | WBLUF0100 |
| Immobilon® Crescendo Western HRP substrate, 100 mL | WBLUR0100 |
| Immobilon® Classico Western HRP substrate, 100 mL | WBLUC0100 |
| Phosphate-buffered saline with 3% nonfat milk, pH 7.4, dry powder | P2194 |
| Phosphate-buffered saline with Tween® 20 surfactant, pH 7.4, tablet | 08057 |
| Ponceau S solution, 0.1% (w/v) in 5% acetic acid, 1 L | P7170 |
| Re-Blot™ Plus Strong Antibody Stripping solution, 10X, 50 mL | 2504 |
| TMB substrate, insoluble (Calbiochem®), 100 mL | 613548 |
| Tris-buffered saline with Tween® 20 surfactant, pH 7.6, tablet | 91414 |
| Tris-glycine buffer 10X Concentrate, 1 L | T4904-1L |

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## Technical Assistance

Visit the tech service page on our web site at www.millipore.com/techservice.

## Standard Warranty

The applicable warranty for the products listed in this publication may be found at www.millipore.com/terms ("Conditions of Sale").

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