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Immobilon-P Transfer Membrane User Guide

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Immobilon-P Transfer Membrane

Introduction

The Millipore Immobilon-P Transfer Membrane is a polyvinylidene fluoride (PVDF) microporous membrane for binding proteins that are transferred from a variety of gel matrices. This membrane is hydrophobic and offers a uniformly controlled pore structure with a high binding capacity for biomolecules. 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.

The Immobilon-P membrane has a nominal pore size of 0.45 micron (µm) and is useful for blotting proteins >10 kDa. It is an ideal substrate for immunodetection. Immobilon-P is compatible with standard blocking agents and detection protocols, including chemiluminescence. Because the membrane is composed of PVDF, it is also compatible with the harsh conditions used in protein sequencing and amino acid analysis. This insert describes how to perform electroblotting using a tank or semi-dry transfer system. It also provides steps on a new rapid immunodetection protocol and technical considerations for protein sequencing applications.

NOTE: If Proteins in the range of 10-20 kDa are to be electroblotted, Immobilon-P and Immobilon-P^{SQ} should be evaluated for optimum detection. Immobilon-P^{SQ} is recommended when electroblotting proteins < 10 kDa.

Contact your local Millipore office for the following:

- TP001, "Protein Blotting Application Guide"
- RP562, "Rapid Immunodetection of Blotted Proteins without Blocking"
- TN051, "Rapid Immunodetection Method on Immobilon-P Using Chemiluminescence"

How to Prepare an SDS-PAGE Gel and the Immobilon-P Membrane

Before starting, have a sodium-dodecyl sulfate-polyacrylamide gel (SDS-PAGE gel) and a complex protein mixture available.

Preparing the Gel

1. Resolve the protein mixture on the gel. The amount of protein loaded depends on the width of the wells, the dimensions of the gel, and the individual protein concentration in the sample. For optimum resolution, consider all these factors when loading.

   CAUTION: Do not overload the gel or the bands may smear during the transfer. For example, 10 to 20 microgram (µg) of a complex protein mixture in each 8-millimeter (mm) lane resolves well on a mini-gel.

2. Open the gel cassette and lift off the gel with a clean razor blade. Then notch a corner of the gel. This provides orientation to the membrane after the transfer.

3. Prepare 100 milliliter (mL) of the appropriate buffer solution for the transfer method. (Prepare 200 mL of buffer for larger gels.) This chart describes buffer compositions for wet tank and semi-dry transfer conditions:

Transfer Method	Buffer	Composition
Tank transfer	Transfer buffer	25 millimolar (mM) Tris base, 192 mM glycine, 10% methanol
Semi-dry transfer	Cathode buffer	25 mM Tris base, 40 mM 6-amino-n-caproic acid, 10% methanol, pH 9.4 NOTE: 6-amino-n-caproic acid may be substituted with glycine.

4. Immerse the gel in the transfer buffer and allow it to equilibrate for 15 minutes.

Preparing the Membrane

Before starting, have a piece of the Immobilon-P membrane, the prepared gel, methanol, and Milli-Q®-grade water available.

1. Prepare 100 mL of transfer buffer for a tank transfer. For a semi-dry transfer, prepare 100 mL of anode buffer II.

Transfer Method	Buffer	Composition
Tank transfer	Transfer buffer	25 mM Tris base, 192 mM glycine, 10% methanol
Semi-dry transfer	Anode buffer II	25 mM Tris, 10% methanol, pH 10.4

2. Cut a piece of the Immobilon-P membrane to the dimensions of the gel. Notch or label one corner of the membrane to correspond to a corner of the gel.
3. Wet the membrane in 100% methanol for 15 seconds. Then transfer it to a container of Milli-Q®- grade water for 2 minutes.

   CAUTION: Use care when handling the membrane to prevent tearing. And do not leave any dry spots that can inhibit the transfer.
4. Equilibrate the membrane for at least 5 minutes in the transfer buffer for a tank transfer or in the anode buffer II solution for a semi-dry transfer.

   NOTE: For alternative buffer systems, equilibrate the membrane in the buffer of choice.
5. Follow the steps in the next section for a tank transfer system. For a semi-dry system, see the "How to Perform a Semi-Dry Transfer" section instead.

How to Perform a Transfer with a Tank System

Steps to transfer proteins from gels using a tank transfer system:

- Prepare the buffer
- Assemble the transfer stack
- Transfer the protein
- Remove the blot

Preparing the Buffer for a Tank Transfer Assembly

Buffer	Composition	Amount
Tank transfer	25 mM Tris base, 192 mM glycine, 10% methanol	A volume sufficient to fill the transfer tank.

Assembling the Transfer Stack for a Tank System

Materials required:

- One tank transfer system
- Two foam pads (for example, Scotch Brite® pads)
- Two sheets of Whatman® 3MM filter paper, cut to the dimension of the gel and soaked in tank transfer buffer for at least 30 seconds
- One glass pipette
- Transfer buffer
- One prepared gel
- One prepared sheet of Immobilon-P membrane

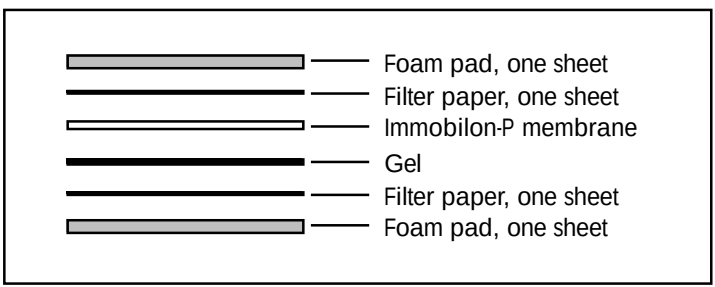
Assembling the Transfer Stack for a Tank System, continued

1. Place a foam pad on one side of the cassette holder. Then place one sheet of filter paper on top of the pad.

   CAUTION: To ensure an even transfer, remove air bubbles by carefully rolling a pipette over the surface of each layer in the stack. Applying excessive pressure may damage the membrane and gel.

2. Place the gel on top of the filter paper. Then put the sheet of the Immobilon-P membrane on top of the gel.

3. Place one sheet of filter paper on top of the stack. Then put a foam pad on top of the filter paper. The stack should look like this:



Transferring the Protein Using a Tank System

1. Close the tank transfer cassette holder. Then place it in the tank blotting apparatus so that the side of the cassette holder with the gel is facing the cathode (-). Add enough transfer buffer to the blotting apparatus to cover the cassette holder.

2. Insert the black cathode lead (-) into the cathode jack. Insert the red anode lead (+) into the anode jack. Then connect the anode lead and cathode lead to their corresponding power outputs.

3. Turn on the system for 1 to 2 hours at 6 to 8 V/cm inter-electrode distance to transfer the proteins to the membrane. The buffer may need to be cooled during the transfer to prevent overheating.

Removing the Blot from the Tank Transfer System

1. Remove the cassette holder from the blotting apparatus. Open the cassette holder. Remove the foam pad and filter papers with forceps.

   NOTE: Let the blot air-dry to improve the protein binding.

2. See the "How to Visualize the Proteins" section further in this document for details on staining. If performing traditional immunodetection with blocking, the membrane must be rewet with methanol and rinsed with water, before putting the membrane in the blocking solution. To visualize the proteins with transillumination or perform a rapid immunodetection, see the "How to Dry the Blotted Membrane" section near the end of this document.

   CAUTION: Before performing transillumination or rapid immunodetection techniques, the Immobilon-P membrane must be dried for specific lengths of time . (See the chart in "How to Dry the Blotted Membrane" section for time details.) The drying enhances protein binding to the surface and reduces background noise.

How to Perform a Transfer with a Semi-Dry System

Materials needed to transfer proteins from the gel using a semi-dry system:

- Prepare the buffers
- Assemble the transfer stack for a single or multiple transfer
- Transfer the protein
- Remove the blot

Preparing the Buffers for a Semi-Dry Transfer Assembly

Prepare anode buffer I, anode buffer II, and cathode buffer solutions:

Buffer	Composition	Amount
Anode buffer I	0.3 M Tris, 10% methanol, pH 10.4	200 mL
Anode buffer II	25 mM Tris, 10% methanol, pH 10.4	200 mL
Cathode buffer	25 mM Tris base, 40 mM, 6-amino-n-caproic acid, 10% methanol, pH 9.4 NOTE: 6-amino-n-caproic acid may be substituted with glycine.	200 mL

Continue to the next section to assemble a stack for a single transfer. For a multiple transfer, see "Assembling the Transfer Stack for a Semi-Dry System (Multiple Transfer)."

Assembling the Transfer Stack for a Semi-Dry System (Single Transfer)

Materials needed to assemble the transfer stack:

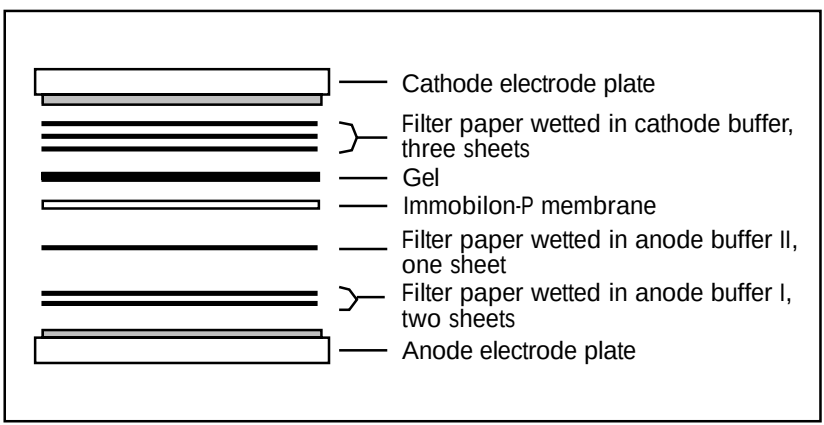
- One semi-dry blotter
- Six sheets of Whatman 3MM filter paper cut to the gel dimensions
- One sheet of prepared Immobilon-P membrane
- One prepared gel
- Prepared anode and cathode buffers

Assembling the Transfer Stack for a Semi-Dry System (Single Transfer), continued

1. Place the anode electrode plate on a level bench top. Then wet two sheets of filter paper in the anode buffer I solution. Place them in the center of the graphite anode electrode plate.

   CAUTION: To ensure an even transfer, remove air bubbles by carefully rolling a pipette over the surface of each layer of the stack. Applying excessive pressure may damage the membrane and gel.

2. Wet a sheet of filter paper in the anode buffer II solution. Place it on top of the first two sheets of filter paper. Place the Immobilon-P membrane on top of the filter paper.
3. Place the gel on top of the membrane. Wet three pieces of filter paper in the cathode buffer solution. Place them on top of the gel.
4. Place the cathode plate cover on top of the assembled transfer stack. The stack should look like this:



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

5. Continue to "Transferring the Protein Using a Semi-Dry System."

Assembling the Transfer Stack for a Semi-Dry System (Multiple Transfer)

This section describes how to assemble a transfer stack for a semi-dry system. Up to six gels may be transferred in a single stack.

1. Locate the materials listed in the previous section "Assembling the Transfer Stack for a Semi-Dry System (Single Transfer)." Additional materials needed include:
   - Dialysis membrane, for multiple transfer stacks, cut to the dimensions of the gel and wet in Milli-Q® grade water

   NOTE: The dialysis membrane should have a molecular weight exclusion small enough to retain the lowest molecular weight protein in the gel.

   - Whatman 3MM sheets cut to the dimensions of the gel
2. Follow steps 1 and 2 from "Assembling the Transfer Stack for a Semi-Dry System (Single Transfer)."
3. Place the gel on top of the membrane.
4. Wet a sheet of filter paper, cut to the dimensions of the gel, in cathode buffer. Then place it on top of the gel.

   NOTE: If you finished adding the last gel to the stack, place two pieces of filter paper, soaked in cathode buffer, on top of the stack. Then skip to step 9. If more gels need to be added to the stack, continue on to step 5.
5. Place a piece of dialysis membrane on top of the filter paper.
6. Wet a sheet of filter paper in anode buffer II and place it on top of the dialysis membrane.
7. Place a sheet of Immobilon-P membrane on top of the filter paper.
8. Return to step 3 to build another transfer stack.
9. Place the cathode plate cover on top of the assembled transfer stack. Then continue to "Transferring the Protein Using a Semi-Dry System."

Transferring the Protein Using a Semi-Dry System

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

Current Density	Time Limit
0.8 mA/cm2	1 to 2 hours
1.2 mA/cm2	1 hour
2.5 mA/cm2	30 to 45 minutes
4.0 mA/cm2	10 to 30 minutes

Removing the Blot from the Semi-Dry System

1. Turn off the power supply and disconnect the system leads when the transfer is complete.
2. Remove the cover. Then peel off and discard the filter papers.
3. Peel off the gel.
4. Peel off the blotted membrane with a pair of forceps.
5. See the next section, "How to Visualize the Proteins," for details on staining. To visualize the proteins with transillumination or perform a rapid immunodetection, see "How to Dry the Blotted Membrane" further in this document.

   CAUTION: Completely dry the Immobilon-P membrane for specific lengths of time before performing transillumination or rapid immunodetection techniques. (See the chart in "How to Dry the Blotted Membrane" section for time details.) The drying enhances protein binding to the surface and reduces background noise.

How to Visualize the Proteins

Once the electrotransfer is complete, the blot may be stained to assess the quality of the transfer using a stain such as Coomassie brilliant blue R, amido black, or Ponceau-S red. If the stain could interfere with subsequent analysis, use transillumination to visualize the proteins. (To determine protein elution from the gel, stain it with Coomassie brilliant blue or a silver stain.) See the following section for details on staining. Skip to "Using Transillumination" for details on transillumination.

Using Staining Methods

This section describes how to stain with:

- Coomassie brilliant blue R
- Amido black
- Ponceau-S red

For details on visualizing proteins with transillumination, see the "How to Visualize with Transillumination" section.

CAUTION: If Coomassie brilliant blue R, amido black, or Ponceau-S red stain will be used to visualize the proteins and the blot is dry, re-wet it in 100% methanol before staining it. This ensures accurate results.

Coomassie Brilliant Blue R Stain

To stain the blot with Coomassie blue, follow these steps.

CAUTION: This stain will interfere with rapid immunodetection. It is not reversible. Follow the steps in the "Ponceau-S Red Stain" section or "How to Visualize with Transillumination" for reversible methods.

1. Incubate the blot in a solution of 0.1% Coomassie brilliant blue R in 50% methanol, 7% acetic acid, for 2 minutes.
2. Destain the blot in 50% methanol, 7% acetic acid, for 10 minutes.
3. Incubate the blot in 90% methanol, 10% acetic acid for 10 minutes to completely destain the background.

Amido Black Stain

To stain the blot with amido black, follow these steps.

CAUTION: This stain will interfere with rapid immunodetection. It is not reversible. Follow the steps in the "Ponceau-S Red Stain" section or "How to Visualize with Transillumination" for reversible techniques.

1. Incubate the blot in a solution of 0.1% amido black in 25% isopropanol, 10% acetic acid for 10 minutes.
2. Destain the blot in the same buffer without amido black for 5 to 10 minutes.

Ponceau-S Red Stain

To stain the blot with Ponceau-S red (reversible stain), follow these steps.

1. Stain the blot in a solution of 0.5% Ponceau-S red, 1% acetic acid, for 1 minute.
2. Destain the blot in Milli-Q grade water to the desired contrast or wash the blot with 0.1 N NaOH to remove the stain completely.

Using the Transillumination Method

Transillumination is a nondestructive, reversible method of determining the presence of the appropriate protein pattern. To perform transillumination on the blot, follow these steps:

1. Let the blot dry completely using one of the drying methods listed in the following section, "How to Dry the Blotted Membrane." Then return to this section.
2. Immerse the dry blot in 20% methanol for 2 minutes.
3. Place the blot on a light box and mask the areas around the blot with a sheet of black paper. The bands appear as clear areas against an opaque background.

   NOTE: Detection sensitivity is comparable to Coomassie brilliant blue R.

How to Dry the Blotted Membrane

After removing the blotted membrane from a tank or semi-dry system, it must be dried before continuing on to any transillumination or rapid immunodetection procedures to ensure optimum results. This section lists four drying options. The length of time for each option varies. As the blot dries, it becomes opaque. Wait the full length of drying time for all of the liquid to evaporate from within the membrane's pore structure.

CAUTION: Thoroughly drying the blot is especially crucial if performing a rapid immunodetection without blocking. If the blot is not dry, any residual water trapped in the pores will cause the membrane to wet out during the procedure, increasing background noise.

To dry the membrane after blotting, select one of these methods:

Drying Method Option	Required Drying Time
Soak the membrane in 100% methanol for 10 seconds to drive out the water. Then place the blot on a piece of filter paper. Wait for the methanol to evaporate.	15 minutes
Place the blot in a vacuum chamber. (Secure the blot between two sheets of filter paper.)	30 minutes
Incubate the blot at 37°C.	1 hour
Place the blot on a lab bench to let it dry at room temperature.	2 hours

See the next section to perform a rapid immunodetection or see the previous section on using transillumination.

How to Perform a Rapid Immunodetection on Immobilon-P Without Blocking

In immunodetection, a specific antibody identifies the location of a membrane-bound antigen. Immunodetection on Immobilon-P does not require blocking or lengthy wash steps if you thoroughly dry the membrane after protein transfer; drying the membrane returns it to its hydrophobic state. Antibodies can then bind specifically to the proteins on the membrane surface, but not to the membrane itself, eliminating the blocking step.

NOTE: Depending on the antibody source and detection system, adjustments may need to be made to the buffer composition and incubation times.

The following procedure is a general overview of the Rapid Immunodetection Procedure utilizing chromogenic detection. Refer to Millipore publication RP562, "Rapid Immunodetection of Blotted Proteins without Blocking" for a more detailed chromogenic detection protocol. Millipore publication TN051, "Rapid Immunodetection Method on Immobilon-P Using Chemiluminescence" details the chemiluminescence detection protocol.

1. Dry the blotted membrane using one of the methods listed in the previous section.

   CAUTION: Thoroughly drying the blot is especially crucial if planning to perform a rapid immunodetection without blocking. If the blot is not dry, any residual water trapped in the pores will cause the membrane to wet out during the procedure, increasing background noise.

2. Incubate the blot for 1 hour with a primary antibody diluted in blocking buffer that contains 0.05% Tween®-20. (Blocking buffer consists of 1% BSA in phosphate-buffered saline [PBS]: 10 mM Na-phosphate, pH 7.2, 0.9% NaCl.)

3. Wash the blot in PBS two times for 10 seconds.

   CAUTION: Exceeding the recommended concentration of detergent or the indicated wash times may reduce the signal-to-noise ratio.

4. Incubate the blot for 30 minutes with a secondary antibody diluted in blocking buffer containing 0.05% Tween-20.

How to Perform a Rapid Immunodetection on Immobilon-P Without Blocking, continued

5. Wash the blot in PBS two times for 10 seconds.
6. Add the substrate to the blot and incubate it until the signal reaches the desired contrast. Then wash the blot in Milli-Q® grade water to stop the reaction.
7. Air-dry the membrane and store it in a dark place.

Considerations for Protein Sequencing Applications

Protein sequencing provides valuable amino acid sequence information; N-terminal sequencing is commonly performed by automated instruments designed for this purpose. The following sections provide information on the most common concerns about using the Immobilon-P membrane for sequencing applications.

Glycine

The glycine present in standard transfer buffers leads to a high background glycine peak in the first few cycles of protein sequencing and in amino acid analysis. To reduce the level of glycine, wet the membrane in 100% methanol and then wash extensively with Milli-Q system water. Alternatively, change the transfer buffer to 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11, 10% methanol. This buffer works well in tank transfer systems, but sometimes gives less efficient transfer in semi-dry systems.

Coomassie Brilliant Blue Stain

Coomassie brilliant blue is compatible with automated protein sequencing chemistries. In general, if the protein band or spot can be visualized by Coomassie staining, there is enough protein for sequencing.

Storage

For long-term storage, dry the blot completely. Then seal it in a plastic bag. Place the bag between two sheets of cardboard. Store the assembly at -20°C or colder to prevent oxidation. The cardboard protects the frozen membrane from breaks or cracks due to handling when removing the membrane from storage. Allow the membrane to thaw to ambient temperature before removing the cardboard.

Protein Elution

For applications requiring protein elution from the membrane, visualize the proteins bands by transillumination or Coomassie staining in solutions free of acetic acid. The acid in staining solutions strengthens the interaction between proteins and the membrane, making elution more difficult. Alternately, individual bands or spots can be eluted from Immobilon-CD after electrophoresis and staining by taking advantage of its ion exchange binding capabilities.

Amino Acid Analysis

For amino acid analysis, always analyze a piece of blank membrane from the same blot to account for any background signals.

Low Molecular Weight Proteins

Proteins with a molecular weight <10 kDa often pass through Immobilon-P during electroblotting; this makes it more difficult to obtain sequence information. For low molecular weight proteins, Millipore recommends the Millipore Immobilon-PSQ membrane. Its smaller pore size and higher internal surface area result in near-quantitative capture of low molecular weight proteins. Immobilon-P^{SQ} is composed of pure PVDF and is fully compatible with protein sequencing. If proteins in the range of 10 - 20 kDa are to be electroblotted, both Immobilon-P and Immobilon-P^{SQ} should be evaluated for optimum detection.

N-Terminal Blockage

Some proteins are blocked at the N-terminus and cannot be sequenced directly. Effective sequencing requires a combination of protein elution, proteolytic or chemical cleavage, and peptide mapping. For these applications, Millipore recommends the Immobilon-CD membrane. This is a cationically derivatized PVDF membrane. The ionic interaction between proteins and the Immobilon-CD surface is easy to disrupt, resulting in efficient elution.

Peptide Mapping

Even though Immobilon-P may be used for peptide mapping by in situ cleavage, hydrophobic peptides often adhere to the membrane surface. Therefore, Millipore recommends the Immobilon-CD membrane for this application. The resulting population of peptides is more representative of the entire protein.

Hydrophobic Proteins

For electrophoresis of hydrophobic proteins (for example, membrane-bound proteins), detergents are essential for good resolution and solubility. But including the detergent in the transfer buffer prevents the protein from binding to the Immobilon-P. Removal of the detergent before electrotransfer often results in protein precipitation in the gel. If detergent must be included in the transfer buffer to provide good transfer, Millipore recommends the Immobilon-P^{SQ} or Immobilon-CD membrane for better results when binding hydrophobic proteins. The smaller pore size and higher internal surface area of Immobilon-PSQ and the cationic surface of Immobilon-CD improve the chances of the protein binding to the membrane.

NOTE: The following sections contain details on choosing the right Immobilon membrane for your application and ordering information.

Guidelines for Choosing an Immobilon PVDF Membrane

This chart lists the membrane of choice for most proteins in specific applications after a western blot. But because of varying protein properties such as charge density, conformation, and hydrophobicity, all proteins do not behave the same way on a membrane surface. Keep in mind that the chart below lists only guidelines. Experiments with a variety of Immobilon membranes may be neccessary to optimize results for your specific application.

Application (After Western Blotting)	Membrane of Choice (Most Proteins)
General immunodetection	Immobilon-P
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}
Peptide mapping	Immobilon-CD
Internal sequencing of N-terminally blocked proteins	Immobilon-CD
General protein elution	Immobilon-CD

Ordering Information

This section lists the catalogue numbers for Immobilon-CD, Immobilon-P, and Immobilon-P^{SQ} membranes. See the Technical Assistance section for information about contacting Millipore Corporation.

Immobilon-P Catalogue Numbers

Membrane Dimensions (0.45 µm pore size)	Catalogue Number
26.5 cm × 3.75 m roll	IPVH 000 10
26 cm × 26 cm sheets	IPVH 304 F0
20 cm × 20 cm sheets	IPVH 202 00
15 cm × 15 cm sheets	IPVH 151 50
10 cm × 10 cm sheets	IPVH 101 00
9 cm × 12 cm sheets	IPVH 091 20
7 cm × 8.4 cm sheets	IPVH 078 50

Immobilon-P^{SQ} Catalogue Numbers

Membrane Dimensions (0.2 µm pore size)	Catalogue Number
26.5 cm × 3.75 m roll	ISEQ 000 10
26 cm x 26 cm sheets	ISEQ 262 60
20 cm x 20 cm sheets	ISEQ 202 00
15 cm x 15 cm sheets	ISEQ 151 50
10 cm x 10 cm sheets	ISEQ 101 00

Immobilon-CD Catalogue Numbers

Membrane Dimensions (0.1 µm pore size)	Catalogue Number
26 cm × 3.75 m roll	ICDM 000 10

Technical Assistance

For more information, contact the Millipore office nearest you. In the U.S., call 1-800-MILLIPORE (1-800-645-5476). Outside the U.S., see your Millipore laboratory products catalogue for the phone number of the office nearest you. You can also look us up on the Internet at our World Wide Web site (http://www.millipore.com) or send e-mail to tech_service@millipore.com.

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