Porablot
PVDF membranes

Technical instructions for blotting procedures

Porablot PVDF

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1 Introduction

porablot PVDF is a naturally hydrophobic fluoropolymer membrane especially designed for Western transfer, protein binding assay and protein sequencing applications. This pure while microporous solid phase support exhibits strong hydrophobic interactions with a wide range of proteins. The immobilized proteins can be used directly for protein sequencing or amino acid analysis and can be visually detected with all common staining reagents including Coomassie Blue, Amido Black, Ponceau S and Indian Ink. Due to the hydrophobic nature of **porablot PVDF** membrane it should be prewetted with methanol before equilibrating with the appropriate transfer buffer.

Electrophoretic transfer of proteins from a polyacrylamide gel to a membrane was first described by Towbin[3]. The protein (Western) transfer technique is a simple, rapid and sensitive method which is used to identify individual proteins in complex mixtures. The procedure can be divided into three parts:

(I) Polyacrylamide gel electrophoresis of protein molecules
(II) Electrotransfer of proteins from the polyacrylamide gel to membranes
(III) Detection of electrophoretically transferred proteins

The versatility of the protein transfer procedure also allows a variety of gel systems to be used together with a wide range of detection systems making this method useful for a number of applications. In this guide we provide general procedures for the electrotransfer and detection of proteins to **porablot PVDF** membranes and also include a section on protein blots.

The following procedures have been developed to take advantage of the unique properties of these membranes, and have given optimum results in our hands.

2 Membrane Handling and Preparation

This membrane is mechanically very strong and resistant towards tearing or cracking, removal from the gel is particularly easy. The membrane should only be handled by the edges using gloves or forceps to prevent membrane contamination. Either scissors or a sharp scalpel must be used to cut the membrane.
The hydrophobic nature of **porablot PVDF** membrane requires that it is prewet with alcohol before equilibrating with transfer buffer; the procedure is as follows: (a modified procedure is used for dot blots).

Step 1: Cut the membrane to the desired size then lay it on the surface of 80 – 100 % (v/v) methanol or ethanol and leave for 3–5 seconds then fully submerge. The membrane will become translucent as it wets.

Step 2: Rinse the membrane with distilled water, totally immerse it in the transfer buffer and equilibrate for 5 minutes.

Step 3: The membrane should not be allowed to dry until blocked with a protein containing solution which renders the membrane hydrophilic.

3 Buffers and Solutions

Electrophoresis Sample Buffer for proteins:

0.125 M Tris-HCl, pH 6.8 containing: 2 % (w/v) (SDS); 10 % (v/v) glycerol; 2 % (v/v) 2-mercaptoethanol; 0.01 % (w/v) Bromophenol Blue

Transfer Buffer:
25 mM Tris base, 0.192 M glycine, 20 % (v/v) methanol, pH 8.3

PBS (pH 7.4):
40 mM disodium hydrogen phosphate; 8 mM sodium dihydrogen phosphate, 150 mM sodium chloride

Casein Blocking Solution:
0.5 % (w/v) casein and 0.05 % Tween 20 in PBS
Heat the solution to 60 ˚C while stirring to dissolve the casein. Cool and filter through a 0.45 µm membrane filter to remove aggregates. Use a freshly prepared solution.

4 Protein Transfer

We recommend to immobilize proteins on the membrane via electrotransfer. Slot blotting or spotting onto PVDF membranes can be performed on pre-wetted membranes, but is not recommended. The optimum amount of protein for detection usually varies between 1 and 10 µg per band.

4.1 Electrotransfer of Proteins

NOTE: This procedure should be carried out in conjunction with manufactures instructions for the use of a given electrotransfer apparatus. This is especially important, if a semi-dry blotting device is used.

Tank blotting

Use the whole gel or cut the gel into sections to be transferred.

Step 1: Cut membrane to the size of gel or gel sections.

Step 2: Gel treament: Equilibrate gel in transfer buffer. Gels may be stained before transfer with Coomassie Blue, or after transfer with Fast Green, Amido Black, or any other appropriate stain. In this case soak the gel for one hour in the transfer buffer.

Step 3: Assemble each gel section into a ”sandwich“ as follows:
1. Saturate MN 218 B or MN 616 B filter paper (2 sheets/gel section) with transfer buffer.
2. Place gel on one sheet of filter paper.
3. Ensure that the membrane is saturated with transfer buffer.
4. Lay the wet membrane on the gel.
NOTE: Roll a clean pipet over the paper and membrane to remove trapped air bubbles.
5. Place the other sheet of wet filter paper over the membrane. Complete the „sandwich“ by placing between two MN 218 B or MN 616 B filter paper. Secure „sandwich“ between two plastic grids.
NOTE: Operation of the cooling system is necessary in order to optimise transfer results.

Step 4: Fill electrotransfer apparatus with transfer buffer. Insert assembly into transfer apparatus with membrane positioned between the gel and anode. Connect heat exchanger to tap water.

Step 5: Connect the tank to the power supply. Start the transfer following the manufacture´s recommendations for current and transfer time.
NOTE: Large proteins (MW >100 Kd) may require longer transfer times.

Step 6: The menbrane can be rinsed in transfer buffer to remove excess gel fragments after transfer.

4.2 Protein Dot Blots

Step 1: Cut the required membrane to the desired size taking appropriate precautions to prevent membrane contamination. If required use a pencil to draw a grid of 1 cm squares to act as a guide when loading samples.

Step 2: Prewet the membrane by laying it on the surface of 80 % (w/v) methanol in distilled water then totally submerge; leave the membranes in the 80 % methanol until prepared to proceed with step 3.

Step 3: Remove the membrane from the 80 % methanol and drain off the excess so that the membrane remains damp. Place the damp membrane on absorbent paper which has been moistened with 80 % methanol. Using a micropipette immediately apply the protein spots using a volume of 1 µL with a protein concentration of between 1–10 mg/mL. After the protein spots have been applied the membrane can either be placed directly in the blocking solution or allowed to dry on a non-absorbent surface for 5 minutes at room temperature then briefly rewetted in 80% methanol before placing in the blocking solution. Proceed with the blocking and staining procedure (Section 5).

5 Detection

After electrotransfer, unlabeled proteins bound to the membrane may be detected by a variety of methods including immunological detection procedures which utilise ^{125}I labeled or enzyme conjugated antibody. If ”direct“ staining of all the separated protein is required then **porablot PVDF** membrane can be stained with either Coomassie Blue or Indian Ink following the procedure given in Appendix 1.

5.1 Blocking

This procedure recommends the use of 0.5 % casein and 0.05 % Tween 20 in PBS as the blocking agent. This has provided consistently good results in both monoclonal and polyclonal antibody detection systems. It should be emphasised that the dilution of detecting antibodies (primary antibody and antibody enzyme conjugates) will affect the level of background. If background is still a problem this can be further reduced by diluting the detecting antibody in 0.1 to 0.5 % casein in PBS and filtering the dilution through a 0.45 µm membrane filter. Alternative blocking agents such as 5 % (w/v) bovine serum albumin in PBS 5 % (w/v), gelatine in PBS or 5 % nonfat dry milk may be tried.

Step 1: Remove membrane from gel surface and place into a heat sealable plastic bag or an appropriate container.

Step 2: Add at least 10 mL of 0.5 % casein or other appropriate blocking solution per 100 cm^2 membrane surface area to the bag or container. Incubate at room temperature (or up to 40 ˚C) for 30–60 minutes with constant agitation.

5.2 Binding of Antibodies

Step 1: Remove the blocking solution from the plastic bag or container.

Step 2: Dilute first antibody in PBS which may contain 0.1–0.5 % casein. Refer to dilution recommended by the manufacturer or optimise dilution by experiment. If ^{125}I labelled antibodies are used, the counts should not exceed 5 x 10^4 cpm/mL.

Step 3: Place 2 mL of antibody solution per 100 cm^2 of membrane in the bag or container.

Step 4: Agitate the bag or container on a rotary shaker (250 rpm) for 30 min to 1 hour at room temperature, then remove unbound antibody as follows:

Step 5: Remove membrane from the bag or container.
Step 6: Briefly dip the membrane in PBS containing 0.05 % (v/v) Tween 20.
Step 7: Place membrane in a larger plastic bag or container. Add 100 mL PBS, 0.05 % Tween 20 per 100 cm^2 membrane surface area.
Step 8: Agitate bag or container on a rotary shaker (100 rpm) for 5 minutes at room temperature, then discard the buffer.
Step 9: Repeat above steps (7 and 8) three times with the final wash being PBS alone.

5.3 Detection by ^{125}1-Labeled Antibodies

This detection procedure has previously been described by Gershoni[1].
Step 1: Blot membrane dry.
Step 2: Autoradiograph to a two-day exposure using an intensifying screen, at -70 °C.

5.4 Immuno Detection

Binding of Secondary Antibodies

Step 1: Dilute the appropriate antibody-enzyme conjugate to the manufacturer's recommended dilution in PBS containing 0.05 % Tween 20 which may contain 0.1–0.5% casein.
Step 2: Place the membrane in a fresh bag or container.
Step 3: Place 2 mL of conjugate solution per 100 cm^2 membrane into the bag.
Step 4: Agitate the bag or container on a rotary shaker (250 rpm) for 1 hour at room temperature.
Step 5: Wash 2 times 5 min with PBS containing 0.05 % Tween 20.

Detection

Step 1: Prepare the substrate solution (see Appendix 3).
Step 2: Equilibrate the membrane with detection buffer.
Step 3: Immediately add the substrate solution to the membrane in the dish. (Use 2 mL of substrate solution per 100 cm^2 of membrane area)
Step 4: Gently agitate for 1–2 minutes or until bands become visible on the membrane.
Step 5: Wash the membrane in distilled water, blot gently and dry at 80 °C for 1–2 minutes. Store protected from the light.
NOTE: If a permanent record is desired, densitometry reading or a photograph should be taken as soon as possible due to instability of the chromophore.

6 Appendices

6.1 References

1) Gershoni, J.M. and Palade, G.E. (1982), Anal. Biochem. **124**:396
2) LaemmLi, U.K., (1970), Nature (London) **227**:680
3) Towbin, H. Staehelin, T., and Gordon, J. (1979), Proc. Nat. Acad.Sci. USA. **76**:4350
4) Southern, E.M. (1975), Mol.Biol. **98**:503 – 517

6.2 APPENDIX 1

Direct staining of proteins on porablot PVDF membrane

Reagents:

Coomassie Blue R Prepare a 0.1 % (w/v) methanol, 10 % (v/v) acetic acid in deionised water. Destain: 10 % (v/v) acetic acid, 40 % (v/v) methanol in deionised water.

Amido Black Prepare a 0.5 % (w/v) solution in 50 % (v/v) methanol, 5 % (v/v) acetic acid in deionised water. Destain: 5 % (v/v) acetic acid, 50 % (v/v) methanol in deionised water.

Ponceau S Dilute 20 mL of concentrate to 200 mL with deionised water. Destain: 5% (v/v) acetic acid in deionised water

Black Indian Ink Prepare a 0.05 % (v/v) solution in the following buffer: 0.15 M NaCl, 10 mM Na_2HPO_4, pH 7.2 containing 0.5 % (v/v) Triton X-100 (PBS-TRITON).

Staining Procedures:

Coomassie Blue After transfer incubate the membrane for 30 minutes using 40 mL stain/100 cm^2 of membrane; destain for 15 minutes or until background is eliminated.

Amido Black After transfer incubate the membrane for 3–15 minutes using 40 mL stain/ 100 cm^2 of membrane; destain for 1–10 minutes.

Ponceau S After transfer incubate the membrane for 10 minutes using 40 mL stain/100 cm^2 of membrane; destain for 1–10 minutes.

Indian Ink After transfer wash the membrane twice for 5 minutes in PBS-TRITON; incubate the membrane in the diluted Indian Ink for 20 minutes; destain using several changes of deionised water.

6.3 APPENDIX 2

EXAMPLE ANTIBODY DETECTION SYSTEMS

Monoclonal Antibody Systems

Step 1: *Sample protein.* Load 0.5 µL of a 2.5 % (w/v) total rat brain homogenate in sample buffer per 5 mm well, on a 10 % SDS-PAGE gel.

Step 2: *Primary antibody.* Mouse monoclonal antibody – anti-β-tubulin diluted 1/100 or 1/1000 in blocking solution. After transfer, incubate the membrane with primary antibody for 1 hour at RT.

Step 3: *Detection:* directly linked polyclonal enzyme conjugate

(a) Goat anti-mouse IgG-alkaline phosphatase diluted 1/1000 incubated with membrane for 30 minutes at room temperature.
or (b) Goat-anti-mouse IgG peroxidase diluted 1/1000 incubated with membrane for 30 minutes at room temperature.

Detection: two stage system:

Rabbit anti-mouse immunoglubin (Ig) diluted 1/1000 incubated with membrane for 30 minutes at room temperature.

(a) Goat anti-rabbit Ig alkaline phosphatase linked diluted1/1000 for 30 minutes at room temperature.
or (b) Goat anti-rabbit Ig peroxidase linked diluted 1/1000 for 30 minutes at room temperature.

Polyclonal Antibody System

Step 1: *Sample protein.* Load 5 µl of human serum albumin (HSA) in sample buffer to give 0.5 or 1 µg per 5 mm well, on a 10 % SDS-PAGE gel.

Step 2: *Primary antibody.* Non-affinity purified Goat anti-HSA polyclonal antibody diluted 1/10,000 in blocking solution. After transfer, incubate the membrane with primary antibody for 1 hour at room temperature.

Detection:

Directly linked polyclonal enzyme conjugate

(a) Rabbit anti-goat peroxidase linked diluted 1/1000 incubated with membrane for 1 hour* at room temperature.
or (b) Rabbit anti-goat alkaline phosphatase linked diluted 1/1000 incubated with membrane for 1 hour* at room temperature.
* 30 minutes can be used.

6.4 APPENDIX 3

EXAMPLE SUBSTRATE SYSTEMS

Alkaline phosphatase

Nitro Blue Tetrazolium (NBT)-5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate

Step 1: NBT stock solution: 75 mg/mL NBT in 70 % (v/v) dimethylformamide (DMF)

Step 2: BCIP stock solution: 50 mg/mL of BCIP in 70 % (v/v) DMF

Step 3: Tris substrate buffer: 0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$,pH 8.5
Immediately before use, add:
33 µL NBT stock solution and
25 µL BCIP stock solution to
7.5 mL Tris substrate buffer

Peroxidase Diminobenzidene (DAB)

DAB stock solution: 0.278 g DAB (1.3 mM) per 100 mL in 50 mM phosphate buffer pH 7.2. Store frozen in aliquots. Dilute 1 in 10 before use.
Immediately before use add 6 µL H_2O_2 (30% v/v) per 10 mL diluted DAB solution giving a final concentration of 0.02 % (v/v).

Alternative substrates:

(a) 3-Amino-9-ethylcarbazole (AEC)
(b) 4-chloronaphthol (4CN)

7 Ordering Information

Description	Size	Pack of	Pore size [µm]	Cat. No.
Nitrocellulose membranes				
porablot NCP	0.3 x 3 m	1 roll	0.45	741280
porablot NCP	200 x 200 mm	10 sheets		741281
Nitrocellulose membranes with supporting tissue				
porablot NCL	0.3 x 3 m	1 roll	0.45	741290
porablot NCL	200 x 200 mm	10 sheets		741291
Amphoteric nylon membranes				
porablot NY amp	0.2 x 3 m	1 roll		741204
porablot NY amp	0.3 x 3 m	1 roll	0.20	741200
porablot NY amp	200 x 200 mm	10 sheets		741201
Positivated nylon membranes				
porablot NY plus	0.2 x 3 m	1 roll		741242
porablot NY plus	0.3 x 3 m	1 roll	0.45	741240
porablot NY plus	200 x 200 mm	10 sheets		741241
porablot NY plus	220 x 220 mm	10 sheets		741243
PVDF membranes				
porablot PVDF	0.25 x 3 m	1 roll	0.20	741260
porablot PVDF	200 x 200 mm	10 sheets		741261