porablot

Nylon membranes

Technical instructions for blotting procedures

porablot NY amp
porablot NY plus

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MACHEREY-NAGEL

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1. Introduction

porablot NY membranes are reinforced nylon 6,6 membranes for use in DNA, RNA and protein transfers. The physical characteristics of **porablot NY** membranes make them especially useful as a transfer medium. **porablot NY** membranes provide excellent band resolution due to their uniform and carefully controlled pore rating. The membranes are inherently hydrophilic and therefore do not require wetting prior to use. **porablot NY** membranes are also heat and solvent-resistant and will not shrink, crack or tear thus allowing multiple hybridization cycles to be performed. **porablot NY amp** is an amphoteric nylon 6,6 membrane with a surface chemistry comprising 50% amino and 50% carboxyl groups. This provides a hydrophilic membrane with an isoelectric point at pH 6.5. **porablot NY amp** membrane has a high binding capacity for biomolecules in transfer applications and offers greater sensitivity, improved nucleic acid retention and superior handling characteristics over traditional nitrocellulose.

porablot NY plus possesses pore surfaces populated by a high density of quaternary ammonium groups making it strongly cationic. The positive surface charge is maintained in the range pH 3 to over pH 10 and promotes strong ionic binding of negatively charged proteins and nucleic acids. **porablot NY plus** is ideally suited to rapid transfer techniques for nucleic acids providing excellent levels of sensitivity. In addition the membrane's immediate immobilisation characteristics make it suitable for prolonged transfer procedures without the risk of nucleic acid diffusion from the membrane.

Membrane handling:

Porablot NY membranes are naturally hydrophilic and do not require pre-wetting before use. The membranes are mechanically very strong and resistant to tearing or cracking making removal from the agarose gel particularly easy. **Porablot NY** membranes should be handled using gloves or forceps to prevent membrane contamination. Either scissors or a sharp scalpel must be used to cut the membrane.

2. Nucleic Acid Transfer Procedures

2.1 Solutions:

20 x SSC	3 M sodium chloride, 0.3 M sodium citrate, pH 7.0 (dissolve 175.3 g NaCl, 88.2 g sodium citrate · 2 H_2O in 800 ml H_2O and adjust pH to 7.0 with NaOH or HCl; add H_2O to 1 l; sterilize by autoclaving)
20 x SSPE	3.6 M sodium chloride, 0.2 M sodium phosphate pH 7.7, 0.02 M EDTA (dissolve 210.4 g NaCl, 27.6 g NaH_2PO_4 · 1 H_2O and 7.4 g Na_2EDTA in 800 ml H_2O; adjust pH to 7.7 with NaOH; add H_2O to 1 l; sterilize by autoclaving)
Digestion solution (plaque & colony lifts)	50 mM Tris-HCl, pH 7.6, 0.1% SDS, 50 mM NaCl, 100 µg/ml proteinase K (50 ml 1M Tris, 10 ml 10% SDS, 10 ml 5 M NaCl; add H_2O to 1 l; add proteinase K just prior to use)
Nonhomologous DNA	10 mg/ml salmon sperm DNA; sonicate for 30 minutes, then boil for 30 minutes, store at –20 °C
100 x Denhardt's solution	2.0% (w/v) "Ficoll" (400 000 MW), 2.0% (w/v) polyvinylpyrrolidone (360 000 MW), 2.0% (w/v) bovine serum albumin (dissolve 20 g Ficoll, 20 g polyvinylpyrrolidone and 20 g bovine serum albumin in 600 ml H_2O; adjust volume to 1 l with H_2O)
10% SDS	dissolve 10 g sodium dodecyl sulfate (SDS) in 100 ml sterile H_2O
Formamide	deionised using a mixed bed ion exchange resin

NOTE: In many experimental systems, especially those using ^{32}P labelled probes, the use of non-homologous DNA and Denhardt's solution may not be required to lower non-specific binding. In these cases the inclusion of 0.1 to 1% SDS and 0.1 to 0.5% N-lauryl sarcosine in hybridization and prehybridization solutions can often provide ample blocking.

2.2. Nucleic Acid Dot/Slot Blotting

with **porablot NY amp** and **porablot NY plus** nylon membranes

The application of nucleic acid in solution directly onto a transfer membrane is termed a dot blot. Both **porablot NY amp** or **porablot NY plus** membranes may be used for dot blots. It is recommended to fix both DNA and RNA dot blots to **porablot NY amp** membrane by UV irradiation although baking can also be employed. DNA loaded in alkali containing buffers should only be baked and ***not*** exposed to UV irradiation. With **porablot NY plus** membrane DNA dot blots need no fixation providing the DNA has been exposed to alkali on the membranes. RNA dot blots on **porablot NY plus** membrane should be fixed either by baking or UV irradiation.

2.2.1 DNA dot blots

Step 1: Solubilise the DNA in either 6 x SSC by heating to 95 °C for 10 minutes then chilling in ice or by dissolving in 0.5 M sodium hydroxide, 1.5 M sodium chloride for 5 minutes prior to blotting. DNA dissolved in 6 x SSC may be used for dot blots on either **porablot NY amp** membrane or **porablot NY plus** membrane; DNA dissolved in 0.5 M sodium hydroxide, 1.5 M sodium chloride is recommended for dot blots on **porablot NY plus** membrane but can also be used for **porablot NY amp**.

Step 2: Spot the dissolved DNA (0.1 pg to 1 ng per spot/slot) onto the appropriate **porablot** membrane in a volume < 1.5 µl. If necessary the spot can be dried and multiple pipettings performed until the desired amount of DNA has been applied.

Step 3: For DNA dissolved in 0.5 M sodium hydroxide, 1.5 M sodium chloride spotted onto either **porablot NY amp** or **porablot NY plus** membrane proceed directly to step 4. For DNA dissolved in 6 x SSC and spotted onto either **porablot NY amp** or **porablot NY plus** membrane place the membrane on filter paper saturated with 0.5 M sodium hydroxide, 1.5 M sodium chloride for 5 minutes. Remove the membrane and place on filter paper saturated with 0.5 M Tris-HCl pH 7.4 for 2 minutes; proceed to step 4.

Step 4: Place the membrane on filter paper (MN 218 B) saturated with 2 x SSC for 5 minutes.

Step 5: For **porablot NY plus** membrane either air dry (the membrane may be stored overnight at 4 °C) or proceed directly with the wet membrane to hybridization procedure. For **porablot NY amp** it is recommended to fix the DNA on the damp or dry membrane by UV irradiation although in many applications baking will lead to a satisfactory result. For DNA loaded in 0.5 M sodium hydroxide, 1.5 M sodium chloride onto **porablot NY amp** baking will give the best results. Refer to hybridization procedure.

2.2.2 RNA dot blots

Step 1: Spot the RNA in 5 x SSPE, 0.1 mg/ml salmon sperm DNA onto the porablot membrane in a volume of < 1.5 µl. If necessary the spot can be dried and multiple pipettings performed until the desired amount of RNA has been applied.

Step 2: Fix the RNA dot blots by baking or UV irradiation. Refer to hybridization procedure.

2.3. DNA Transfer (Southern Blotting)

with **porablot NY amp** and **porablot NY plus** nylon membranes

2.3.1 Capillary transfer (neutral transfer)

Electrophoresis and pretreatment of gel for transfer

Run DNA on an agarose gel with a running buffer of TAE or TBE. Use a sample buffer containing bromophenol blue.

When electrophoresis is complete fragment the DNA by incubating the gel briefly (< 20 minutes) in 0.25 M HCl. Rinse gel with distilled water and denature DNA by soaking gel in 1.5 M NaCl/ 0.5 M NaOH two times for 20 minutes each. Neutralize the gel by soaking in 1 M Tris/pH 7.5,1.5 M NaCl two times for twenty minutes each.

Transfer conditions:

Use a transfer buffer of 10 x or 20 x SSPE or SSC. Cut 3 pieces of filter paper (MN 827 B, MN 218 B) 7 cm longer than the glass plate to be used for the capillary transfer. Saturate the filter paper with transfer buffer and place on top of a glass plate. Place gel on top of the filter paper, and the membrane over the gel. Roll a clean pipette over the membrane to remove trapped air bubbles (**porablot NY membranes need not be pre-wetted and will wet instantly on contact with the gel)**. Place 5 pieces of filter paper cut to the size of the gel over the assembly. Throughout the transfer, do not allow the paper on the top of the gel to contact the paper below the gel. This is done by placing strips of Parafilm around the sides of the gel. Place the glass plate and the gel assembly on top of a glass baking tray filled with transfer buffer. Allow the bottom layer of filter paper (MN 827 B, MN 218 B) to overhang into the transfer solution in the glass baking tray. Place a 5 cm stack of paper towels on top of the gel assembly and secure with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA molecules to the membrane. Secure plastic wrap over the entire assembly and place in a cold room for 3 hours to overnight. If the paper towels become saturated with transfer buffer, replace them with dry ones. After transfer, stain a part of the gel with 0.5 µg/ml ethidium bromide to check transfer efficiency. Remove the membrane from the gel surface. Do not rinse the membrane. The preferred method of fixation for **porablot NY amp** is baking or UV irradiation. The preferred method of fixation for **porablot NY plus** is baking for 15 minutes at 80 °C and then rinsing in 2 x SSC. Refer to hybridization procedure.

> **NOTE:** Transfer of partially cleaved DNA fragments may be complete in as little as 2 hours, and this will be facilitated by changing the paper towels once during transfer.

2.3.2 Capillary transfer (alkaline transfer)

For porablot NY plus membranes an improved alkaline transfer may be used.

In contrast to the standard protocol the gel is not neutralized after the denaturation step. Furthermore the procedure uses only 0.4 M NaOH as the transfer solution.

After alkaline transfer to **porablot NY plus** rinse the membrane in 2 x SSC for 5 minutes and either proceed directly to hybridization or airdry (the membrane may be stored overnight at 4 °C).

2.3.3 Vacuum blotting

with **porablot NY amp** and **porablot NY plus** nylon membranes

NOTE: This procedure should be carried out in conjunction with the manufacturer's instructions for the use of a given vacuum blotting apparatus.

Vacuum blotting systems employ a low pressure vacuum to transfer nucleic acids from the electrophoresis gel to the transfer membrane. Transfer can be completed in less than one hour with very high transfer efficiency. Because of the short transfer time there is less diffusion giving sharper more focussed bands. Both **porablot NY amp** and **porablot NY plus** membranes may be used in vacuum blotting; in the following example **porablot NY plus** membrane has been employed.

Electrophoresis

Run DNA on an agarose gel with a running buffer of TAE or TBE. Use a sample buffer containing bromophenol blue.

Preparation of the gel and transfer

Step 1: Assemble the vacuum transfer apparatus ensuring that the mask is cut such that the electrophoresis gel overlaps the window by approximately 5 mm.

Step 2: Cut the **porablot NY plus** membrane to the appropriate size. Place the dry sheet of **porablot NY plus** membrane so that it is exactly positioned within the window of the mask.

Step 3: When electrophoresis is complete transfer the gel directly to the **porablot NY plus** membrane in the vacuum transfer apparatus taking care to accurately position the gel and avoid trapping air bubbles between the gel and membrane. Ensure that the mask is clamped down then turn on the vacuum pump to immobilise the gel.

Step 4: Without waiting for the vacuum to reach the working level, pour on 0.25 M HCI to fragment the DNA. Stabilise the vacuum at 40 cm H_2O. Incubate the gel until the bromophenol blue turns yellow (~ 30 min). Ensure that the gel remains completely covered during the depurination step.

Step 5: Remove solution from the gel and pour on 1.5 M NaCl/0.5 M NaOH to denature the DNA. Incubate the gel until the bromophenol blue turns blue (~ 30 min). Ensure that the gel remains completely covered during the denaturation step.

Step 6: Remove solution from the gel and pour on 20 x SSC. Ensure that during the subsequent transfer time (40 – 120 minutes) the gel remains completely covered with transfer buffer.

Step 7: Remove all the transfer buffer and with the vacuum still on, carefully peel off the gel leaving the **porablot NY plus** membrane still in place. Turn off the vacuum and remove the membrane. Do not rinse the membrane. The preferred method of fixing is by baking for 15 minutes at 80 °C and then rinsing in 2 x SSC. Refer to hybridization procedure.

2.4 RNA Transfer (Northern Blotting)

with **porablot NY amp** and **porablot NY plus** nylon membranes

NOTE: When working with RNA it is important to keep the equipment and reagent solutions free from RNase contamination. Gloves should be worn at all times. It is recommended that 0.1% SDS is incorporated in all solutions except the 20 x SSC transfer buffer.

The following procedure is suitable for both **porablot NY plus** and **porablot NY amp**. **porablot NY** membranes are recommended for rapid transfer techniques such as vacuum transfer and electrotransfer.

Conditions for electrophoresis

Run RNA under denaturing conditions in a glyoxal, formaldehyde or methyl mercuric hydroxide gel.

Transfer Procedures

Capillary transfer (neutral and alkaline) of RNA (see page 5 for detailed information).
For **porablot NY amp** membrane the preferred method of fixing the RNA is by UV irradiation of the damp or dry membrane. For **porablot NY plus** the RNA may be fixed by baking or UV irradiation.

NOTE: Overnight transfer is recommended for RNA to ensure complete recovery of higher molecular weight RNA from the gel.

2.4.1 Vacuum transfer of RNA

Refer to the general notes on the vacuum blotting procedure given at page 6. After electrophoresis carefully slide the gel from the glass support plate onto the **porablot NY** membrane in the vacuum transfer apparatus. Take care to accurately position the gel and avoid trapping air bubbles between the gel and the membrane. Ensure that the mask is clamped down then turn on the vacuum pump to immobilise the gel. Without waiting for the vacuum to reach working level pour 20 x SSC transfer buffer onto the center of the gel. Use sufficient solution to completely cover the gel. Stabilise the vacuum at 40 cm H_2O. Ensure that during the subsequent transfer time (40 – 120 minutes) the gel remains completely covered with transfer buffer.
Remove all the transfer buffer by aspirating with a pipette tilting the apparatus to allow any excess fluid to flow from the concave centre of the agarose gel. With the vacuum still on carefully peel off the gel leaving **porablot NY** membrane still in place. Turn off the vacuum and remove the membrane. Do not rinse the membrane. For **porablot NY amp** the preferred method of fixing the RNA is by UV irradiation of the damp or dry membrane. For **porablot NY plus** the RNA may be fixed by baking or UV irradiation

2.4.2 Electrotransfer of RNA

Cut the **porablot** membrane to the exact size of the gel. Place the gel on two sheets of filter paper (MN 827 B, MN 218 B) which have been pre-wetted with 1 x electrotransfer buffer (20 x electrotransfer buffer: 0.06 M citric acid, 0.08 M disodium hydrogen phosphate). Place the dry **porablot NY** membrane onto the gel surface taking care not to trap any air bubbles. Cover the **porablot NY** membrane with two sheets of filter paper pre-wetted with 1 x electrotransfer buffer. Assemble the membrane and gel in the electroblotting unit. Transfer according to manufacturers instructions. After transfer is complete remove the membrane from the module. Do not rinse the **porablot NY** membrane. For **porablot NY amp** the preferred method of fixing the RNA is by UV irradiation of the damp or dry membrane. For **porablot NY plus** the RNA may be fixed by baking or UV irradiation. Refer to hybridization procedure.

2.5. Colony Lifts, Plaque Lifts and Replica Plating

with **porablot NY amp** membranes

It is recommended to use **porablot NY amp** 0.2 µm for colony and plaque lifts and for replica plating. To avoid contamination the membrane should be pretreated in the following way:
Boil the membranes in 1 mM EDTA for 5 minutes; thoroughly rinse in double distilled water; interleaf the membranes with chromatographic filter paper (MN 218 B) and wrap in aluminium foil; autoclave for 20 minutes and vacuum dry.

Colony and plaque lifts on porablot NY amp

The petri dish, with colonies or clearly visible plaques, should be precooled in a +4 °C refrigerator for 30 minutes. Using aseptic technique carefully lay the **porablot NY amp** membrane disc onto the agar surface. Make location marks by piercing the membrane with a sterile needle. Carefully remove the membrane disc in a single movement and place on filter paper (MN 218 B) saturated with 0.5 M NaOH, 1.5 M NaCl ***with the colony or plaque side uppermost.*** Leave for 5 minutes then remove the membrane disc and place briefly on dry filter paper to remove excess fluid. Neutralise by placing on filter paper saturated in 0.5 M Tris-HCl pH 7.4,1.5 M NaCl for 5 minutes, rinse in 2 x SSC. Fixation may be by UV irradiation or baking.

Replica plating

Bacterial colonies containing plasmids and cosmids can be directly plated on **porablot NY amp** membrane. The membranes must be pretreated and autoclaved as above to avoid contamination. Using aseptic technique carefully lay the **porablot NY amp** membrane disc onto the surface of the agar in the petri dish ensuring that there are no air bubbles trapped between the membrane and the agar. Prepare an appropriate dilution of the bacterial suspension and directly plate onto the membrane (use 0.5 ml of suspension for 82 mm discs and 1.2 ml for 132 mm discs).
After a suitable incubation time at 37 °C very small colonies (< 0.5 mm) should be visible. Remove the membrane disc and place ***colony side up*** on a sterile filter paper mounted on a glass plate. Take a fresh sterile **porablot NY amp** membrane disc and position this exactly over the first laying it down in a single movement. Take care not to realign or the colonies will be blurred. Cover with a second sterile filter paper and glass plate. Apply strong hand pressure on the upper glass plate then remove it and the filter paper. Using a sterile needle make location marks by piercing the two membranes while still stuck together. Carefully separate the membranes and place each respective membrane ***colony side up*** on a fresh agar petri dish. The original colonies regrow on the first membrane disc; this plate is kept in a refrigerator as the master plate. The colonies on the second membrane (the replica) usually take slightly longer to grow (4 – 6 hours). Remove the membrane from the agar plate once very small colonies can clearly be seen. Denature and neutralise the replica membrane using the method given in steps at page 5. Fixation may be by UV irradiation or baking. Refer to hybridization procedure.

NOTE: After fixation proteins are removed by digestion for 6 to 12 hours at 37 °C in digestion solution (see page 3). The yellow colored colony spots will disappear during this treatment. Rinse the membrane disc in 2 x SSC.

3. Nucleic Acid Hybridization Procedures

This section covers prehybridization and hybridization procedures for Southern and Northern transfers including dot blots and colony and plaque transfers.

3.1 Solutions

Stock DNA solution	Denature nonhomologous DNA (10 mg/ml salmon or herring sperm DNA in water) by sonicating for 30 minutes then boiling for 30 minutes. Store frozen in small aliquots at –20 °C
Hybridization solution Southerns	Denature an aliquot of nonhomologous DNA by heating to 100 °C for 10 minutes then snap cooling on ice. Add at a final concentration of 100 µg/ml to the DNA hybridization solution comprising: 5 x Denhardt's solution, 5 x SSC, 0.1% (w/v) SDS
Hybridization solution Northerns	Denature an aliquot of nonhomologous DNA by heating to 100 °C for 10 minutes then snap cooling on ice. Add at a final concentration of 100 µg/ml to the RNA hybridization solution comprising 50% deionized formamide, 5 x SSPE, 5 x Denhardt's, 0.1% (w/v) SDS
Rinse solution	2 x SSC
Wash buffer	2 x SSC, 0.1% SDS. NOTE: This should be optimised for a given probe system; the most stringent would be 0.1 x SSC, 0.1% SDS at 50 – 65 °C for 100% homologous probe systems

NOTE: Denhardt's solution may not be required in some systems; for these applications the use of 0.1% SDS solution has been found adequate.

3.2 Preparation of the probe

The exact method will depend on the nature of the probe and the existing laboratory procedure; radiolabeled probes can be denatured by heat alone (100 °C for 10 minutes in the presence of 100 µg/ml nonhomologous DNA and then snap cool on ice) or by alkali denaturation by adding 1/10 volume 1 M NaOH to the probe in the presence of 100 µg/ml nonhomologous DNA and heating at 65 °C for 10 minutes; neutralize the DNA solution by the addition of 1/10 volume 1 M HCl.

NOTE: Biotinylated probes must not be subjected to alkaline denaturation which will cleave the biotin from the probe.

3.3 Prehybridization

Seal the membrane in a plastic bag with 2 to 4 ml of appropriate hybridization solution per 100 m^2 of membrane. Incubate the bag at 65 °C for Southerns and 42 °C for Northerns for 15 minutes to 1 hour.

3.4 Hybridization

Open the bag containing the membrane and remove excess hybridization solution. Roll a pipette over the bag to remove as much of the solution as possible. Add the denatured labeled probe to an appropriate volume of fresh hybridization solution. To calculate this volume use 2 ml per 100 cm^2 of membrane. Add the appropriate hybridization solution containing the labeled probe to the membrane and reseal the bag.
For Southern transfers immerse the bag at 65 °C for the duration of hybridization (2 hours to overnight). For Northern transfers immerse the bag at 42 °C for the duration of hybridization (4 hours to overnight).

3.5 Washing

Step 1: After hybridization, remove the membrane from the plastic bag; rinse briefly in rinse solution (2 x SSC). Place the membrane in a fresh plastic bag.
Step 2: Add 250 ml of wash buffer at room temperature (2 x SSC, 0.1% SDS) per 100 cm^2 of membrane.
Step 3: Agitate the bag vigorously (200 rpm) for 20 minutes at room temperature, then discard the buffer.
Step 4: Repeat steps 2 and 3 two times. The membrane is now ready for autoradiography (or detection of biotinylated probe with an avidin or streptavidin conjugate).

3.6 Rehybridization

Both **porablot NY amp** and **porablot NY plus** membranes are easily reprobed. For rehybridization we recommend UV irradiation as the method to fix DNA and RNA to **porablot NY amp** membrane and RNA to **porablot NY plus** membrane. DNA should be fixed to **porablot NY plus** membrane by baking at 80 °C. The preferred stripping method is boiling in 0.1% aqueous SDS.

> **NOTE:** It is important that the membrane is not allowed to dry before stripping off the probe. Certain systems may require more rigorous treatment to remove the probe; boiling in 0.1% SDS may be extended for up to 30 minutes.

The procedures given below are intended for radiolabeled probes. Special conditions are required for biotinylated probes which can be successfully reprobed on nylon membranes. Refer to specific manufacturer's protocols for reprobing using non-radioactive probe systems. Pour boiling 0.1% aqueous SDS solution onto the membrane and shake for a few minutes. Discard the solution and immediately add fresh boiling 0.1% SDS solution. Allow to cool to 40 °C or room temperature.
Detection with non-radioactive labeled oligonucleotides shows sometimes a higher unspecific background! SDS has already been shown to be very useful in blocking the unspecific binding of probes to nylon membranes in nucleic acid hybridiziations. Therefore it is recommended to increase the amount of SDS from 0.1% to 1 – 2% in the hybridization buffer.

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4. Protein Transfer Procedures

with **porablot NY amp** and **porablot NY plus** membranes

4.1 Introduction

porablot NY membranes are reinforced nylon 6,6 microporous membranes for use in a variety of transfer applications. **porablot NY** membranes have high protein binding capacities providing increased sensitivity and are inherently hydrophilic. The **porablot NY amp** membrane is recommended as a general purpose transfer membrane while the **porablot NY plus** membrane with its positive surface charge promotes strong ionic binding of negatively charged proteins.
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** 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.

4.2 Membrane Handling and Preparation

All these membranes are mechanically very strong and resistant to tearing or cracking; removal from the gel is particularly easy. The membranes 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.
porablot NY membranes are naturally hydrophilic and while the dry membrane can be laid directly onto the gel it may be preferred to pre-wet these membranes before use by laying on the surface of the transfer buffer for a few seconds then fully submerging to complete the wetting process.

Step 1: Cut the membrane to the desired size then lay it on the surface of 100% methanol and leave for 3 – 5 seconds then fully submerge.
Step 2: Remove the membrane from the methanol and immediately totally immerse it in the transfer buffer and equilibrate for 5 – 15 minutes.
Step 3: The membrane should not be allowed to dry until blocked with a protein containing solution which renders the membrane hydrophilic.

4.3 Solutions

Electrophoresis sample buffer for proteins	0.125 M Tris-HCI 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 I	0.025 M Tris-HCI, 0.192 M glycine pH 8.3 containing 20% (v/v) methanol
Transfer buffer II	10 mM Tris-HCI, pH 7.5
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 in PBS either 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; or, at room temperature prepare a 0.5% (w/v) dispersion of casein in PBS; while stirring continuously raise the pH to 12 using dropwise addition of 5 M sodium hydroxide solution; the solution will clear. Immediately lower the pH to 7.4 by dropwise addition of 2 M sodium dihydrogen phosphate solution; filter through a 0.45 µm membrane filter. **Always use a freshly prepared solution.**

4.4 Blocking Procedure

This procedure recommends the use of 0.5% casein in PBS as the blocking agent. This has provided consistently good results in both monoclonal and polyclonal antibody detection systems. It should be emphasized 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 at least 30 minutes with constant agitation.

4.5 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: For **porablot NY amp, porablot NY plus** membranes place the dry membrane on a non-absorbent support. Using a micropipette apply the protein spots using a volume of between 1 – 3 µl with a protein concentration of between 1 – 10 mg/ml. Air dry for five minutes at room temperature. Proceed to procedures for blocking (page 13) and immunodetection (chapter 5).

Use of vacuum dot blot apparatus

NOTE: Protein solutions can be loaded under vacuum; refer to the manufactures operating instructions for a given apparatus.

In all the following procedures the blocking reagents, primary antibody and conjugate dilutions should be re-filtered down to 0.2 µm to prevent non-specific effects due to protein aggregates in these solutions.

Step 1: Cut the required membrane to the desired size (e.g. 9 x 12 cm) taking appropriate precautions to prevent membrane contamination. For **porablot NY amp** and **porablot NY plus** membranes wet the membrane by laying in the surface of PBS then fully submerge. Proceed to step 2 with the wet membrane.

Step 2: Carefully assemble the membranes on the vacuum dot blot apparatus taking care to ensure that the membrane is well sealed to prevent leakage or side diffusion.

Step 3: Using volumes between 10 – 100 µl load the desired dilutions of proteins into the wells. Any negative control wells should be loaded with a similar volume of PBS.

Step 4: The loaded protein solutions should be left for 30 minutes then pulled through using a vacuum of 600 to 700 mbar.

Step 5: Block the membrane by loading each well with 100 µl of 0.5% casein solution in PBS and incubating for 30 minutes. Pull the blocking solution through under vacuum and rinse each well with 100 µl of PBS while still under vacuum.

Step 6: Load each well with 100 µl of an appropriate dilution of primary antibody and incubate for 1 hour; pull through under vacuum.

Step 7: Wash the membrane by loading each well with 100 µl of 0.1% (v/v) Triton X-100 in PBS; pull through under vacuum. Repeat this wash one more time then release the vacuum and load each with 100 µl of PBS only; pull through under vacuum.

Step 8: Load each well with 100 µl of an appropriate dilution of enzyme conjugated antibody (see Appendix 1) and incubate for 1 hour; pull through under vacuum.

Step 9: Repeat the wash procedure given in step 7 and then load each well with 100 µl of distilled water; pull through under vacuum.

Step 10: Prepare the required substrate (see Appendix 2) ensuring that the substrate working solution is prefiltered to 0.2 µm. Load each well with 100 µl of substrate solution and incubate for 5 minutes, pull through under vacuum.

Step 11: With the vacuum still on wash each well twice with 100 µl volume of distilled water.

Step 12: Release the vacuum and disassemble the apparatus; remove the membrane and air dry at room temperature.

4.6 Electrotransfer of Proteins

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

4.6.1 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 treatment: Wet gel surface with transfer buffer I. 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 I.

Step 3: Assemble each gel section into a "sandwich" as follows:
1. Saturate MN 218 B or MN 827 B filter paper (2 sheets per gel section) with the appropriate 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 827 B filter paper. Secure "sandwich" between two plastic grids.
NOTE: Operation of the cooling system is necessary in order to optimize 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: Apply a current of 0.3 A for 2 hours to an 18 cm x 1.5 mm x 16 cm gel.
NOTE: Large proteins (MW > 100 Kd) may require longer transfer times.

5. Immunodetection of Proteins

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. Anionic stains cannot be used with **porablot NY amp** or **porablot NY plus** membranes. In these cases staining can be achieved using the biotinylation/avidin-peroxidase procedure.

5.1 Blocking: (see page 13)

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 optimize dilution by experiment. If ^{125}I labeled antibodies are used, the counts should not exceed 5 x 10^4 cpm/ml.
Step 3: Place at least 8 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 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 0.1% (v/v) Triton X-100.
Step 7: Place membrane in a larger plastic bag or container. Add 100 ml PBS, 0.1% Triton X-100 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}I 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 ELISA Detection Procedure:

Step 1: Dilute the appropriate antibody-enzyme conjugate to the manufacturer's recommended dilution using PBS which may contain 0.1 – 0.5% casein.
Step 2: Place the membrane in a fresh bag or container.
Step 3: Place at least 8 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, then remove unbound conjugate as detailed in steps 5 – 9 above except that the final wash should be in double distilled water.
Step 5: Prepare the substrate solution (see Appendix 2).
Step 6: Remove the membrane from the double distilled water, wash and place it in an appropriately sized container, e.g. a petri dish.
Step 7: Immediately add the substrate solution to the membrane in the dish. Use 10 ml of substrate solution per 100 cm^2 of membrane area.
Step 8: Gently agitate for 1 – 2 minutes or until bands become visible on the membrane.
Step 9: Wash the membrane in distilled water, blot gently and dry at 80 °C for 1 – 2 minutes. Store protected from 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.

5.5 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. Natl. Acad. Sci. USA. **76,** 4350
4) Southern, E.M. (1975) Mol. Biol. **98,** 503 – 517

5.6 Appendix 1

Examples for Antibody Detection Systems

5.7.1 Monoclonal antibody system

The following model system is routinely used with **porablot NY plus** membrane.

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 room temperature.

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 immunoglobulin (Ig) diluted 1/1000 incubated with membrane for 30 minutes at room temperature.

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

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

5.7.2 Polyclonal antibody system

The following system is routinely used with **porablot NY plus**

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

5.7 Appendix 2

Examples for Substrate Systems

5.8.1 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

5.8.2 Peroxidase and diaminobenzidine (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).

5.8.3 Alternative substrates:

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

6. 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
porablot NCP	∅ 132 mm	50 disks		741282
porablot NCP	∅ 82 mm	50 disks		741283
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
porablot NCL	∅ 132 mm	50 disks		741292
porablot NCL	∅ 82 mm	50 disks		741293
Amphoteric nylon membranes				
porablot NY amp	0.2 x 3 m	1 roll	0.20	741204
porablot NY amp	0.3 x 3 m	1 roll		741200
porablot NY amp	200 x 200 mm	10 sheets		741201
porablot NY amp	∅ 132 mm	50 disks		741202
porablot NY amp	∅ 82 mm	50 disks		741203
Positivated nylon membranes				
porablot NY plus	0.2 x 3 m	1 roll	0.45	741242
porablot NY plus	0.3 x 3 m	1 roll		741240
porablot NY plus	200 x 200 mm	10 sheets		741241
porablot NY plus	220 x 220 mm	10 sheets		741243
porablot NY plus	∅ 132 mm	50 disks		741244
PVDF membranes				
porablot PVDF	0.25 x 3 m	1 roll	0.20	741260
porablot PVDF	200 x 200 mm	10 sheets		741261