porablot

Nitrocellulose membranes

Technical instructions for blotting procedures

porablot NCP
porablot NCL

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MACHEREY-NAGEL

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1. Introduction

porablot NCP is a 100% pure cast nitrocellulose. This assures a high binding capacity (100 µg/cm^2) and sensitivity. Pure nitrocellulose exhibits the highest sensitivity with very low background in all transfers, especially in protein blotting. Unlike PVDF, nitrocellulose wets out naturally, does not require methanol, and will not turn hydrophobic during the transfer process. Excellent results will be obtained with all detection systems: antibody/antigen, radiolabeled, biotinylated, and chemiluminesscent, giving you a great amount of flexibility in designing your procedure.

porablot NCL is a supported pure nitrocellulose membrane. It has two advantageous properties: The specific binding characteristics of nitrocellulose and the dimensional strength of nylon. The inert web prevents curling, cracking and tearing even after baking. This makes **porablot NCL** an ideal tool for RNA and DNA transfers. These properties are especially important in colony and plaque lifts and multiple reprobings or any other procedure requiring rigorous handling.

porablot NCL ensures a high binding capacity of approximately 100 µg/cm^2. This membrane provides excellent bond resolution due to its uniform and carefully controlled pore rating. The membranes are inherently hydrophilic and, therefore, do not require wetting prior to use.

2. Southern (DNA) and Northern (RNA) Hybridizations

with **porablot NCP** and **porablot NCL** membranes.

2.1 Gel Preparation

Southern Blots Run DNA on an agarose gel with a running buffer of TAE or TBE. If necessary, fragment gel in 0.25 N HCl. Rinse gel with distilled water and denature DNA by soaking gel in 1 M NaCl/0.5 M NaOH two times for 20 minutes each. Neutralize the gel by soaking in 0.5 M Tris/pH 7.5, 1.5 M NaCl two times for twenty minutes each.

Northern Blots Run RNA under denaturing conditions in a Glyoxal, Formaldehyde, or Methyl Mercuric Hydroxide gel. Gel should be 0.8 – 1.5% agarose, 2.5 – 5.0 mm thick. Stain with 33 µg/ml acridine orange in 10 mM $NaPO_4$/pH 6.5, then destain 3 x 15 minutes in buffer, or stain with ethidium bromide, 1 µg/ml in 50 mM NaOH for 25 minutes, then destain in 200 mM Na_4OAc/pH 4.0, 2 x 20 minutes.

Gel Pretreatment

Southerns: Not necessary.

Northerns: Pretreatment, such as fragmentation of RNA, is unnecessary.

2.2 Membrane Preparation

Float membrane on distilled water, then immerse until wet thoroughly. If wetting is not immediate, heat water until just under boiling temperature. Soak in the transfer buffer until use.

2.3 Capillary Transfer

Use a transfer buffer of 20 x SSPE or SSC.

Cut three pieces of filter paper 7 cm (MN 827 B; MN 218 B) 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 bubble. Place five 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.

2.4 Alternative Transfer Systems

Vacuum blotting, semi-dry electroblotting, bidirectional transfers, and positive pressure blotting systems can all be used with **porablot NC** membranes. Follow manufacturers instructions, and contact MN Technical Services with any questions.

2.5 Immobilization

After blotting, wash the membranes in 5 x SSPE at 60 °C for 5 minutes. This is an optional step to remove residual agarose.

Place the membrane between two sheets of dry filter paper (MN 218 B) and bake in a vacuum oven at 80 °C for 1 to 2 hours.

2.6 Hybridization Procedure

Hybridization is most commonly done in heat-sealable bags in order to conserve solution and protect researchers from exposure to radioactivity. All hybridization solutions should be filtered before use e.g. with a 0.22 µm cellulose acetate filter. (NOTE: Use only cellulose acetate filters; other membrane types may not perform comparably). Low binding cellulose acetate filters will filter prehybridization and hybridizaton solutions without nonspecifically binding essential components of these solutions.

2.6.1 Prehybridization

This step should always be carried out at the temperature of the hybridization. Place the membrane in a heat-sealable bag without the probe in 0.1 ml/cm^2 of the following prehybridization buffers:

Southern Prehybridization Solution	5x SSPE
	50% formamide
	5x Denhardt's solution
	0.5% SDS
	100 µg/ml denatured DNA
	10% dextran sulfate
Northern Prehybridization Solution	5x SSPE
	50% formamide
	5x Denhard's solution
	0.1 – 0.5% SDS
	100 µg/ml denatured DNA
	10% dextran sulfate

Nitrocellulose membranes (**porablot NCP, porablot NCL**) should be wet with 1 x SSPE, 0.1% SDS before prehybridization step. Shake 1 to 2 hours.

2.6.2 Hybridization

Remove prehybridization solution completely from bag, and add the hybridization solution. Hybridization temperature should be determined by the presence of formamide in the hybridization solution.

Hybridization Temperature Chart

Temperature	% Formamide	Hybridization Solution
42 °C	50%	Low-Temperature Hybridization Solution
65 °C	0%	High-Temperature Hybridization Solution

Low-Temperature Hybridization Solution for Southern Blots

50% formamide (47%)
5x Denhardt's solution
6x SSPE
0.2% SDS
100 – 200 µg/ml denatured fragmented DNA
10% dextran sulfate

Low-Temperature Hybridization Solution for Northern Blots

50% formamide
5x Denhardt's solution
5x SSPE
0.2% SDS
100 – 200 µg/ml denatured fragmented DNA
10% dextran sulfate

High-Temperature Hybridization Solution for Southern Blots

5x Denhard's solution
6x SSPE
0.5% SDS
50 µg/ml denatured fragmented DNA
10% dextran sulfate

High-Temperature Hybridization Solution for Northern Blots

5x Denhard's solution
5x SSPE
0.5% SDS
100 µg/ml denatured fragmented DNA
10% dextran sulfate

Dextran sulfate is a rate enhancer for probes larger 200 base pairs and should not be used with oligonucleotide probes.

Clean probe solutions by adding a small amount of hybridization solution to the probe, and filter it through a cellulose acetate syringe filter, to eliminate any contaminants before they come into contact with the transfer membrane.

Denature the probe by boiling in TE buffer for 5 minutes, or incubate with 0.1 volume of 1 N NaOH at 37 °C for 5 minutes. Place on ice. Add the probe to the hybridization solution in the heat-sealable bag and reseal. Probe concentration should not exceed 20 ng/ml. Single copy genes or low copy message may require 1 – 5 x 10^6 cpm/ml. Probes should be labeled no more than 24 hours before hybridization. Hybridize 12 hours to overnight.

Important: If the membrane is to be rehybridized, do not allow it to dry past this point. This will cause irreversible binding to the membrane.

2.6.3 Post Hybridization

Wash temperature should be 25 °C below the T_m (melting temperature of the hybrid). If the homology between the probe and membrane bound DNA is inexact, the wash temperature should be lower.

Stringency Washing Procedure

NOTE: With nitrocellulose membranes, an initial low stringency washing procedure is recommended. Use 0.5 ml of wash solution per square centimeter for all membranes. After washing, remove excess moisture with paper towels.

Low Stringency (for inexact matching)
2 x 15 minutes with 1 x SSC, 0.1% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.1% SDS at 37 °C

Medium Stringency
2 x 15 minutes with 5 x SSC, 0.5% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.5 – 1.0% SDS at 37 °C
1 x 15 minutes with 0.1 x SSC, 1.0% SDS at 37 °C

High Stringency (for perfect hybrids)
2 x 15 minutes with 5 x SSC, 0.5% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.5 – 1.0% SDS at 37 °C
3 x 15 minutes with 0.1 x SSC, 1.0% SDS at 65 °C

2.6.4 Autoradiography

Wrap the membrane in plastic wrap and autoradiograph at –70 °C in a cassette with an intensifying screen while slightly damp. Expose the membrane for 25 – 60 hours.

2.6.5 Probe Removal

Do not allow the membrane to dry if a rehybridization step is intended.
For **porablot NCP** (unsupported nitrocellulose): Boil 0.1 x SSPE or SSC, 0.1% SDS and add blot after removing from heat. Check for residual probe activity.
For **porablot NCL** (supported nitrocellulose): Boil in distilled water for 5 minutes. Check for residual probe activity. If necessary, boil for an additional 5 minutes.

3. Nucleic Acid Dot/Slot Blotting

with **porablot NCP, porablot NCL** nitrocellulose membranes.

3.1 Sample Preparation

Purify DNA or RNA by standard methods and suspend in 50 µl of TE buffer/pH 8. For RNA adjust the pH to 7.5.

DNA: Denature DNA with the addition of 0.1 vol of 3 M NaOH. Incubate for 1 hour at 60 °C. Cool and add 1 vol 2 M NH_4OAc/pH 7, or 6 x SSC.

RNA: Denature RNA by adding 35 µl of 20 x SSC, and 20 µl of 37% formaldehyde. Incubate for 15 minutes at 60 °C.

Wet membrane in distilled water, then soak in the appropriate buffer.

DNA: Soak in 1 M NH_4OAc/pH 7, or 6 x SSC.

RNA: Soak in 20 x SSC.

3.2 Sample Application

If using a filtration manifold, cut two pieces of filter paper to the size of the manifold plate and soak them in:

DNA: 6 x SSC

RNA: 20 x SSC

Place filter paper (MN 218 B), then membrane onto the sample plate and secure the unit. Apply low vacuum, and wash wells with 500 µl of 1 M NH_4OAc/pH 7, or 6 x SSC for DNA, 500 µl of 20 x SSC for RNA. Apply sample (not to exceed 10 µg DNA or RNA per well), under low vacuum. Remove membrane. If working with RNA, rinse each well again with 500 µl of 20 x SSC.

If dotting directly onto the membrane, apply 2 – 5 µl samples to membrane placed on two sheets of dry filter paper. Allow to dry.

3.3 Immobilization

Immobilize DNA by baking in a vacuum oven at 80 °C for 15 minutes – 1 hour. Remove when dry.

3.4 Hybridization

Hybridize by standard methods (see Southern and Northern procedures listed on page 2).

3.5 Detection

Detect with isotopic or colorimetric probes. Densitometric analysis can be carried out on nitrocellulose membranes after clearing with paraffin oil.

4. Colony Hybridizations

with **porablot NCP, porablot NCL** nitrocellulose membranes.

4.1 Membrane Preparation

If necessary, sterilize the membrane between two pieces of filter paper (MN 218 B) in the autoclave for 15 minutes. Allow the membrane to wet on the agar surface. Mark the membrane so that future orientation is assured.

4.2 Colony Transfer

Step 1: Incubate cell colonies and plaques at 37 °C until they are 0.5 – 1.0 mm in diameter. Alternatively, the cell colonies may be grown after the membrane and appropriate cell media have been added to the plate.

Step 2: Replication. After 30 – 60 seconds, use blunt ended forceps, lift the membrane from the surface of the plate and place it colony side up on two sheets of dry filter paper (MN 218 B). Carefully place a fresh membrane over the one with the colonies, and apply gentle pressure with a replication tool or glass plate. Mark the filters in three or more asymetric locations with a sterile needle. Wrap the filters in aluminium toil and store them at –20 °C at least for two hours. Thaw the filters for 2 – 3 minutes and peel the filters apart (FAST!). Avoid smearing the colonies when separating the membranes. To amplify plasmids, transfer the membrane to an agar plate with 200 – 250 µg/ml of chloramphenicol at 37 °C for 10 hours.

4.3 Isolation

Peel the membrane and immerse it, DNA side up on filter paper prewet with 0.5 N NaOH for 5 – 7 minutes to denature the nucleic acid. Neutralize the membrane on filter paper saturated with 1 M Tris-HCl/pH 8.0 for 5 minutes. Incubate on filter paper saturated with 1 M Tris-HCl/pH 8.0, 1.5 M NaCl for 5 minutes. Wash in 2 x SSC.

4.4 Immobilization

Bake membranes until dry for 15 – 30 minutes at 80 °C. Nitrocellulose membranes must always be baked in a vacuum oven.

4.5 Hybridization

Use 5 – 10 ml of prehybridization buffer solution per membrane with gentle agitation. Prehybridize for 1 hour in a heat-sealable bag.

Prehybridization Buffer 50% formamide
5 x SSPE
1 – 3 x Denhardt's solution
0.1 – 0.5% SDS
100 – 200 µg/ml denatured DNA

Hybridize in freshly made prehybridization buffer with 1 – 5 x 10^6 cpm/ml probe, or approximately 5 – 20 ng/ml. Incubate for 12 – 20 hours at 45 °C.

4.6 Detection

Autoradiograph on X-ray film at –70 °C for 48 hours or more.

4.7 Probe Removal

See probe removal details in the appropriate sections of his protocol (See Southern and the Northern reprobing procedures on page 4).

5. Plaque Lifts

with **porablot NCP, porablot NCL** nitrocellulose membranes.

5.1 Membrane Preparation

If necessary, sterilize the membrane between two pieces of filter paper (MN 218 B) in the autoclave for 15 minutes. Allow the membrane to wet on the agar surface. Mark the membrane so that future orientation is assured.

5.2 Plaque Transfer

Step 1: Cells should be plated with phage in soft agarose and incubated at 37 °C until plaques are 0.2 mm. The plate should not show confluent lysis. Chill the plates for 30 minutes at 4 °C to allow the top agarose to harden.

Step 2: Place membrane on the plate in complete contact with the agarose and assure orientation by marking. Be careful not to trap air bubbles. The filters should be handled with gloved hands! Mark the filter (needle) and the plate (pen) in three or more asymmetric locations. Allow phage to transfer for 15 minutes. Increase the transfer time if many transfers are to be performed.

5.3 Isolation

Peel the membrane and immerse it, DNA side up on filter paper prewet with 0.5 N NaOH, 1.5 M NaCl for 5 – 7 minutes to denature the nucleic acid.

5.4 Immobilization

Bake membranes until dry for 15 – 30 minutes at 80 °C. Neutralize and incubate the membrane on filter paper saturated with 0.5 M Tris-HCl/pH 8.0, 1.5 M NaCl. Place the membrane on sheets wet with 2 x SSC and blot dry.

5.5 Hybridization

Use 5 – 10 ml of prehybridization buffer solution per membrane with gentle agitation. Prehybridize for 1 hour in a heat-sealable bag.

Prehybridization Buffer 50% formamide
5 x SSPE
1 – 3 x Denhardt's solution
0.1 – 0.5% SDS
100 – 200 µg/ml denatured DNA

Hybridize in freshly made prehybridization buffer with 1 – 5 x 10^6 cpm/ml probe, or approximately 5 – 20 ng/ml. Incubate for 12 – 20 hours at 45 °C.

5.6 Detection

Autoradiograph on X-ray film at –70 °C for 48 hours or more.

5.7 Probe Removal

See probe removal details in the appropriate sections of this protocol (See Southern and Northern reprobing procedures on page 4).

6. Western Blotting

with **porablot NCP, porablot NCL** membranes

Protocols are often provided for Western blotting onto nitrocellulose and nylon membranes, but MN recommends that most Western blot procedures be performed on **porablot NCP, porablot NCL** or **porablot PVDF** membranes to reduce the potential for high backgrounds.

6.1 Gel Preparation

Gels may be stained before transfer with Coomassie Blue, or after transfer with Fast Green, Amido Black, or any other appropriate stain. Soak the gel for 1 hour in a transfer buffer made of 25 mM Tris-HCl/pH 8.0, 0.15 M glycine, 20% methanol.

6.2 Membrane Preparation

Completely soak the membrane in deionized water, and then in transfer buffer.

6.3 Transfer

6.3.1 Electroblotting

Assemble the membrane and gel in the electroblotting unit. Place the membrane on the anode (positive) side of the gel. Transfer according to manufacturers instructions. Remove and wash thoroughly with transfer buffer.

6.3.2 Capillary Blotting

Prepare gel assembly by the method of Southern (see page 1). Transfer for 2 hours to overnight. Use transfer buffer of 10 mM Tris-HCl/pH 7.5. After the transfer step, determine transfer efficiency by staining the blot or gel by standard methods.

6.4 Blocking Procedures

Step 1: First Wash

Block the blot in PBS buffer (0.9% NaCl, 10 mM sodium phosphate/pH 7.2) containing 5% nonfat dry milk for 1 hour, with gentle agitation. Tween-20 may also be added to enhance blocking.

Step 2: Primary Antibody Binding

Remove the PBS buffer solution from blot completely. Dilute the first antibody in 50 ml of fresh PBS buffer solution. Incubate the blot in the PBS blocking buffer/antibody solution for 1 hour at 37 °C with gentle agitation. Use a ratio of 5 – 10 ml of solution to 100 cm^2 of membrane.

Step 3: Second Wash

Wash the membrane in 100 ml of fresh PBS buffer solution (without antibody) with 0.1 – 0.3% Tween-20. Agitate in a shaker for 5 minutes. Repeat the wash step 2 times. (Note: Increasing the number of short washes reduces the potential for high backgrounds).

6.5 Detection

Thoroughly remove the PBS buffer solution and overlay the blot with an anti-species (second) antibody, or with protein A (radiolabelled or enzyme linked) for 1 – 2 hours at room temperature with gentle agitation. The final concentration of radiolabelled second antibody solution should be 1 – 2 x 10^5 cpm/ml of PBS buffer solution. Enzyme-linked second antibody solutions should be made at a 1:1000 titer in PBS buffer solution. Repeat the wash step described in the procedure above.

6.6 Signal Development

The choice of signal development method is dependent on the type of probe used. Radiolabeled probes are developed and quantitated by autoradiography. Enzyme-conjugated labels (horseradish peroxidase or alkaline phosphatase) are developed and quantitated with the appropriate substrate solution.

6.7 Probe Removal (if necessary)

Do not allow the filter to become dry, or irreversible binding of the probe will result.
Wash the membrane at 60 °C for 30 minutes in 0.05 M sodium phosphate/pH 6.5, 10 M urea, 0.1 M 2-mercaptoethanol, or wash the membrane in 0.2 M glycine-HCl, 0.5 M NaCl for 5 minutes. Rinse in 0.1 M NaOH or 0.5 M Tris for 10 minutes.

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
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