GENESCREEN^{T} &
GENESCREEN PLUS® &
GENESCREEN PLUS®-NR

HYBRIDIZATION
TRANSFER MEMBRANES*
TRANSFER AND DETECTION
PROTOCOLS

For Laboratory Use
***Method of Use U.S. Patent 4,455,370**
CAUTION: A Research Chemical for Research Purposes Only

INTRODUCTION

The PerkinElmer Life Sciences line of nylon-based membranes, GeneScreen and GeneScreen Plus, have been developed to provide the highest sensitivity, performance and reproducibility. Each batch of GeneScreen and GeneScreen Plus is applications tested.

GeneScreen is a supported nylon membrane with high flexibility and strength preventing the membrane from distorting with use. The membrane is ideally suited to withstand the conditions necessary to strip and reprobe. GeneScreen has a high nucleic acid binding capacity when compared to nitrocellulose.

GeneScreenPlus is a supported, positively charged nylon membrane. The support makes GeneScreen Plus resistant to cracking and tearing. The charged surface gives it a higher nucleic acid binding capacity than neutral membranes.

This manual contains detailed protocols for the most common membrane applications. Changes in times, temperatures, pH, or buffers may be necessary depending on the characteristics of your sample.

I. DNA

A. Electrophoresis

Native double-stranded DNA is separated by electrophoresis in an agarose gel with either 1X TEN or 1X TAE buffer (See IV. D. Recipes) at a constant voltage. The voltage depends upon the distance between the electrodes in the apparatus. Consult the manufacturer's instructions for recommended voltages. The agarose concentration will depend upon the size of the fragments being separated.

Range of Separation for Different Amounts of Agarose[4]

% (w/v) Agarose	Efficient range of separation of linear DNA molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3

B. Alkaline Transfer Protocol

There are published protocols for the capillary transfer of single and double stranded DNA to nylon membranes. By using 0.4 N NaOH in place of the traditional high salt solution of the Southern[3] protocol, you can denature, transfer and fix the DNA in a single step. Denaturing the DNA leads to increased hydrophobicity and therefore results in stronger binding to the hydrophobic membrane.

Cut the membrane to exactly the same size as the gel.

Pre-wet the membrane in distilled water for a few seconds.

3 Equilibrate the membrane in 0.4 N NaOH for 10-15 minutes.

Agitate the gel in 0.25 N HCl for approximately 10 minutes.

5. Denature the DNA by soaking the gel in 0.4 N NaOH for 30 minutes.

Set up a capillary blot using 0.4 N NaOH as the transfer solution. Be sure to remove all bubbles between the filter paperwick, the gel and the membrane.

Allow the buffer to flow through the membrane by capillary action for 3-18 hrs. Change the blotting paper or add transfer buffer as necessary. Use the minimum time necessary to achieve the required sensitivity.

After transfer, wash the membrane in excess 2X SSC for 1-2 minutes.

Fix the DNA to the membrane. (See III. Fixation.)

C. Salt Transfer Protocol

Cut the GeneScreen or GeneScreen Plus to exactly the same size as the gel.

Pre-wet with distilled water for a few seconds.

Equilibrate the membrane in 10X SSC for 15 minutes.

Agitate the gel in 0.25N HCl for approximately 10 minutes.

Briefly rinse the gel with dH_20 to remove the excess HCl.

Agitate the gel in 0.4 N NaOH/0.6 M NaCl for 30 minutes.

7. Agitate the gel in 1.5 M NaCl/0.5 M Tris-HCl pH 7.5 for 30 minutes.

Set up a capillary blot using 10X SSC as the transfer solution. Be sure to remove all bubbles between the filter paper wick, the gel and the membrane.

After the transfer is complete remove the membrane from the gel and agitate the membrane in 0.4 N NaOH for 1 minute. This will denature the DNA.

10. Neutralize the membrane in 0.2 M Tris-HCI pH 7.5/1X SSC for 1 minute.

11. Fix the DNA to the membrane. (See III. Fixation.)

D. Electroblotting of DNA

Electroblotting of DNA with GeneScreen and GeneScreen Plus may be performed using any standard transfer apparatus. Always consult the equipment manufacturer's recommendations for transfer buffers and voltages.

E. DNA Dot/Slot Blot

Cut either GeneScreen or GeneScreen to the exact size of the slot/dot manifold.

Wet the membrane in 0.4 M Tris-HCl, pH 7.5 and soak for 5 minutes.

3. Place the wet membrane on the manifold and assemble according to manufacturers recommendations. The manifold should be well cleaned prior to use. We recommend cleaning all equipment with AbSolve^{T} Glassware Cleaner, Cat. No. NEF971.

4. Denature DNA for 10 minutes at room temperature in a solution of 0.25 N NaOH, 0.5 M NaCl.

5. Dilute DNA samples on ice to desired concentrations in 0.1 X SSC, 0.125 N NaOH. (200 µL samples are typical for most blotting manifolds.)

6. Load the samples into the manifold. Allow solution to remain on the membrane without suction for 30 minutes.

7. Apply a light suction to the manifold for approximately 30 seconds or until the loading buffer is drawn through the wells.

8. Carefully release the vacuum. If the vacuum is released too quickly the loading buffer may reverse flow in the manifold causing artifacts to appear on the membrane.

9. Remove the membrane from the manifold and neutralize it by washing the membrane in a solution of 0.5 N NaCl, 0.5 M Tris-HCl, pH 7.5.

10. Fix the DNA to the membrane. (See III Fixation)

F. Aqueous Hybridization Buffer for DNA

These protocols were optimized using random primer or nick-translation labeled probes. If you are using oligo probes, the times, temperatures and stringency of hybridization and washes need to be adjusted.

1. Wet membrane in 2X SSC for approximately 1 minute.

2. Place the membrane in a heat sealable bag with 50 l of prehybridization buffer per cm^2 of membrane

Prehybridization and Hybridization Buffer

8.46 mL dH_20

1 gm Dextran Sulphate, Na salt (MW 500,000)

1 mL 10% SDS

Heat at 65°C for 30 minutes.
Add 0.58 gm of NaCl and heat an additional 15 minutes.
(This buffer may be prepared in bulk and stored in the freezer)

NOTE: The use of 1X-5X Denhardt's. (See IV.D. Recipes) in the hybridization buffer is optional. It is possible to hybridize in the absence of Denhardt's without the loss of signal or increase in background.

Prehybridize for 1 hour to overnight with agitation in a 65°C water bath.

Hybridization:

Prepare 1 mL of probe solution for 10 mL of hybridization buffer.

Probe Solution

0.9 mL dH_20
0.1 mL 5 mg/mL sheared carrier DNA
(e.g., herring sperm DNA, salmon sperm DNA)
10^7 dpm of ^{32}P or ^{35}S labeled probe

Denature the probe solution by heating for 10 minutes at 95-100°C. Chill on ice for at least 15 minutes before adding to the prehybridization buffer. This will make the probe concentration in the hybridization solution ~10^6dpm/mL.
The specific activity of the labeled probe should be 10^8-10^9 dpm/µg for optimum sensitivity. The hybridization buffer should not exceed 10 ng probe/mL and should contain approximately 10^5-10^6 dpm/mL.

If ^{35}S labeled probes are used, we recommend the addition of 10 mM dithiothreitol to the prehybridization and hybridization solutions to reduce background.

5. Reseal the bag and agitate the membranes for 4 hours to overnight with agitation in a 65°C water bath.
6. Wash the membrane with excess 2X SSC for 5 minutes at room temperature. Repeat.
7. Wash at 60°C for 15-30 minutes with 2X SSC, 1.0% SDS. Repeat.
8. Wash at room temperature for 15-30 minutes with 0.1 X SSC.
9. After each wash (Steps 7 and 8) monitor the blot for background. The purpose of the wash is to remove non-specifically bound probe (or background). When the background is low enough, further washing is unnecessary and may result in removing specifically bound signal.
10. After the final rinse, take the damp membrane and wrap it securely in plastic wrap. Do not allow the membrane to dry.

Expose the blot to x-ray film. If the background is still too high, the membrane can be washed again. Once the membrane dries it will be impossible to remove non-specifically bound probe. (See IV. B. Imaging with *KODAK* X-OMAT Blue Autoradiography Film and Intensifying Screen.)

50% Formamide, Hybridization Buffer for DNA

The use of formamide will lower the melting temperature of any double stranded DNA. A 50% formamide solution will cause a
30°C drop in the T_m. Therefore hybridization is done at a much reduced temperature, usually 42°C.

1. Wet membrane in 2X SSC for approximately 1 minute.

2. Place the membrane in a heat sealable bag with 50 l of prehybridization buffer per cm^2 of membrane.

   Prehybridization Buffer

1%	SDS
2X	SSC
10%	Dextran Sulphate, Na salt (MW 500,000)
50%	deionized formamide

   NOTE: The use of 1X-5X Denhardt's in the hybridization buffer is optional. It is possible to hybridize in the absence of Denhardt's without loss of signal or increase in background.

3. Prehybridize for 30 minutes to overnight with agitation at 42-45°C.

Hybridization

Prepare 1 mL of probe solution for 10 mL of prehybridization buffer.

Probe Solution

0.9 mL dH_20

0.1 mL 5 mg/mL sheared carrier DNA
(e.g. herring sperm DNA, salmon sperm DNA)

10^7 dpm of ^{32}P or ^{35}S labeled probe

Denature the probe solution by heating for 10 minutes at 95-100°C. Chill on ice for at least 15 minutes before adding to the prehybridization buffer. This will make the probe concentration in the hybridization solution ~ 10^6 dpm/mL.
The specific activity of the labeled probe should be 10^8-10^9 dpm/ g for optimum sensitivity.

The hybridization butter should not exceed 10 ng probe/mL and should contain approximately 10^5-10^6 dpm/mL. If ^{35}S-labeled probes are used, we recommend the addition of 10 mM dithiothreitol to the prehybridization and hybridization solutions to reduce background.

5. Reseal the bag and agitate for 6 hours to overnight at 42-45°C.

Wash the membrane with excess 2X SSC for 10 minutes at room temperature.

7. Wash at the hybridization temperature for 20 minutes with 2X SSC, 1.0% SDS. Repeat.

Wash at the hybridization temperature for 20 minutes with 0.2X SSC, 1.0% SDS. Repeat.

After each wash (Steps 7 and 8) monitor the blot for background. The purpose of the wash is to remove non-specifically bound probe (or background). When the background is low enough, further washing is unnecessary and may result in removing specifically bound signal.

For ^{32}P probes, wrap the damp membrane securely in plastic wrap. Do not allow the membrane to dry. For ^{35}S probes the membrane must be dry before film exposure.

Expose the blot to x-ray film. For ^{32}P, if the background is still too high, the membrane can be washed again. Once the membrane dries it will be impossible to remove non-specifically bound probe. (See IV. B. Imaging with *KODAK* X-OMAT Blue Autoradiography Film and Intensifying Screen.)

H. Chemiluminescence Detection with Fluorescein Labeled Probes & GeneScreen Plus-NR

This is a simplified protocol for detection. For complete protocols and troubleshooting information, consult the manuals supplied with the kits.

Hybridization

1. If the membranes with the target DNA on them are dry, wet them in 5 X SSC. Add prehybridization buffer (0.1 mL/cm^2) and prehybridize at least 1 hour at the at 65°C.

   Prehybridization and Hybridization Buffer

   5 X SSC
   0. 1% (w/v) SDS
   0.5% (w/v) Blocking Reagent (supplied with kit)
   5% (w/v) Dextran Sulfate

Remove the prehybridization fluid and add fresh hybridization solution containing the denatured probe. To denature probe, boil for 3-5 minutes then chill the probe on ice for 5-10 minutes. A final probe concentration of 25 ng/mL of hybridization buffer is suggested. Sheared denatured carrier DNA should also be added at a final concentration of 50-100 g/mL. Use 0.05 mL cm^2 to 0.1 mL of hybridization buffer per cm^2 of membrane.

Hybridize overnight at 65°C in a shaking water bath. Hybridizations with long probes are usually carried out at 65°C, but this temperature should be adjusted according to the size of the probe.

4. Wash with 2X SSC, 0.1% SDS for 15 min. at 65°C followed by 0.2X SSC, 0.1% SDS for 15 minutes at 65°C. It is important in nonradiometric detection to wash with large volumes to decrease nonspecific signals. Use at least 1 mL of wash buffer per cm^2 of membrane.

Detection

1. Rinse the membrane in Buffer 1.
   Buffer 1
   0.1M Tris-HCl pH 7.5
   0.15M NaCl

Block the membrane in Buffer 2 for 1 hour at room temperature.

Buffer 2
0.5% (w/v) Blocking Reagent (supplied with kit) in Buffer 1.
Gently heat the solution until the reagent has been dissolved.
Cool to room temperature before use.

3. Remove the blocking solution and rinse the membrane briefly in Buffer 1.

Dilute the antifluorescein HRP conjugate 1:1,000 in Buffer 2. Place the membrane in the antibody conjugate solution and incubate at room temperature for 1 hour.

5. Wash the membrane 4 times for 5 minutes each in Buffer 1.

Preparation of Chemiluminescence Reagent

1. Prepare the Chemiluminescence Reagent by mixing equal volumes of the Luminol Reagent and the Oxidizing Reagent immediately before use. Use at least 0.125 mL of reagent per cm^2 of membrane.

2. Remove the membrane from the last wash solution and place in a clean incubation dish. Immediately pour the Chemiluminescence Reagent onto the membrane.

3. Gently agitate the membrane for one minute making sure the blot is completely covered with the substrate solution.

4. Remove the membrane and gently blot off excess solution on filter paper. Place the damp membrane between a polypropylene sheet protector with the black interleaf removed. Wipe any reagent from the front of the sheet protector before exposing the blot to film or the *KODAK* Image Station. Do not allow the membrane to dry.

Film Exposure

1 . Place the membrane, DNA side up, in a metal film cassette.

2. Expose the x-ray film for 5 minutes and develop.

3. Repeat the film exposure, varying the exposure time as needed for optimal sensitivity. A 2 hour exposure results in adequate sensitivity for most applications but sensitivity may be increased by allowing the exposure to go overnight.

***KODAK* Image Station Exposure**

Place the membrane, DNA side down, on the platen surface.

Close instrument lid.

Expose and repeat as necessary or use the predict feature to calculate optimal exposure time.

II. RNA

A. Electrophoresis

As with DNA, RNA will migrate at different rates in relation to its molecular weight. It is necessary to insure that the RNA does not hybridize to another RNA molecule or form secondary structures. The most common way to be sure that the RNA remains single stranded is to react the free amines of the bases with formaldehyde. The resulting Schiff base is not capable of hydrogen bonding between complementary bases, which reduces the likelihood of two strands of RNA hybridizing or forming secondary structures. Formaldehyde is the preferred denaturant because it is less toxic, less expensive and more stable than other common RNA denaturants such as glyoxal and methyl mercuric hydroxide.

Precautions

- To guard against ribonuclease contamination, all stock solutions should be sterilized prior to use. All glassware, plastic tubes and tips should be cleaned with AbSolve, Cat. No. NEF971, or autoclaved.

- Formaldehyde is TOXIC. All operations involving formaldehyde should be conducted in a fume hood.

- Formamide is an irritant and can be harmful if absorbed through the skin. Wear gloves and exercise caution.

We recommend the use of horizontal gels for this procedure.
This protocol is based on the methods of Lehrach[1] and Davis,
et al.[6]. A l% agarose gel will usually be sufficient. For mRNA smaller than 2 kb you may need a 1.5% agarose gel.

Gel Composition

1% agarose

0.66 M formaldehyde

1X MOPS Buffer (See section IV.D. Recipes)

1. Dissolve 1 gm agarose in 85 mL dH_20 and heat to boiling.

2. Cool, while stirring, to approximately 70°C.

3. Add 10 mL of 10X MOPS Buffer.

4. While in a fume hood, add 5.4 mL of 37% w/v formaldehyde to the gel solution.

5. Mix thoroughly and pour gel.

6. Allow gel to harden at least 30 minutes before use.

7. Sample Preparation:

   Loading Buffer

Formamide	720 l
10X MOPS	160 l
37% Formaldehyde	260 l
dH_20	180 l
80% glycerol	100 l
Bromophenol Blue (saturated solution)	80 l

   Make the loading buffer fresh every 1-2 weeks and discard if the color changes. You may also aliquot and store at -20°C. Dry down 10-15 g sample of total RNA [or 1-2 µg of poly(A+) RNA). Add 20 µL of loading buffer to each dry sample and denature for 2 minutes at 95°C, then cool on ice.

8. Using 1X MOPS as a running buffer, load samples and run gel until Bromophenol blue migrates three fourths of the gel. In our experience, buffer circulation during the run is not necessary when horizontal gels are used.

B. Northern Transfer of RNA

1 Soak the gel in 5 volumes of distilled water for ~5 minutes to remove the formaldehyde from the gel. Repeat 4 times.

2. Cut the GeneScreen or GeneScreen Plus to the exact size of the gel.

3. Place membrane in distilled water for a few seconds until fully hydrated

4. Soak membrane in 10X SSC or 10X SSPE buffer for 15 minutes.

5. Set up a capillary blot using 10X SSC or 10X SSPE as the transfer solution. Be sure to remove all bubbles between the filter paper wick, the gel and the membrane.

6. Transfer for 16-24 hours changing blotting paper frequently and adding more buffer as needed.

Carefully remove the filter paper without disturbing the membrane.

8. Carefully lift the membrane away from the gel with plastic forceps.

9. Rinse the membrane briefly in 2X SSPE to remove residual agarose. Place the membrane RNA side up on a piece of filter paper to dry.

10. Fix RNA to the membrane and then reverse the formaldehyde reaction. (See III Fixation.)

C. RNA Dot/Slot Blot

Precautions

- To guard against ribonuclease contamination, all stock solutions should be sterilized prior to use. All glassware, plastic tubes and tips should be cleaned with AbSolve Glassware Cleaner, Cat. No. NEF971 or autoclaved.

- Formaldehyde is TOXIC. All operations involving formaldehyde should be conducted in a fume hood.

- Formamide is an irritant and can be harmful if absorbed through the skin. Wear gloves and exercise caution.

1. Sample preparation

Combine in a sterile microcentrifuge tube:

deionized formamide	17.5 µL
37%(w/v) formaldehyde	6.0 µL
5X MOPS, pH 7.0	3.5 µL
RNA	variable

Sterile distilled water to a final volume of 35 µL.

Mix gently and incubate at 60°C for 15 minutes. Place mixture on ice and add 35 µL of TE buffer. (See IV. D. Recipes). Add l µL of Bromophenol Blue dye solution if desired. Mix well and keep on ice until ready to apply to membrane.

Cut either GeneScreen or GeneScreen Plus to the exact size of the slot/dot manifold.

3. Wet the membrane in distilled water for 15 minutes.

4. Place the wet membrane on the manifold and assemble according to manufacturers recommendations.

5. Pre-wash each well of the manifold with 100 l of TE buffer. Apply slight suction to draw buffer through the membrane.

6. Add RNA samples to manifold wells. Maximum binding occurs if solutions are allowed to remain on the membrane for 30 minutes without any applied suction. After 30 minutes, apply gentle suction to draw the solution through the membrane.

Fix the RNA to the membrane. (See III. Fixation.)

D. Hybridization Buffer for RNA

1. Prehybridize membrane in a minimum volume of prehybridization solution (approximately 50 µL/cm^2) at 42°C for 2-4 hours. This is conveniently done in a heat sealable plastic bag.

Prehybridization Solution

5X	SSPE
50%	(w/v) deionized formamide
5X	Denhardt's Solution
1%	SDS
10%	Dextran Sulphate, Na salt (MW 500,000)

Optional: 100 g/mL denatured sheared nonhomologous DNA, e.g., salmon sperm or calf thymus.

NOTE: Heat prehybridization solution to 42°C before adding to the bag containing the membrane.

If nonhomologous DNA was included in the prehybridization solution, remove the solution from the bag containing the membrane.

Add fresh hybridization solution to the bag (approximately 50 µl/cm^2 membrane). Hybridization solution is identical to prehybridization solution except nonhomologous DNA is omitted.

4. Add denatured ^{32}P or ^{35}S-labeled probe. For optimum signal to background ratio, the quantity of added probe should be determined experimentally. We recommend starting with $5x10^5$ dpm purified probe per mL hybridization solution and optimizing further if necessary.

Heat seal the bag and hybridize 16-24 hours at 42°C with gentle agitation.

NOTE: If a less stringent hybridization is desired, use a lower percentage of formamide in the prehybridization and hybridization solutions. If ^{35}S-labeled probes are used, we recommend the addition of 10 mM dithiothreitol to the prehybridization and hybridization solutions to reduce background.

Wash membrane in 200 mL of 2X SSPE at room temperature for 15 minutes. Repeat. After each wash (Steps 6-8) monitor the blot for background. The purpose of the wash is to remove non-specifically bound probe (or background).
When the background is low enough, further washing is unnecessary and may result in removing specifically bound signal.

7. Wash membrane in 400 mL of 2X SSPE, 2% SDS at 65°C for 45 minutes. Repeat.

Wash membrane in 200 mL of 0.1 X SSPE at room temperature for 15 minutes. Repeat.

9. Gently blot the excess liquid from membrane. Place membrane in plastic bag or plastic wrap and autoradiograph for desired length of time. (See IV. B. Imaging with *KODAK* X-OMAT Autoradiography Film and Intensifying Screen.)
If reprobing is desired, it is important that the membrane not be allowed to dry. Drying will cause the probe to become irreversibly bound to the membrane.

III. FIXATION

UV-crosslinking nucleic acids to nylon membranes is an easy and efficient method to ensure complete immobilization of RNA or DNA. Covalently binding nucleic acids permits higher stringency washes to strip probes or reduce background. Prehybridization can be done immediately following crosslinking. There is no need to wait 1-3 hours for the membrane to dry (Exception: Formaldehyde reversal with RNA blots).

CAUTION

ALWAYS WEAR APPROPRIATE EYE AND FACE PROTECTION WHEN USING A UV LIGHT SOURCE.

UV Crosslinking is recommended for fixing nucleic acids to GeneScreen and GeneScreen Plus.

We recommend crosslinking the membrane while wet. A dry membrane requires much less exposure than a wet membrane. DNA on completely dehydrated membranes requires a 90% lower optimal UV dose. Exposure times for dry membranes are very short and difficult to control[2].

A. UV Crosslinking of Nucleic Acids

1. After transfer is complete, rinse membrane in 2X SSPE or 2X SSC (See IV. D. Recipes) to remove residual agarose. Any agarose remaining on the membrane will lead to high auto radiographic background.

2. Place the wet membrane on a piece of wet filter paper. This will prevent the membrane from drying.

There are many commercially available instruments for UV crosslinking membranes. These are the best option for fixing nucleic acids. Follow the manufacturers' instructions.

If manual cross-linking is desired, we recommend that a meter be purchased in order to calibrate the lamp on a regular basis. Adjusting the exposure time or distance from the lamp will compensate for the natural change in energy output as the lamp ages. It is very easy to over crosslink the nucleic acids on the membrane. If this happens, the nucleic acids may not be available for hybridization and the signal will
be depressed. We have found that at 254 nm, a dose of 1,200 W/cm^2 at the surface of a wet membrane for approximately 1 minute, 40 seconds is optimal. This is equal to
1.2×10^5 $W\text{-}sec/cm^2$ or 0.12 $Joules/cm^2$
(1 Joule = 10^6 W-sec).

4. GeneScreen need not be dried before or after cross-linking. You can proceed directly to the prehybridization step or the membrane may be air-dried and stored for future use. For RNA it may be desirable to bake the membrane to reverse the formaldehyde reaction. (See III.B. Baking Membranes.)

If membrane is baked or air dried, wet in 2X SSPE or 2X SSC before proceeding to prehybridization.

B. Baking Membranes

When fixing RNA to GeneScreen Plus, there is no need to bake the membrane in order to fix the RNA to the membrane. However, it may be necessary to bake the membrane in order to reverse the formaldehyde reaction used to separate the RNA molecules. In our experience, this step is sometimes unnecessary, but we recommend including it if there are problems with high background or weak signal. Although nylon membranes are not flammable and do not require a vacuum, you may wish to use a vacuum to trap any residual formaldehyde fumes.

1. Rinse the membrane in 2X SSC or 2X SSPE to remove residual agarose. Any agarose remaining on the membrane may cause a high background.

2. Place the membrane on a clean piece of filter paper to remove excess buffer.

Bake the membrane at 80°C for 1-2 hours. While it is not required, the membranes will dry faster under a vacuum.
At this point the membranes may be stored for future use.

Wet in 2X SSC or 2X SSPE before proceeding to prehybridization.

IV. APPENDICES

Stripping DNA Probes

GeneScreen and GeneScreen Plus are the ideal membranes
for experiments requiring probe stripping and multiple rehybridizations because of their increased durability,
strength, and high binding capacity.

PerkinElmer has tested two protocols which will, for the majority of samples, remove at least 95% of ^{32}P labeled DNA probes. Due to the positive charge and high binding capacity of GeneScreen Plus, some ^{32}P probes may be difficult to strip without significant loss of target. They are most effective when the target DNA is covalently bound to GeneScreen by UV-crosslinking. If the membranes have been allowed to dry after hybridization, it may not be possible to strip the probe. Since membranes which have been probed with ^{35}S must be dried before the film exposure, it is not possible to strip ^{35}S probes.

^{32}P Labeled Probes

Method 1:
Boil membranes for 10-30 minutes in a solution of 0.015 M sodium chloride, 0.0015 M sodium citrate (0.1 X SSC), 1 % SDS.

Method 2:
Boil for 10-30 minutes in a solution of 10 mM Tris-HCI,
pH 7.5-8.0, 1 mM EDTA, 1% SDS.

Fluorescein Labeled Probes

Wash membranes for 30 minutes at the hybridization temperature in a solution of 0.2 N NaOH, 0.1% SDS.

NOTES:

- After hybridization the membrane must not be allowed to dry. If the membrane dries, even partially, the probe may be irreversibly bound.
- To keep membranes moist during the film exposure, place it in a heat sealed bag or wrap it securely in plastic wrap immediately after the final washing step. Proceed with the film exposure and stripping protocol as quickly as possible.
- If the membranes are to be stored for long periods of time before rehybridization, the probe should be stripped and the membranes stored dry.
- If the target DNA concentration is less than 5 ng/lane, the boiling time can usually be reduced to 10-20 minutes.
- If the probe is difficult to remove, the above procedures can be repeated with fresh solutions.
- For maximum removal of ^{32}P labeled probe, the SDS concentration should always be at least 1%. For nucleic acids which have been UV-crosslinked to GeneScreen, the SDS concentration can be increased to 3-5% without loss target DNA.

B. Imaging with Autoradiography Film

When using probes labeled with ^{35}S and ^{33}P PerkinElmer's EN^{3}HANCE^T Spray Surface Autoradiography Enhancer
(Cat. No. NEF970) is recommended. EN^{3}HANCE Spray significantly reduces exposure times and permits the detection of extremely low levels of radioactivity. For direct exposures, blot membrane dry with filter paper and allow to dry completely. Expose dry membrane directly to x-ray film. Since the membrane must be dried, the ^{35}S-labeled probes can not be efficiently stripped from GeneScreen and GeneScreen Plus.

When using ^{32}P labeled DNA probes the use of *KODAK* X-OMAT Blue Autoradiography Film with Kodak Intensifying Screen is recommended.

KODAK X-OMAT Blue Autoradiography Film will produce superior results with the *RENAISSANCE*® Chemiluminescence Detection Reagents and other substrate systems.

C. Methylene Blue Staining of RNA

1. After baking, briefly wet the membrane in 1 X SSPE.

Immerse the wet membrane in a solution of 0.02% methylene blue, 0.5 M Sodium Acetate, pH 5.2.

Allow membrane to sit in the staining solution for
10 minutes, or until bands become visible.

4. Destain the membrane for 15 minutes in 1 X SSPE or for
5-10 minutes in 20% ethanol. Change the solution every
few minutes as it becomes colored.

Photograph or mark the bands of interest with a pencil before proceeding to the next step. The RNA bands will appear dark blue against a lighter blue background.

6. Remove stain before beginning hybridization steps by washing the membrane in 0.2X SSPE, 1% SDS for 15 minutes at room temperature. Change solution as it becomes colored. Staining
the membrane after hybridization is not recommended since
the SDS in the wash solutions interferes with the binding of the methylene blue to the RNA.

Recipes

<u>100X Denhardt's Solution</u>

Polyvinylpyrrolidone (MW 40,000)	1 gm
Bovine Serum Albumin	1 gm
Ficoll 400	1 gm

Add distilled water to make 50 mL and sterile filter.
Store at 4°C or aliquot and store frozen.

<u>0.5 M EDTA</u>

EDTA-Na_2-$2H_20$	93.05 gm
dH_20	300 mL

Mix well and add 10 N NaOH to pH 8.0
(EDTA will not dissolve until ~pH 7)
Add distilled water to make 500 mL

<u>Formamide deionized</u>
Add 50 mL of formamide to 5 gm of a mixed-bed resin (e.g., Bio-Rad® AG 501-X8). Stir gently for 30 minutes at 4°C and filter twice through Whatman® No. 1 filter paper. For best results prepare fresh daily. Deionized formamide may also be prepared in bulk, aliquoted and stored at -20°C.

10X MOPS Buffer:

0.4 M Morpholinopropanesulfonic acid	83.7 gm
0.1 M Sodium Acetate-$3H_20$	13.6 gm
10 mM EDTA-Na_2-$2H_20$	3.72 gm

Adjust pH to 7.2 with NaOH
Add distilled water to make 1 liter

Prehybridization Buffer for ^{32}P or ^{35}S labeled DNA probes

dH_20	8.46 mL
Dextran Sulphate, Na salt (MW - 500,000)	1 gm
10% SDS	1 mL

Heat at 65°C for 30 minutes. Add 0.58 gm of NaCl and heat 15 minutes. This buffer may be prepared in bulk and stored in the freezer. If ^{35}S-labeled probes are used, add 10 mM dithiothreitol to the prehybridization and hybridization solutions to reduce background.

20X SSC

3 M NaCl	175.35 gm
0.3 M Sodium Citrate Dihydrate	88.23 gm

Add distilled water to make 1 liter

20X SSPE

3 M NaCl	175.3 gm
0.2 M NaH_2PO_4-H_20	27.6 gm
0.02 M EDTA-Na_2	
(0.5 M stock solution)	40 mL
dH_20	800 mL

Adjust pH to 7.4 with NaOH
Add distilled water to make 1 liter

40X TAE

1.6 M Tris	193.6 gm
0.8 M Sodium Acetate-$3H_20$	108.9 gm
40 mM EDTA-Na_2-$2H_20$	15.2 gm

Adjust to pH 7.2 with glacial acetic acid
Add distilled water to make 1 liter

TE Buffer
10 mM Tris-HCl, pH 7.5
1 mM EDTA, pH 8.0

10X TEN Buffer

120 mM Tris	14.54 gm
60 mM Na Acetate, anhydrous	4.92 gm
3 mM EDTA, disodium dihydrate	1.12 gm

Adjust to pH 7.5 with glacial acetic acid
Add distilled water to make 1 liter

DNA Tracking Dye

Glycerol	500 µL
0.1 M EDTA	200 µL
1% Xylene Cyanole FF	60 µL
1% Bromophenol blue	60 µL

1 M Tris-HCl (pH 7.5)	180 µL

E. References

1 Lehrach, et al., *Biochemistry*, **16**, 4743-4751 (1977).

2 Church, G. M., Gilbert, W., PNAS, **81**, 1991-1995 (1984).

3 Southern, E. M., *Journal of Molecular Biology*, **98**, 503-517 (1975).

4 Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989).

5 Reed, K. C., Mann, D. A., *Nucleic Acids Research*, **13**, 7207-7221 (1985).

6 Davis, L. G., Dibner, M. D., Battey, J. F., *Basic Methods on Molecular Biology*, Elsevier (1986).

V. ORDERING INFORMATION

GeneScreen^{T} Hybridization Transfer Membranes

NEF972	30 x 45 cm	5 Sheets
NEF983	30 cm x 3 m	1 Roll
NEF984	20 x 20 cm	10 Sheets
NEF985	22 x 22 cm	10 Sheets
NEF1018	20 cm x 3 m	1 Roll

GeneScreen Plus Hybridization Transfer Membranes/GeneScreen Plus NR

NEF976	30 x 45 cm	5 Sheets
NEF986	20 x 20 cm	10 Sheets
NEF987	22 x 22 cm	10 Sheets
NEF988	30 cm x 3 m	1 Roll
NEF993	6.4 x 22.8 cm	20 Sheets
NEE994	8.5 x 12.4 cm	20 Sheets
NEF1017	20 cm x 3 m	1 Roll
NEF-1016	30 cm x 3 m	1 Roll

KODAK X-OMAT BLUE Autoradiography Film

NEF595	100 Sheets of 14" x 17"
NEF596	100 Sheets of 8" x 10"

Nucleic Acid Products

NEL201	DNA Thunder Chemiluminescence Reagent (for 2,500 cm^2 of membrane)
NEL202	DNA Thunder Chemiluminescence Reagent (for 5,000 cm^2 of membrane)
NEL204	DNA Thunder Chemiluminescence Reagent PLUS (for 2,500 cm^2 of membrane)
NEL205	DNA Thunder Chemiluminescence Reagent PLUS (for 5,000 cm^2 of membrane)
NEL413	Fluorescein dUTP, 25 nmol
NEL414	Fluorescein UTP, 25 nmol

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