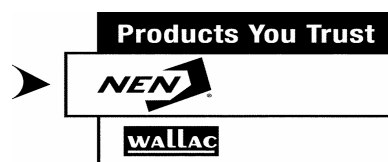


NEN Life Sciences, Inc.



**Colony/Plaque Screenä
Hybridization Transfer Membranes
Transfer and Detection Protocols
Catalog Number
NEF978/NEF978Y/ NEF978A
NEF978X/NEF990/NEF990A/
NEF993/NEF994**

Manufactured by Pall[®]

For Laboratory Use

***Method of Use U.S. Patent 4,455,370**

CAUTION: A Research Chemical for Research Purposes Only

INTRODUCTION	1
I. COLONY OR PLAQUE LIFTS	1
II. FIXATION	3
A. Air drying	3
B. Autoclave	3
III. DETECTION	4
A. Aqueous Hybridization Buffer	4
B. 50% Formamide Hybridization Buffer	6
C. Chemiluminescence Detection with Fluorescein Labeled Probes	9
IV. STRIPPING DNA PROBES	12
V. APPENDICES	12
A. Replica Plating of Bacteria	12
B. Imaging with Autoradiography Film	15
C. Recipes	16
D. References	17
VI. ORDERING INFORMATION	18

INTRODUCTION

NEN Life Sciences, Inc. Products line of nylon-based membranes have been developed to provide the highest sensitivity, performance and reproducibility.

Colony/Plaque Screen[®] are circles of GeneScreen Plus[®], a supported, positively charged nylon membrane, cut to commonly used sizes of petri plates. The support makes the membrane resistant to cracking and tearing. The optional Orientation Holes and lift tabs make these discs easy to handle and allow for accurate alignment of the master plate, membrane and autoradiograph.

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

I. COLONY OR PLAQUE LIFTS

The following procedure was developed using low density colony or plaque lift plates, i.e. 50-200 colonies or plaques per 82 mm plate. The procedure is an adaptation of the procedures used by Grunstein and Hogness(2), and Benton and Davis(3). See II-B. Autoclaving, for a protocol which combines the lysing and fixing steps.

- 1. Plates should be chilled to 4° C for 1 hour prior to lifting.**
- 2. Carefully place the dry membrane disc onto the agar plate. To prevent trapping air under the membrane, bend the membrane and place the trough of the disc on the center of the plate. There is no need to force the edges down because as the disc wets, it will flatten itself onto the agar plate. DO NOT attempt to move disc once it has made contact with the agar.**

- 3. Allow the disc to sit on the agar plate for 2-3 minutes. Mark disc position by stabbing a needle through it and the agar at appropriate positions. If Colony/Plaque Screen with Orientation Holes are used, number the holes with a ball point pen before placing on the agar plate. A sterile pasteur pipette equipped with a rubber bulb is then used to extract agar plugs from the plate through the three asymmetrically punched holes in the filter. The holes in the master plate will be used to align the plate with the autoradiograph. See V-B. Imaging with Kodak X-OMAT Blue Autoradiography Film.**
- 4. While disc is in contact with plate, lay out a sheet of plastic wrap. Pipette a 0.75 mL pool of 0.5 N NaOH onto the plastic wrap.**
- 5. Carefully remove membrane disc from plate with plastic forceps. Place disc with colony or plaque side up onto the pool of 0.5 N NaOH. Stretch plastic wrap to insure that the disc wets evenly. After 2 minutes, remove disc and place on blotting paper (colony or plaque side up).**
- 6. Repeat step 5 with a new sheet of plastic wrap and fresh 0.5 N NaOH.**
- 7. On a new sheet of plastic wrap, pipette a 0.75 mL pool of 1.0M Tris-HCl, pH 7.5. Place disc with colony or plaque side up onto the pool of buffer. After 2 minutes, remove disc and place on blotting paper (colony or plaque side up).**
- 8. Repeat step 7 with a new sheet of plastic wrap and fresh 1.0 M Tris-HCl, pH 7.5.**
- 9. Fix the DNA to the membrane. See section II. Fixation.**

II. FIXATION

A. Air drying

- 1. Place the membrane on clean filter paper to remove excess buffer.**
- 2. After excess buffer is removed, place on a piece of dry filter paper and allow the membrane to completely dry (this step may take several hours in a humid climate). At this point the membranes may be stored for future use.**
- 3. Before prehybridization, wet the membrane in 2X SSC or 2X SSPE to facilitate even wetting.**

Note: In a humid environment air drying may not always be complete. Under those circumstances baking is recommended to minimize possible loss of target nucleic acids.

B. Autoclave

The following procedure may be advantageous for researchers performing multiple lifts. Because this procedure combines the lysing and fixing steps, it may save the researcher time, effort and hands-on manipulations required by other fixing procedures.

- 1. Remove the lift from the agar plate and place nucleic acid side face up on a clean sheet of filter paper.**
- 2. After completing the desired number of lifts, cover lightly (avoid disturbing target) with a second sheet of clean filter paper and place the samples in the autoclave.**

3. Set the timer to 1 minute and the temperature to 100° C with quick or dry exhaust.
4. Remove the samples and proceed to the prehybridization.

III. DETECTION

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

A. Aqueous Hybridization Buffer

1. Wet membrane in 2X SSC for approximately 1 minute.
2. Place the membrane in a heat sealable bag with 50 mL of prehybridization buffer per cm² of membrane.

Prehybridization Buffer:

8.46 mL H₂O
1 gm Dextran Sulphate, Na salt
 (MW ~500,000)
1 mL 10% SDS
10mM dithiothreitol (only if ³⁵S is used)
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 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 1 hour to overnight with agitation in a 65° C water bath.
4. Hybridization
Prepare 1 mL of probe solution for 10 mL of hybridization buffer.

Probe Solution

0.9 mL H₂O
0.1 mL 5 mg/mL sheared carrier DNA
(e.g. herring sperm DNA, salmon sperm DNA)
10⁷ dpm ³²P or ³⁵S 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 ~10⁶dpm/mL. The specific activity of the labeled probe should be 10⁸-10⁹ dpm/mg for optimum sensitivity. The hybridization buffer should not exceed 10ng probe/mL and should contain approximately 10⁵-10⁶ dpm/mL. If ³⁵S-labeled probes are used, add 10mM dithiothreitol to the prehybridization and dehybridization solutions to reduce background.

5. Reseal the bag and agitate the membranes for 4 hours to overnight at 65° C.
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 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. Once the membrane dries, it will be impossible to remove non-specifically bound probe.
11. Expose the blot to x-ray film. If the background is still too high, the membrane can be washed again. See Appendix V-B. Imaging with Kodak X-OMAT Autoradiography Film.

B. 50% Formamide Hybridization Buffer

The use of formamide will lower the melting temperature of any double stranded DNA. A 50% formamide solution will cause an ~30° C drop in the T_m .

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

2. Place the membrane in a heat sealable bag with 50 mL of prehybridization buffer per cm² of membrane

Prehybridization Buffer

1%	SDS
2X	SSC
10%	Dextran Sulphate
50%	doionized formamide
10mM dithiothreitol (only if ³⁵ S is used)	

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 in a 42-45° C water bath.
4. Hybridization
Prepare 1 mL of probe solution for 10 mL of hybridization buffer.

Probe Solution

0.9 mL	H ₂ O
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 ~10⁶ dpm/mL. The specific activity of the labeled probe should be 10⁸-10⁹ dpm/mg for optimum sensitivity. The hybridization buffer should not exceed 10 ng probe/mL and should contain approximately 10⁵-10⁶ dpm/mL.

- 5. Reseal the bag and agitate the membranes 6 hours to overnight at 42-45° C.**
- 6. Wash the membrane with excess 2X SSC for 10 minutes at room temperature. This will remove excess hybridization buffer.**
- 7. Wash at the hybridization temperature for 20 minutes with 2X SSC, 1.0% SDS. Repeat.**
- 8. Wash at the hybridization temperature for 20 minutes with 0.2X SSC, 1.0% SDS. Repeat.**
- 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 if reprobing is desired. Once the membrane dries, it will be impossible to remove bound probe.**
- 11. Expose the blot to x-ray film. If the background is still too high, the membrane can be washed again. See Appendix V-B. Imaging with Kodak X-OMAT Autoradiography Film.**

C. Chemiluminescence Detection with Fluorescein Labeled Probes

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/cm²) 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 w/kit)
5% (w/v)	Dextran Sulfate

2. 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 50-100mg/mL. Use 0.05 ml/cm² to 0.1 mL of hybridization buffer per cm² of membrane.

3. 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 non-radiometric detection to wash with large volumes to decrease non-specific signals. Use at least 1 mL of wash buffer per cm² of membrane.

Detection

Blocking Membrane

1. Rinse the membrane in Buffer 1.
Buffer 1

0.1 M Tris-HCl pH 7.5
0.15 M NaCl

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

Buffer 2

0.5% (w/v) Blocking Reagent 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.

4. Dilute the antiluorescein HRP conjugate 1:1000 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 cm² 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. 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.

IV. STRIPPING DNA PROBES

1. Agitate the disc in ~ 100 mL of 0.4 N NaOH at 42° C for 30 minutes.
2. Agitate the disc in ~ 100 mL of 0.1 X SSC, 0.1 % SDS, 0.2 M Tris-HCl, pH 7.5, at 42° C for 30 minutes.
3. Remove the disc, place the membrane on clean filter paper to remove excess buffer, wrap in plastic and autoradiograph for an appropriate period of time to determine if sufficient probe has been removed. In our experience, this procedure removes between 90-95% of labeled probe.

V. APPENDICES

A. Replica Plating of Bacteria

There are several ways in which to replica plate bacteria. One method requires that the bacteria are first spread on the agar plate and grown overnight at 37° C. The bacteria are then lifted from the plate by using a dry Colony/Plaque Screen membrane. With the second method, the bacteria are initially grown on a membrane placed on the agar plate. Both methods are described below.

Method 1

- 1. Plate bacteria on the agar plate containing selective media and grow until they are 1-2 mm in diameter. If only a moderate number of colonies are expected, then incubating overnight is sufficient.**
- 2. Cool the plate to 4°C for 1-2 hours. Take a dry Colony/Plaque Screen membrane and lay it onto the plate containing the bacterial colonies. Press lightly to insure complete contact between the filter and the colonies, but not so heavily as to smear the colonies.**
- 3. Lift off the filter and place it colony side up on to a damp piece of filter paper. All the bacterial colonies will stick to the dry filter leaving virtually nothing on the agar plate. This filter is referred to as the master filter. If the filter is not already marked, place 3 asymmetrically spaced holes around the perimeter of the filter by stabbing it with a needle.**
- 4. Wet a new Colony/Plaque Screen membrane by placing it onto a fresh agar plate.**
- 5. Place the damp filter against the colonies of the master filter. Make matching holes in the replica filter to align colonies.**
- 6. Place filter, bacteria side up, on a fresh agar plate containing appropriate antibiotics.**
- 7. Repeat with a second replica.**
- 8. Put plates at 37°C and allow the colonies to grow until they have rounded up: 6 hours to overnight.**

Method 2

1. Place a filter on a new agar plate containing the appropriate antibiotic.
2. Dilute the bacteria to be plated onto 100 mL of Luria-Bertani Medium and pipette onto the center of the filter. Spread the bacteria to evenly cover the entire filter.

Luria-Bertani Medium(I)

bacto-trytone	10 gm
bacto-yeast extract	5 gm
NaCl	10 gm
dH ₂ O	950 mL

Shake until the solutes have dissolved. Adjust the pH to 7 with NaOH. Add dH₂O to make 1 liter. Sterilize by autoclaving.

3. Incubate overnight at 37° C.
4. Remove membrane and place colony side upon a damp piece of filter paper. Place 3 asymmetrically spaced holes around the perimeter of the filter by stabbing it with a needle.
5. Wet a Colony/Plaque Screen membrane on a new agar plate.
6. Place the damp filter against the colonies of the master filter. Make matching holes in the replica filter to align colonies.
7. Place filter, bacteria side up on a fresh agar plate containing appropriate antibiotics.
8. Repeat with a second replica.

9. Put plates at 37°C and allow the colonies to grow until they have rounded up: 6 hours to overnight.

B. Imaging with Autoradiography Film

When using probes labeled with ^3H , ^{14}C , ^{35}S , and ^{33}P , PerkinElmer EN³HANCE Spray Surface Autoradiography Enhancer (Cat. No. NEF970) is recommended. EN³HANCE Spray significantly reduces exposure times and permits the detection of extremely low levels of radioactivity. Expose dry, enhanced membrane directly to x-ray film at -70°C. For direct exposure time of ^{35}S , blot the membrane dry with paper towel and allow to dry completely. Expose the dry membrane directly to the x-ray film. Since the membrane must be dried, ^{35}S -labeled probes cannot efficiently be stripped from Colony/Plaque Screen.

When using ^{32}P labeled DNA probes, the use of Kodak X-OMAT Blue Autoradiography Film and a 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.

In all cases, the membranes may be labeled using NEN's UltEmit Autoradiography Marker (Cat. No. NEF980). This pen contains a long-decaying phosphorescent ink. It is insoluble in most hybridization buffers. It is activated (and reactivated) by ordinary room light. The UltEmit marker is extremely useful when aligning colony or plaque lifts with the autoradiograph. Discs of Colony/Plaque Screen with Orientation Holes are numbered with a ball point pen and applied to the agar plate as described in this manual. A sterile pasteur pipette equipped with a rubber bulb is then used to extract agar plugs from the plate through the three asymmetrically punched holes in the filter. Hybridization and washing of the membrane discs are performed as recommended in this manual.

When the discs dry, dots of UltEmit ink are put on the alignment holes of each disc to mark the orientation of the filter. The final autoradiogram shows the number of each disc and precisely marks the position of alignment holes on the master plate. Colonies or plaques are easily and rapidly identified by placing the autoradiogram on a light box and aligning the holes in the master plate with their corresponding signals on the x-ray film.

Positive colonies or plaques are selected by noting the corresponding position of the signal on the autoradiogram which is clearly visible through the translucent agar plate.(4)

C. Recipes

0.5 M EDTA

EDTA-Na₂-2H₂O 93.05 gm
dH₂O 300 mL

Mix well and add 10 N NaOH to pH 8.0 (EDTA will not dissolve until pH 7) Add dH₂O to make 500 ml.

20X SSPE

3 M	NaCl	175.3 gm
0.2 M	NaH ₂ PO ₄ -H ₂ O	27.6 gm
0.02 M	EDTA-Na ₂ (0.5 M stock sol'n)	40 mL
Add dH ₂ O		800 mL
Adjust pH to 7.4 with NaOH		
Add dH ₂ O to make 1 liter		

20X SSC

3 M NaCl 175.35 gm
0.3 M sodium citrate dihydrate 88.23 gm

Add distilled water to make 1 liter

D. References

- 1. Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989).**
- 2. Grunstein, M. and Hogness, D., PNAS, 72,3961 (1975).**
- 3. Benton, W. and Davis, R., Science, 196, 1980 (1977).**
- 4. Johnson, D.H., NEN[®] Research Tips #E94757.**

Colony/Plaque, Screen[®], GeneScreen Plus[®], RENAISSANCE[®], ENHANCE[®] UltEmit[®], and AbSolve[®] are trademarks of NEN Life Sciences, Inc.

Dextran Sulphate is a product of Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

VI. ORDERING INFORMATION

Colony/Plaque Screenä

NEF978	82mm	50 Discs
NEF978A	137mm	50 Discs
NEF978X	82mm	50 Discs with orientation notches and lift tabs
NEF978Y	137mm	50 Discs with orientation notches and lift tabs
NEF990A	137mm	50 Discs
NEF1010	82mm	50 Discs [Neutral CPS]
NEF1012	137mm	50 Discs [Neutral CPS]
NEF1013	132mm	50 Discs [Neutral CPS]
NEF1014	87mm	50 Discs [Neutral CPS]

Kodak X-OMAT Blue Autoradiography Film

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

RENAISSANCE Nucleic Acid Products

NEL103	Renaissance Chemiluminescence Plus
NEL104	Renaissance Chemiluminescence Plus
NEL105	Renaissance Chemiluminescence Plus
NEL201	Chemiluminescence Reagent for 2,500 cm ² of membrane
NEL202	Chemiluminescence Reagent for 5,000 cm ² of membrane
NEL413	Fluorescein dUTP, 25 nmol
NEL414	Fluorescein UTP, 25 nmol

NAME AND PLACE OF MANUFACTURE

Manufactured by Pall, for:

NEN Life Sciences, Inc.
549 Albany Street
Boston, MA 02118-2512
USA
800-551-2121, 617-482-9595
techsupport@perkinelmer.com

European Headquarters
NEN Life Sciences, Inc.
Imperlastraat 8
BE-1930 Zaventem
Belgium
techsupport.europe@perkinelmer.com

Country	Telephone
Belgium	0800 94 540
Denmark	80 88 3477
France	0800 90 77 62
Germany	0800 1 81 00 32
Italy	0800 79 03 10
Netherlands	0800 02 23 042
Norway	800 11 947
Spain	900 873 255
Sweden	020 79 07 35
Switzerland	0800 55 50 27
United Kingdom	0800 89 60 46

PC2506-0403