Membranes

INSTRUCTION MANUAL

Catalog #420100–#420105 (Duralon-UV™ Membranes),
#420106–#420108 (Nitrocellulose Membranes),
#420111–#420115 (Duralose-UV™ Membranes),
and #420130 (PVDF Membranes)
Revision #042001b

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Membranes

MATERIALS PROVIDED

Membrane	Size	Quantity	Catalog #
Duralon-UV™ membranes	20 × 20 cm	20 sheets	420100
	300 × 30-cm roll	1 roll	420101
	137-mm circles[a]	50 circles	420102
	82-mm circles[a]	50 circles	420103
	11 × 18 cm[b]	20 sheets	420104
	11 × 10 cm[b]	20 sheets	420105
Nitrocellulose Membranes	82-mm circles[a]	50 circles	420106
	137-mm circles[a]	50 circles	420107
	300 × 30-cm roll	1 roll	420108
Duralose-UV™ membranes	82-mm circles[a]	50 circles	420111
	137-mm circles[a]	50 circles	420112
	300 × 30-cm roll	1 roll	420113
	11 × 18 cm[b]	20 sheets	420114
	11 × 10 cm[b]	20 sheets	420115
PVDF Membranes	20 × 20 cm	5 sheets	420130

[a] These membrane sizes are suitable for colony and plaque lifts.

[b] These membrane sizes are suitable for transfer of DNA from agarose gels by pressure blotting using the PosiBlot® 30–30 pressure blotter and Pressure Control Station.

INTRODUCTION

Stratagene offers four distinct membranes for nucleic acid immobilization and hybridization techniques and for protein transfer: Duralon-UV™ membranes, Nitrocellulose Membranes, Duralose-UV™ membranes and Polyvinyldifluoridine (PVDF) Membranes. Each membrane has a pore size of 0.45 μm.

Revision #042001b

STRATAGENE'S MEMBRANES FOR NUCLEIC ACID TRANSFER AND HYBRIDIZATION

Nucleic acids can be bound to Stratagene's Duralon-UV membranes, Nitrocellulose Membranes and Duralose-UV membranes by the traditional 2-hour vacuum oven baking method or by rapid ultraviolet (UV) crosslinking. Being noncharged, these membranes have increased binding capacity, decreased background and can be crosslinked instead of baked. The Stratalinker® UV crosslinker covalently crosslinks nucleic acids to membranes in ~30 seconds. Each lot of these membranes is manufactured and tested to exacting specifications and is chosen for maximum signal-to-noise ratio when used in conjunction with the Stratalinker UV crosslinker. These membranes are Triton®-free to ensure excellent plaque and colony recovery.

Nitrocellulose Membranes

Nitrocellulose has traditionally been used for all standard blotting applications. Stratagene's Nitrocellulose Membranes exhibit the uniform wettability, low background, and 80 µg of RNA or DNA/cm^2 binding capacity of pure nitrocellulose transfer membranes. These membranes must be prewet first with distilled water (dH_2O) and then with transfer buffer for thorough transfer. If a membrane does not wet completely, boil the membrane briefly. Nitrocellulose can be sterilized by steam autoclaving. When baking Nitrocellulose Membranes, use a *vacuum* oven. Ultraviolet crosslink only *damp* membranes, as nitrocellulose is highly flammable. Reprobing of nitrocellulose can be difficult due to its inherent fragility (fragility increases after baking).

Cleanly cut the nitrocellulose with a razor or similarly sharp instrument to avoid nicks and tears that will eventually enlarge. Avoid handling Nitrocellulose Membranes with bare hands, as the proteins and oils associated with fingerprints interfere with the proper wetting of the membrane and may increase the background after hybridization. Alkaline transfers cannot be performed using Nitrocellulose Membranes. It is best to choose nitrocellulose for experiments requiring low background from a single probing. Either side of Stratagene's Nitrocellulose Membranes can be used. For best retention of nucleic acid during blotting, use 20× SSC buffer (see *Preparation of Media and Reagents*) as a transfer medium.

Nitrocellulose Membranes should be stored at a constant temperature and in a dark, cool place devoid of solvent vapors to prevent these membranes from drying out and becoming brittle. Nitrocellulose has wetting agents that could dry out if stored at room temperature in dry climates. A 4°C cold room is a convenient way to maintain constant temperature and humidity.

Note *Nitrocellulose Membranes cannot be used with the Illuminator™ nonradioactive detection system or with alkaline transfers.*

Duralose-UV Membranes

Duralose-UV membranes are reinforced nitrocellulose membranes that have the tensile strength and reliability of nylon membranes, while retaining the lower background generally observed with nitrocellulose membranes. Each lot of Duralose-UV membranes binds 80–100 µg of DNA or RNA/cm^2 and is 500× stronger than ordinary nitrocellulose membranes. These supported nitrocellulose membranes allow several reprobings and do not require the extensive blocking necessary with nylon membranes for visualization of colorimetric reactions. As with conventional nitrocellulose, alkaline transfer methods cannot be used with Duralose-UV membranes, and gloves should be worn when handling these membranes. For best retention of nucleic acids during blotting use 20× SSC buffer as a transfer medium.

Note *Duralose-UV membranes cannot be used with the Illuminator nonradioactive detection system or with alkaline transfers.*

Duralon-UV Membranes

Duralon-UV membranes are noncharged nylon membranes that exhibit exceptional strength, thermal stability and increased sensitivity in comparison with conventional nitrocellulose membranes. Each lot of Duralon-UV membranes will bind at least 500 µg of DNA or RNA/cm^2 (compared to 80 µg/cm^2 characteristic of nitrocellulose membranes), including fragments as small as 10 bp, and can be reprobed several times. These easy-to-handle membranes should not tear, crack, shrink or curl whether wet or dry and may be stored at room temperature. Completely cut through the polymer supporting threads before separating a measured piece from the remainder of the Duralon-UV membrane sheet.

Baking or UV crosslinking is required for fixing the nucleic acids onto these noncharged membranes. Duralon-UV membranes can be used in alkaline transfer systems. The high binding capacity may increase the background after probing. Duralon-UV membranes are an ideal choice for hybridization of low-copy-number sequences, unusually small fragments or when multiple probings are necessary.

STRATAGENE'S NUCLEIC ACID HYBRIDIZATION SOLUTION

QuikHyb® Hybridization Solution

QuikHyb® hybridization solution is a rapid hybridization solution developed at Stratagene. This unique hybridization solution reduces the time required for nucleic acid hybridization down to 1–2 hours. QuikHyb hybridization solution can be used for Northern, Southern and slot blots and is compatible with any conventional membrane used for nucleic acid transfer.

STRATAGENE'S MEMBRANES FOR PROTEIN TRANSFER

PVDF Membranes

PVDF membranes are a practical alternative to traditional nitrocellulose membranes in protein transfer and Western blotting applications. These membranes exhibit stronger signals, higher signal-to-noise ratios, greater mechanical strength and a higher protein binding capacity of 125 µg/cm^2. In addition, these membranes are chemically stable, allowing use of a wide range of solvents for rapid destaining, and are compatible for use in conventional protein staining and immunoblotting protocols.

SOUTHERN BLOTTING

Hybridization of Long DNA Probes Using Stratagene's Nucleic Acid Transfer and Hybridization Membranes

Transfer of the Target DNA onto the Membrane

1. Electrophorese the DNA samples in an agarose gel, stain the gel with ethidium bromide (EtBr) and photograph under UV light with a ruler for later reference.

2. Depurinate the DNA by soaking the gel in 0.25 N HCl for 5–15 minutes, depending on the thickness of the gel (e.g., 5 minutes for a 3-mm-thick gel and 15 minutes for a 4- to 6-mm-thick gel). This fragments the DNA and facilitates transfer of DNA through the gel and onto the membrane and is especially important for the transfer of large DNA fragments.

3. Rinse the gel in dH_2O *to complete the depurination process* and denature the DNA by soaking the gel in a solution of 1.5 M NaCl and 0.5 M NaOH for 15–30 minutes.

4. Neutralize by soaking the gel in a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 15–30 minutes.

5. Briefly soak the gel in transfer buffer [e.g., either 10–20× SSPE (see *Preparation of Media and Reagents*), 10–20× SSC buffer or 25 mM sodium phosphate].

   Note *For Nitrocellulose Membranes or Duralose-UV membranes, use 20× SSC buffer as the transfer buffer to obtain maximum DNA retention.*

6. Prewet the membrane in dH_2O and then in transfer buffer. If a portion of the membrane resists wetting, pour boiling dH_2O over the membrane and rock gently.

7. Transfer DNA to the membrane by traditional capillary blotting[1] or by pressure blotting using Stratagene's PosiBlot 30–30 pressure blotter and Pressure Control Station.

8. After the transfer is complete, mark the location of the gel wells on the membrane and then, after re-staining the gel in EtBr, check the gel under UV light to determine if any DNA has failed to transfer.

9. Either bake the *damp* membrane under vacuum for 2 hours at 80°C or UV crosslink the transferred DNA to the membrane using the Stratalinker UV crosslinker, which takes ~30 seconds.

10. Place the membrane in fresh transfer buffer to gently remove any agarose gel residue and then on blotting paper to carefully blot off any excess transfer buffer.

For nylon membranes, the blot may be rinsed briefly in dH_2O to remove excess salt and any fragments of agarose debris.

Prehybridization of the Membrane for Use with Long DNA Probes

Note *Stratagene recommends using QuikHyb hybridization solution for performing the prehybridization step for long DNA probes. When using QuikHyb hybridization solution, follow the protocols outlined in Stratagene's* QuikHyb® Hybridization Solution Instruction Manual.

The prehybridization and hybridization solutions are identical for long DNA probes. A prehybridization step greatly reduces nonspecific probe hybridization (background or noise) and is particularly important for high-binding-capacity membranes such as Duralon-UV membranes. The reagents should be of the highest quality and the formamide should be properly deionized to ensure low backgrounds.

Conventional prehybridization and hybridization solutions[2] or either of the following prehybridization and hybridization solutions may be used.

Prehybridization and Hybridization Solution A

2× 1,4-piperazine-diethanesulfonic acid (PIPES) buffer[§]
50% deionized formamide
0.5% (w/v) sodium dodecyl sulfate (SDS)[‖]
100 µg/ml of sonicated or sheared, denatured salmon sperm DNA[#]

Prehybridization and Hybridization Solution B

6× SSC buffer or 6× SSPE buffer
5× Denhardt's reagent[§]
0.5% (w/v) SDS[‖]
100 µg/ml of sonicated or sheared, denatured salmon sperm DNA[#]
50% deionized formamide (optional)

> **Note** *Prehybridize the Duralose-UV membranes or the Nitrocellulose Membranes in 0.05 ml of prehybridization solution/cm². Prehybridize the Duralon-UV membranes in 0.25 ml of prehybridization solution/cm².*

1. Combine all the reagents except the salmon sperm DNA and warm the prehybridization solution to ~50°C. Denature the salmon sperm DNA by boiling for 5 minutes and add the salmon sperm DNA to the warmed prehybridization solution.

2. Seal the crosslinked membrane with the prehybridization solution in a heat-sealable hybridization bag, minimizing trapped air bubbles. Massage the hybridization bag to completely wet the membrane. Alternatively, the membranes can be incubated in hybridization tubes or bottles instead of in heat-sealable hybridization bags if using a hybridization oven.

3. Incubate the hybridization bag at 42°C (if using a prehybridization solution that contains deionized formamide) or at 68°C (if using a prehybridization solution that does not contain deionized formamide) for at least 1 hour with constant agitation. The prehybridization temperature should always equal the hybridization temperature (see *Hybridization of Radiolabeled DNA Probes*).

[§] See *Preparation of Media and Reagents*

[‖] For Duralon-UV membranes, increase the SDS in the prehybridization and hybridization solution A to 1% (w/v).

[#] The salmon sperm DNA serves as a blocking agent for possible nonspecific probe binding sites and will function only as such when it is sonicated or sheared and then denatured.

Hybridization of Radiolabeled DNA Probes

During the prehybridization period, a DNA fragment probe may be generated by nick translation or by random primed synthesis. Freshly labeled probes result in the lowest background. The DNA to be labeled should be free of contaminating DNA, RNA and proteins. After radiolabeling of the probe, unincorporated radiolabel should be removed to provide the highest hybridization signal-to-background ratio. Excess radionucleotides are efficiently removed from labeled probes using Stratagene's NucTrap® probe purification columns and Push Column Beta Shield Device.

A reasonable specific activity for the probe is $\geq 10^8$ dpm/µg for nick translation protocols and $\geq 10^9$ dpm/µg for random oligonucleotide labeling procedures. For most applications, 1×10^6–5×10^6 cpm/ml of labeled probe may be required.

1. Double-stranded DNA (dsDNA) must be denatured to serve effectively as a probe; therefore, boil the probe for 5 minutes with the salmon sperm DNA and then add the probe directly to the prehybridized membrane without removing the prehybridization solution. Alternatively, the prehybridization solution can be poured off and the denatured probe and salmon sperm DNA can be added to fresh, warmed hybridization solution and then added to the blot.

2. Massage the hybridization bag to distribute the probe evenly.

3. Hybridize with constant agitation for 12–18 hours at the following temperatures:

Probe-to-target sequence homologies	Hybridization temperatures	
	For hybridization solutions with 50% deionized formamide	For aqueous hybridization solutions without 50% deionized formamide
95–100%	42°C	68°C
90–95%	37°C	68°C
85–90%	32°C	68°C

If the homology is <85%, refer to Reference 1.

Post-Hybridization Washes of Long DNA Probes

1. Pour off the hybridization solution into a suitable liquid radioactive waste container. Wash the blot once for 10 minutes at room temperature in a small volume of a 1× SSC buffer and 0.1% (w/v) SDS wash solution to remove any probe not hybridized to the blotted DNA and to remove the hybridization solution.

2. Wash the membrane as indicated below:

High-Stringency Washes

For 95–100% homology between the probe and the target, wash three times for 15 minutes each in a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution at 55–65°C.

Medium-Stringency Washes

For 90–95% homology, wash three times for 15 minutes each in a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution at 40–50°C.

Low-Stringency Washes

For 85–90% homology, wash three times for 15 minutes each in a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution at room temperature to 37°C.

3. When the washes are complete, briefly blot the membrane on blotting paper and either seal the damp (not wet) membrane in a heat-sealable hybridization bag or in plastic wrap. Expose the membrane to autoradiographic film at –80°C in an X-ray cassette with an intensifying screen. Exposure time is determined principally by the amount of target DNA on the blot, the specific activity of the probe and the stringency of the post-hybridization washes.

Note that exposure of the hybridized and washed membrane while damp allows for more stringent washes to be performed later, if necessary, and allows for the probe to be stripped from the blot for hybridization with another probe.

Hybridization of Oligonucleotide Probes Using Stratagene's Nucleic Acid Transfer and Hybridization Membranes

Transfer of the DNA onto the Membrane

Treat the DNA within the gel and blot the DNA from the gel onto the chosen membrane as indicated previously in *Transfer of the Target DNA onto the Membrane*.

Prehybridization of Southern Blots for Oligonucleotide Probes

Note *Stratagene recommends using QuikHyb hybridization solution for performing the prehybridization step for oligonucleotide probes. When using QuikHyb hybridization solution, follow the protocols outlined in Stratagene's* QuikHyb® Hybridization Solution Instruction Manual.

Prehybridize the membrane in the following prehybridization solution at 0.05–0.1 ml/cm^2 for 1–2 hours at 42°C with constant agitation in a heat-sealable hybridization bag:

6× SSC buffer
5× Denhardt's reagent
20 mM NaH_2PO_4
500 µg/ml of sheared or sonicated, denatured salmon sperm DNA (see *Prehybridization of the Membrane for Use with Long DNA Probes*)

Hybridization of Oligonucleotide Probes to Southern Blots

During the prehybridization period, an oligonucleotide probe may be produced using T4 polynucleotide kinase (PNK) and high-specific-activity [γ-^{32}P]ATP (>3000 Ci/mmol). Stratagene offers a KinAce-It™ kinasing kit. Unincorporated radiolabel must be removed from the probe prior to hybridization (see *Hybridization of Radiolabeled DNA Probes*). It is not necessary to denature the oligonucleotide probes. An acceptable specific activity for an oligonucleotide probe is $\geq 10^9$ cpm/µg. The oligonucleotide probe concentration should be at least 0.5×10^6 cpm/ml.

1. Pour off the prehybridization solution and add the probe to the hybridization bag with the minimum volume of the following hybridization solution:

   6× SSC buffer
   20 mM NaH_2PO_4
   0.4% (w/v) SDS[‖]
   500 µg/ml of sonicated or sheared, denatured salmon sperm DNA (see *Prehybridization of the Membrane for Use with Long DNA Probes*)

2. Massage the hybridization bag to evenly distribute the probe. Incubate 12–18 hours at 42°C with constant agitation.

[‖] The SDS concentration can be increased to 1% (w/v) for Duralon-UV membranes.

Post-Hybridization Washes of Oligonucleotide Probes

Calculate the approximate melting temperature (T_m) of the oligonucleotide probe ≤18 bases in length by using the following formula[2]:

Note *The first method below overestimates the* T_m *of hybrids involving longer nucleotides. The second formula works only for* Na^+ *concentrations of ≤1 M.*

Oligonucleotides Shorter than 18 Bases

$$T_m = 2°C(A + T) + 4°C(G + C)$$

Oligonucleotides 14 Bases and Longer (up to 60–70 Nucleotides)

$$T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) – (600/N)$$

where N is the chain length.

1. Pour the hybridization solution into a suitable liquid radioactive waste container.

2. Wash the membrane once at room temperature for 10 minutes with a 6× SSC buffer and 0.1% (w/v) SDS wash solution to remove any unbound probe and the hybridization solution.

3. Wash the membrane twice for 15 minutes each at a temperature 10°C below the calculated T_m.

4. Wash the membrane several times for 15 minutes each at a temperature 5°C below the calculated T_m, if necessary, in a 6× SSC buffer and 0.1% (w/v) SDS wash solution.

5. Blot any excess solution off the membrane with blotting paper and expose the damp membrane for 1–24 hours at –80°C as described in the *Post-Hybridization Washes of Long DNA Probes*.

Note that only the damp membranes can be rewashed or stripped of the original probe and rehybridized to a new probe. Once a membrane has dried following hybridization, the probe is irreversibly bound to the membrane.

Stripping of Probes for Reuse Using Stratagene's Nucleic Acid Transfer and Hybridization Membranes

The probes can be stripped off the Southern blots only if the membranes have been kept damp.

Duralon-UV Membranes

For Duralon-UV membranes, use **one** of the three methods listed below to remove the residual probe.

1. Wash the membrane for 30 minutes at 65°C in the following wash solution:

   50% formamide
   6× SSPE buffer or 6× SSC buffer

2. Pour a boiling solution of 0.1× SSC buffer and 0.1% (w/v) SDS or a boiling solution of 0.1× SSPE buffer and 0.1% (w/v) SDS over the Southern blot and wash the membrane for 15 minutes (after removing the membrane from heat). Repeat several times if necessary.

3. Wash the membrane for 15 minutes at 65°C in dH_2O.

Duralose-UV Membranes

For Duralose-UV membranes, use **one** of the four methods listed below to remove the residual probe.

1. Boil the membrane in dH_2O for 5 minutes.

2. Wash the membrane for 1 hour at 90–95°C in the following wash solution:

   0.5× Denhardt's reagent
   25 mM Tris-HCl (pH 7.5)
   0.1% (w/v) SDS

3. Wash the membrane for 15 minutes at 65°C in dH_2O.

4. Pour a boiling solution of 0.1× SSC buffer and 0.1% (w/v) SDS or a boiling solution of 0.1× SSPE buffer and 0.1% (w/v) SDS over the Southern blot and wash the membrane for 15 minutes (after removing the membrane from heat). Repeat several times if necessary.

Nitrocellulose Membranes

For Nitrocellulose Membranes, pour a boiling solution of 0.1× SSPE buffer and 0.1% (w/v) SDS or a boiling solution of 0.1× SSC buffer and 0.1% (w/v) SDS over the Southern blot and wash the membrane for 15 minutes (after removing the membrane from heat). Repeat several times if necessary.

Check the Southern blot with a Geiger counter for residual radioactivity. Blot off any excess buffer, seal the damp membrane in plastic wrap or in a heat-sealable hybridization bag and expose the hybridization bag to autoradiographic film.

To reprobe the stripped Southern blot, the investigator must again prehybridize the Southern blot as described in the following sections: *Prehybridization of the Membrane for Use with Long DNA Probes* and *Prehybridization of Southern Blots for Oligonucleotide Probes*.

NORTHERN BLOTTING

Transfer of RNA onto the Membrane

To allow a more complete transfer for Northern blots, it is important to either run a vertical gel or a thin horizontal gel.

Pretreatment of Formaldehyde Gels Prior to Northern Transfer

1. Following electrophoresis, stain the formaldehyde gel in EtBr (5 µg/ml) and then destain the formaldehyde gel in deionized water. Photograph the formaldehyde gel under UV light with a ruler for later reference.

2. If the Northern blot will be probed for transcripts ≤~2 kb, rinse the formaldehyde gel in two changes of transfer buffer (10–20× SSC buffer) to remove the formaldehyde.

3. If the Northern blot will be probed for transcripts >~2 kb or if the formaldehyde gel is >1% agarose, perform the following pretreatments:

    a. Soak the formaldehyde gel in a 0.05 N NaOH and 0.15 M NaCl solution for 20–30 minutes.

    b. Neutralize the formaldehyde gel in a 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl solution for 30 minutes.

    If using a vertical gel apparatus (1.5- to 3-mm-thick gels), the soaking and neutralizing steps may be performed for 15 minutes each.

4. Prewet the membrane first in dH_2O and then in transfer buffer (if using Nitrocellulose Membranes or Duralose-UV membranes, blot in 20× SSC buffer). Never submerge a dry membrane in SSC buffer, since this may cause uneven wetting and blemishes in the membrane.

5. Using 10× SSC buffer, transfer the RNA to a solid support by traditional capillary method or by pressure blotting using the PosiBlot 30–30 pressure blotter and Pressure Control Station. When blotting onto Nitrocellulose Membranes or Duralose-UV membranes, use 20× SSC buffer as the transfer buffer.

6. After transfer, mark the location of the wells on the membrane. Place the membrane on blotting paper to remove any excess buffer. Either bake the *damp* membrane under vacuum for 2 hours at 80°C or UV crosslink the transferred RNA to the membrane using the Stratalinker UV crosslinker.

For nylon membranes, the blot may be rinsed briefly in dH_2O to remove excess salt and any fragments of agarose debris.

Prehybridization of Northern Blots

Note *Stratagene recommends using QuikHyb hybridization solution for performing the prehybridization step. When using QuikHyb hybridization solution, follow the protocols outlined in Stratagene's* QuikHyb® Hybridization Solution Instruction Manual.

Using 0.1–0.5 ml/cm^2, prehybridize the membrane in the following solution for ~1 hour at 42°C with constant agitation in a heat-sealable hybridization bag:

50% deionized formamide
10% dextran sulfate
1% (w/v) SDS
1 M NaCl
100 µg/ml of denatured, sonicated salmon sperm DNA

Hybridization of Northern Blots

The hybridization solution is the same as the prehybridization solution listed in the previous section. The probe concentration should be 5×10^5–5×10^6 cpm/ml. Incubate the hybridization bag overnight at 42°C.

Post-Hybridization Washes of Northern Blots

1. Pour the hybridization mixture into a suitable liquid radioactive waste container.

2. Wash the membrane one time at room temperature for 15 minutes with a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution to remove any unbound probe and the hybridization solution.

3. Wash the membrane according to the suggestions in step 2 of *Post-Hybridization Washes of Long DNA Probes* for high-, medium- and low-stringency washes.

4. Blot any excess solution off the membrane with blotting paper and expose the damp membrane with an intensifying screen overnight at –80°C.

SCREENING

Plaque Lifts

Plating the Phage

1. The agar plates used to plate the phage should be ~2 days old for best results. If only freshly poured plates are available, dry the plates out in a 37°C incubator for several hours with the lids askew.

2. Determine the titer of the phage stock before plating for plaque lifts. Avoid confluent plates of plaques (>50,000 pfu/150-mm plate). Ideally, individual plaques should be discernible.

   Include a lift from a blank plate (plaque free, only top agar and plating cells) for each hybridization bag.

3. Add 48–50°C top agar to the phage preabsorbed to fresh plating cells, roll the tube back and forth between the palms of the hands gently but quickly to mix and pour the top agar onto an agar plate on a level surface. Avoid vortexing or other methods that introduce air bubbles into the solution. Let the plates set for 20 minutes.

4. Incubate the inverted plates for 8–16 hours at 37°C. The size of the plaques obtained is phage and/or host dependent.

Lifting the Plaques

Up to four plaque lifts per plate are possible. Always handle the membranes with gloved hands.

1. After incubation, shake off the condensation from the plate lids if necessary and chill the plates inverted at 4°C for 2 hours. This will help prevent the top agar from sticking to the membranes.

2. Prior to performing plaque lifts, label the membrane of choice with a pen. Label each membrane with the plate number and a designation of the order of the lift from each plate. For example, if three membranes are to be lifted from plate one, label the membranes 1A, 1B, and 1C, respectively. To ensure future orientation, mark the first membrane to be lifted with a series of asymmetrically placed dots ~5 mm from the membrane edge. For example, if the circular membrane was a clock face, one dot would be marked between one and three o'clock, two dots would be marked between five and seven o'clock and three dots would be marked between nine and twelve o'clock.

3. Place the first membrane onto the surface of the plate without trapping air bubbles. Gently curve the membrane in half and touch its center to the center (line of diameter) of the plate and allow the wetting action to pull the remainder of the membrane onto the plate. Once the membrane is thoroughly wet, punch holes through the pen-marked dots and into the agar with a 16- or 18-gauge syringe needle.

4. Hold the plate up to a light and, on the underside of the plate, use a lab marker to note the position of the needle holes.

5. After 2 minutes, carefully remove the first membrane with forceps and place the membrane into the denaturing solution (see *Treating the Plaque-Lift Membranes*).

6. Lay the next membrane carefully onto the top agar and let the membrane wet. Again hold the plate up to a light (membrane side forward) and use the lab maker dots as a guide to repeat the needle holes through the membrane. Remove the membrane after 5 minutes and immerse the membrane in denaturing solution.

   Repeat for one or two more membranes as necessary. If the top agar sticks, try lifting the membrane from the plate in a different area or try chilling the plates at 4°C before proceeding with duplicate lifts.

Treating the Plaque-Lift Membranes

1. Denature each membrane for 2 minutes at room temperature by submerging the membranes in a denaturing solution of 1.5 M NaCl and 0.5 M NaOH. Gently rub off any clinging agar residue from the membranes with gloved fingertips. Residual agar will contribute to the background. Separate the membranes so that the entire surface is exposed to the denaturing solution.

2. Neutralize the membranes for 5 minutes by submerging the membranes in a neutralizing solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) at room temperature. Do not allow the membranes to stick together.

3. Rinse the membranes briefly in a solution of 0.2 M Tris-HCl (pH 7.5) and 2× SSC buffer at room temperature.

4. Blot the membranes on blotting paper and bake the membranes between sheets of blotting paper for 2 hours at 80°C under vacuum or UV crosslink the damp membranes in the Stratalinker UV crosslinker (see *Transfer of the Target DNA onto the Membrane*).

5. Cover the master plates with Parafilm® laboratory film and store the inverted master plates at 4°C for subsequent phage isolation.

Prehybridization of Plaque-Lift Membranes

For long DNA probes, use hybridization solution A from *Prehybridization of the Membrane for Use with Long DNA Probes*. For oligonucleotide probes, use the prehybridization solution from *Prehybridization of Southern Blots for Oligonucleotide Probes*.

Prehybridize with 2–3 ml of solution/137-mm membrane and 0.8–1.2 ml/87-mm membrane. Membranes that will be hybridized with the same probe (up to 20) may be placed in the same heat-sealable hybridization bag. To ensure that all the membranes are thoroughly wet, individually wet each membrane in a tray of prehybridization solution before placing the membrane in the hybridization bag. Squeeze out any air bubbles between the membranes before sealing the hybridization bag. Prehybridize the membranes for 2 hours at the appropriate hybridization temperature (see *Hybridization of Radiolabeled DNA Probes* or *Hybridization of Oligonucleotide Probes to Southern Blots*).

Hybridization of Plaque-Lift Membranes

Refer to *Hybridization of Radiolabeled DNA Probes* or *Hybridization of Oligonucleotide Probes to Southern Blots* for the solutions and probes. For long-stranded probes, use ~0.2 × 10^6 cpm/filter and, for oligonucleotide probes, use 1 × 10^6–1 × 10^7 cpm/filter.

Post-Hybridization Washes of Plaque-Lift Membranes

Refer to the *Post-Hybridization Washes of Long DNA Probes* and *Post-Hybridization Washes of Oligonucleotide Probes* for the post-hybridization procedure.

Colony Lifts

Note that colony screening should be performed on duplicate sets of membranes. This protocol is used to make multiple replica plates of transformations. The original or master membrane can be saved to pick colonies which have been identified by the screening of replica membranes.

Plating of Transformed Bacterial Cells

1. Spread the transformants in liquid culture directly onto Nitrocellulose Membranes already positioned on LB agar plates (see *Preparation of Media and Reagents*) containing an appropriate antibiotic. Approximately 200 µl of liquid culture/137-mm membrane and ~100 µl of liquid culture/82-mm membrane is adequate. Leave a border at the edge of the membrane, which is free of bacteria.

2. Incubate the plates at 37°C until the colonies are 0.5–1 mm in diameter. The plates should not be confluent with colonies; ideally, individual colonies should be discernible. To minimize the background, the colonies must be ≤1 mm in diameter. These membranes will serve as the master membranes.

Replicating the Membranes

1. Remove the master membranes and place the membrane colony side up on blotting paper.

2. Carefully place a second Nitrocellulose Membrane that has been prewet on an LB agar plate containing an appropriate antibiotic onto the master membrane. Mark the membrane with a needle as described in step 2 of *Lifting the Plaques*, after applying gentle pressure with a weighed glass plate or replicating tool.

3. Carefully peel the membranes apart to avoid smearing the colonies and place the replica membrane colony side up on an LB agar plate containing an appropriate antibiotic. Repeat this procedure with a second prewetted membrane.

4. Store the master membrane inverted at 4°C on an LB agar plate containing an appropriate antibiotic with the colony side up. It may be necessary to incubate the master membrane on a fresh LB agar plate containing an appropriate antibiotic for 3–4 hours to regenerate the colonies.

Growing the Replicated Colonies

Incubate the replicated membranes on an LB agar plate containing an appropriate antibiotic at 37°C until the colonies are 1 mm in diameter.

For low-copy-number plasmids or for plasmids containing inserts >10 kb, grow the replica membranes on LB agar plates containing chloramphenicol (0.15 µg/ml) (see *Preparation of Media and Reagents*) overnight. This may double the colony size. Chloramphenicol will amplify the plasmids within the cells. This step is unnecessary with the pBluescript® phagemid, since the pBluescript phagemid has a high copy number.

Treating the Replica Membranes

1. Prepare three trays each containing several sheets of blotting paper. Saturate the trays with the following solutions. Avoid excess fluid.

   Tray 1
   A solution of 0.5 M NaOH
   Tray 2
   A solution of 1 M Tris-HCl (pH 7.5)
   Tray 3
   A solution of 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl

2. Place the replica membranes colony side up on the wet blotting paper in Tray 1 for 5 minutes and then blot the membranes on dry blotting paper colony side up for 5 minutes.

3. Place the replica membranes onto the blotting paper in Tray 2 for 2 minutes and then blot the membranes dry for 2 minutes. Place the replica membranes into Tray 3 for 10–15 minutes and then blot the membranes dry for 2 minutes.

4. Either UV crosslink the DNA for ~30 seconds or bake the membranes between pieces of blotting paper at 80°C for 1.5–2 hours to fix the DNA to the membrane.

5. Prehybridize and then hybridize the membrane as outlined in *Hybridization of Long DNA Probes Using Stratagene's Nucleic Acid Transfer and Hybridization Membranes* for long DNA fragment probes and *Hybridization of Oligonucleotide Probes Using Stratagene's Nucleic Acid Transfer and Hybridization Membranes* for oligonucleotide probes.

Post-Hybridization Washes of Colony Lifts

Refer to *Post-Hybridization Washes of Long DNA Probes* and *Post-Hybridization Washes of Oligonucleotide Probes* for the post-hybridization wash procedure.

WESTERN BLOTTING

Transfer of Proteins from SDS–Polyacrylamide Gels to PVDF Membranes

Transfer of proteins from SDS–polyacrylamide gels to a solid support such as the PVDF Membranes can be achieved using one of the following methods: tank electroblotting or semidry electroblotting (see *Tank Electroblotting* and *Semidry Electroblotting*).

Tank Electroblotting

Note *Wear gloves at all times when handling the SDS–polyacrylamide gel, Whatman® 3MM paper and PVDF Membranes. Oils and secretions from the skin may prevent the transfer of proteins from the gel to the membrane.*

1. Following electrophoresis, place the SDS–polyacrylamide gel in transfer buffer (see *Preparation of Media and Reagents*) and allow the gel to equilibrate for 15–20 minutes prior to blotting.

2. Cut two pieces of Whatman 3MM paper and one piece of the PVDF Membrane to the exact size of the SDS–polyacrylamide gel. If the Whatman 3MM paper and the membrane are larger than the gel, the overhanging edges of the Whatman 3MM paper and the membrane may touch, possibly preventing the transfer of the protein from the gel.

3. Soak the two pieces of Whatman 3MM paper in a shallow tray containing a small amount of transfer buffer.

4. Prewet the PVDF Membrane in 5–10 ml of 100% (v/v) methanol for 5 seconds and then soak the membrane in 500 ml of dH_2O for 5 minutes to remove the methanol.

5. Equilibrate the PVDF Membrane with 500 ml of transfer buffer for 10–15 minutes prior to use in blotting.

   Note *The PVDF Membrane must remain wet at all times. If the PVDF Membrane dries out, rewet the membrane in methanol and dH_2O as described in step 4 above and proceed with the protein transfer.*

6. Assemble the transfer cassette as follows:

   a. Lay the bottom half of a plastic cassette flat on the laboratory bench.

   b. Place one piece of moistened Whatman 3MM paper from step 3 above onto the plastic cassette. Using a glass pipet as a roller, squeeze out any air bubbles.

c. Remove the glass plates holding the SDS–polyacrylamide gel from the electrophoresis tank. Carefully position the gel exactly on top of the wet Whatman 3MM paper. Remove any trapped air bubbles using a gloved hand.

d. Place the wet PVDF Membrane from step 4 above onto the gel. Again make sure that the PVDF Membrane is exactly aligned and that no air bubbles are trapped between the membrane and the gel.

e. Overlay the second sheet of moistened Whatman 3MM paper from step 3 above exactly on top of the PVDF Membrane. Remove any trapped air bubbles.

f. Place a porous pad on top of the second sheet of Whatman 3MM paper in order to complete the cassette assembly and to ensure uniform membrane contact with the gel.

7. Insert the assembled cassette into the transfer apparatus. In order for transfer from the gel to the membrane to take place, it is essential that the membrane side of the cassette assembly faces the anode (+) electrode when the cassette is inserted into the transfer apparatus.

8. Fill the transfer apparatus with 4–5 liters of transfer buffer and connect the cooling coil to a suitable circulating water bath.

9. Transfer the proteins from the SDS–polyacrylamide gel to the PVDF Membrane at 70 V for 1–2 hours.

10. After transfer is complete, disconnect the cooling coil and disassemble the cassette assembly.

11. Transfer the PVDF Membrane to a tray containing Amido black or Coomassie® brilliant blue. Stain the membrane for 10 minutes and follow with a rapid destaining in a 50% (v/v) methanol and 10% (v/v) acetic acid solution for 10 minutes. Following destaining, wash the membrane in dH_2O.

Semidry Electroblotting

Following electrophoresis, place the SDS–polyacrylamide gel in transfer buffer and allow the gel to equilibrate for 15–20 minutes prior to blotting. For the semidry electroblotter to be used, consult the respective manufacturer's instructions for details on the assembly procedure and recommended buffers.

Notes *When assembling the transfer cassette, be sure to remove all trapped air bubbles in order to avoid bare spots on the PVDF Membrane.*

If transferring proteins from thin SDS–polyacrylamide gels, place the moistened PVDF Membrane directly onto the gel while the gel is still on the electrophoretic glass plate. Invert the glass plate and carefully place the PVDF Membrane and the gel onto the moistened Whatman 3MM paper. Remove the glass plate and continue assembly.

The transfer times are dependent on the size of the proteins and on the percentage of the gel used (see the table below). In most cases, complete transfer is usually achieved within 30 minutes; however, transfer times should never exceed 1 hour.

Protein size range	Estimated transfer time
50,000 Da	15 minutes
50,000–200,000 Da	30 minutes
Up to 250,000 Da	40 minutes

Storage of Western Blots

Western blots can be dried and stored at 4°C for use in any conventional immunostaining assays. Western blots are stable for up to 1 year when stored dry. To rewet the membrane, first prewet the PVDF Membrane in a small volume of 100% (v/v) methanol for 1–2 seconds and then soak the membrane in a large volume of dH_2O to remove the methanol. If methanol exposure needs to be avoided, block the protein background and then air-dry the membrane. Blocking the PVDF Membrane coats the membrane with hydrophilic protein and allows for easy rewetting of the membrane in antibody incubation solution. In order to use this method, the PVDF membrane must be stored at 4°C.

PREPARATION OF MEDIA AND REAGENTS

20× SSC Buffer (per Liter)

175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of water
10.0 N NaOH
Adjust to pH 7.0 with a few drops of a 10.0 N NaOH
Adjust volume to 1 liter with water

20× SSPE (per Liter)

174.0 g of NaCl
27.6 g of NaH_2PO_4
7.4 g of EDTA-disodium salt (pH 8.0)
13.0 ml 5 M NaOH
Adjust pH to 7.4 with NaOH
Adjust volume to 1 liter with water

10× PIPES Buffer

4.0 M NaCl
0.1 M PIPES (pH 6.5)

50× Denhardt's Reagent (per 500 ml)

5 g of Ficoll
5 g of polyvinylpyrrolidone
5 g of BSA (Fraction V)
Water to 500 ml
Filter through a disposable filter
Dispense into aliquots and store at –20°C

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Chloramphenicol Agar (per Liter)

1 liter of LB agar
Autoclave
Cool to 55°C
Add 30 mg of filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

Transfer Buffer

25 mM Tris-HCl (pH 8.2)
192 mM glycine
5% (v/v) methanol

REFERENCES

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

ENDNOTES