GE Healthcare

Amersham Hybond-N Hybond-NX

Nylon membranes optimized for nucleic acid transfer

Product Booklet

Codes:	Hybond-N	Hybond-NX	
	RPN82N	RPN82T	82 mm
	RPN87N	RPN87T	87 mm
	RPN132N	RPN132T	132 mm
	RPN137N	RPN137T	137 mm
	RPN119N		11.9 x 7.8 cm
	RPN1210N		12 x 10 cm
	RPN1510N		15 x 10 cm
	RPN1520N	RPN1520T	15 x 20 cm
	RPN2020N	RPN2020T	20 x 20 cm
	RPN2222N		22.2 x 22.2 cm
	RPN3050N		30 x 50 cm
	RPN203N	RPN203T	20 cm x 3 m
	RPN303N	RPN303T	30 cm x 3 m

Page finder

1. Legal

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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.* Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

We recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes wash immediately with water.

Note that the procedures requires the use of:

Sodium Dodecyl Sulfate: irritant
Formaldehyde: toxic substance
Formamide: toxic substance
Ethidium Bromide: mutagenic substance
Sodium Hydroxide: corrosive
Hydrochloric Acid: corrosive
Dimethyl Sulphoxide: toxic substance

This product may also be used with radioactive materials.

Please follow the manufacturers' safety data sheets relating to the safe handling and use of these reagents.

2.2. Storage

Membranes should be stored in a clean, dry atmosphere away from excessive heat, light and noxious fumes. The membranes should be handled wearing gloves or using blunt ended forceps to prevent contamination.

2.3. Stability

Membranes are stable for up to three years. Membranes should be kept in the bags in which they are received. Performance is consistent when stored under the recommended conditions.

2.4. Expiry

Expiry information can be found on the external packaging.

3. Components

Hybond-N (neutral nylon)

RPN82N	82 mm diam, 50 discs
RPN87N	87 mm diam, 50 discs
RPN132N	132 mm diam, 50 discs
RPN137N	137 mm diam, 50 discs
RPN119N	11.9 x 7.8 cm, 50 sheets
RPN1210N	12 x 10 cm, 20 sheets
RPN1510N	15 x 10 cm, 20 sheets
RPN1520N	15 x 20 cm, 10 sheets
RPN2020N	20 x 20 cm, 10 sheets
RPN2222N	22.2 x 22.2 cm 10 sheets
RPN3050N	30 x 50 cm, 5 sheets
RPN203N	20 cm x 3 m, 1 roll
RPN303N	30 cm x 3 m, 1 roll

Hybond-NX (neutral nylon)

RPN82T	82 mm diam 50 discs
RPN87T	87 mm diam, 50 discs
RPN132T	132 mm diam, 50 discs
RPN137T	137 mm diam, 50 discs
RPN1520T	15 x 20 cm. 10 sheets
RPN2020T	20 x 20 cm, 10 sheets
RPN203T	20 cm x 3 m, 1 roll
RPN303T	30 cm x 3 m, 1 roll

4. Other materials required

Equipment

- Agarose gel electrophoresis apparatus, for example GE Healthcare. HE33 Mini or HE99X Max submarine gel electrophoresis systems
- Microwave
- Hybond Blotting Paper
- Absorbent paper towels
- Trays/dishes
- Glass plates • 750 g weight
- Pipettes, for example, Gilson™ Pipetman™ P20, P200, P1000 and P5000.
- Assorted laboratory glassware
- Oven, at 80°C or UV transilluminator
- Orbital shaker
- SaranWrap™ or similar cling film

5. Critical parameters

- Storage
  Membranes should be stored in a clean, dry atmosphere away from excessive heat, light and noxious fumes.
- Handling
  The membranes should be handled wearing gloves or using blunt ended forceps to prevent contamination. All membranes should be cut using a clean sharp scissors to avoid damage to the membrane edges.
- Wettability
  The wettability of the membranes is important in achieving a consistent performance. Nylon membranes are hydrophilic and do not require pre-wetting before use in blotting procedures. Wetting is however advised for large blots (>100 cm^2) or when multiple blots are hybridized together. Wet the membrane first in water then equilibrate in an appropriate buffer.
- Fixation
  The fixation procedure can significantly affect the eventual sensitivity of a system. Sub optimal fixation reduces the amount of available target sequences, particularly following stripping. Nucleic acid may be fixed using heat or UV crosslinking. It is essential that fixation times and/or energy settings are optimized when UV crosslinking, details are provided in the Additional information section of the pack leaflet. Hybond-N and Hybond-NX are not suitable for alkali fixation or blotting procedures, which can result in diffuse signal. Hybond-XL gives better results for these procedures due to its positive charge.
- Low volume hybridization (less than 70 µl/cm^2)
  Hybond-NX has been designed for this purpose. Volumes of at least 30 µl hybridization buffer per cm^2 of membrane should be used with this membrane.

6. Description

All Hybond™ membranes from GE Healthcare are manufactured specifically for life science applications. Production runs are carefully controlled and the product exhaustively screened to ensure that only the most consistent product reaches the user. All Hybond membranes are identical on both sides.

There are two distinct manufacturing methods, resulting in membranes with different characteristics.

a. Unsupported, where the active substrate is cast as a pure sheet. Hybond ECL™ is an example. Due to their fragile nature, unsupported membranes should be handled with care.

b. Supported, where the active substrate is cast onto an inert 'web'or support. Hybond-C extra, Hybond-N, Hybond-NX, and Hybond-N+ all fall into this class.

Supported nylon membranes have a high binding capacity for nucleic acid, in addition to high tensile strength. For applications requiring a high degree of sensitivity and/or reprobing these types of membranes are an ideal choice. Due to its high protein binding capacity, nylon requires extensive blocking prior to detection with antibodies to avoid high backgrounds. Consequently, nylon membranes are not recommended for use in Western blotting.

7. Solutions required for nucleic acid electrophoresis and blotting

All reagents should be of AnalaR™ grade where possible.

10 x nucleic acid loading dye mix	40 mg Bromophenol blue 40 mg Xylene cyanol 2.5 g Ficoll™ 400 Add approximately 8 ml of distilled water. Mix to dissolve. Make up to a final volume of 10 ml. Store at room temperature for up to 3 months.
50 x TAE (DNA electro-phoresis buffer)	242 g Trizma™ base 18.6 g Ethylenediaminetetra-acetic acid (EDTA), sodium salt Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 8 with glacial acetic acid (~57 ml/l). Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.
Depurination solution	11 ml HCl 989 ml Distilled water Mix. Store at room temperature for up to 1 month.
Denaturation buffer	87.66 g NaCl 20 g NaOH Add approximately 800 ml of distilled water. Mix to dissolve. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.

Neutralization buffer	87.66 g NaCl 60.5 g Trizma base Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 7.5 with concentrated hydrochloric acid. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.
Nucleic acid transfer buffer (20 x SSC)	88.23 g Tri-sodium Citrate 175.32 g NaCl Add approximately 800 ml of distilled water. Mix to dissolve. Check the pH is 7–8. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.
TE buffer	1.21 g Trizma base 0.372 g EDTA, sodium salt Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 8 with concentrated Hydrochloric Acid. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.
10 x MOPS buffer	41.2 g 3-(N-morpholino)propanesuphonic Acid (MOPS) 10.9 g Sodium Acetate, 3-hydrate 3.7 g EDTA, sodium salt Add approximately 800 ml of nuclease free distilled water. Mix to dissolve. Adjust to pH 7 with NaOH (prepared in nuclease free distilled water). Make up to a final volume of 1000 ml. Filter sterilize. Store at room temperature protected from light.

100 x Denhardt's solution

Do not use if the solution appears yellow in color.

2.0 g Bovine Serum Albumin
2.0 g Ficoll 400
2.0 g Polyvinylpyrrolidone
Add approximately 50 ml of distilled water. Mix to dissolve. Make up to a final volume of 100 ml. Store at -15°C to -30°C for up to 3 months.

8. Quality control

Hybond-N and Hybond-NX are QC tested using related GE Healthcare products and protocols to ensure maximum compatibility and optimum performance.

Description:
Nylon hybridization transfer membrane.

Quality control assay:
Hind III restricted human genomic DNA, separated using DNA agarose, is Southern blotted onto Hybond-N/NX and hybridized with N-ras proto-oncogene probe.

Specifications:
Detection of 0.5 pg of target DNA. Background below 0.15 OD. Hybridization volume 35 µl/cm^2 for Hybond-NX or 125 µl/cm^2 for Hybond-N.

Labelling and detection:
Performed using the appropriate system: Megaprime™ random prime labelling kit with Redivue™ ^{32}P-dCTP label, radioactive signal detected using Hyperfilm™ MP.

Storage instructions:
Store in a clean dry environment.

9. Blotting protocols

The capillary blotting technique first described by Southern (1) in 1975 is still the most widely used technique for transferring separated nucleic acid fragments from an agarose gel to a solid support. Today a large number of variations on this original procedure exist (2, 3, 4), often reflecting the membrane to be used and the apparatus (5, 6) available to the researcher. This booklet limits itself to the capillary blotting technique and is representative of the procedure used at GE Healthcare laboratories.

Details of the gel treatments required before the transfer of DNA or RNA may be found on page 17. Figure 1 (page 18) is a diagrammatic representation of the transfer apparatus used in this technique.

9.1. Protocol for capillary blotting

Protocol	Notes
1. Cut a sheet of membrane to an appropriate size.	**1.** The membrane should be cut with clean scissors.
2. Half fill a tray or glass dish of a suitable size with the transfer buffer. Make a platform and cover with a wick made from three sheets of Hybond blotting paper saturated in transfer buffer.	**2.** At least 800 ml of buffer is required for a 20 x 20 cm gel and a dish 24 x 24 cm. Ensure the wick ends are immersed in the transfer buffer.
3. Place the treated gel on the wick platform. Avoid trapping any air bubbles between the gel and the wick. Surround the gel with cling film to	**3.** Air bubbles block the transfer of nucleic acid to the membrane. They may be removed at any stage by rolling a clean pipette or

Protocol	Notes
3. *Continued.* prevent the transfer buffer being absorbed directly into the paper towels.	**3.** *Continued.* glass rod over the surface.
4. Place the membrane on top of the gel. Avoid trapping any air bubbles.	**4.** Do not attempt to move the membrane once it has touched the gel surface.
5. Place three sheets of Hybond blotting paper cut to size and saturated in transfer buffer, on top of the membrane. Avoid trapping any air bubbles.	
6. Place a stack of absorbent towels on top of the Hybond blotting paper at least 5 cm high.	
7. Finally, place a glass plate and a weight on top of the paper stack. Allow the transfer to proceed overnight. The weight should not exceed 750 g for a 20 x 20 cm gel.	**7.** Small fragments (0.5–1.5 Kb) are rapidly transferred upwards in a few hours, larger fragments (>10 Kb) require at least overnight transfer. The efficiency of transfer of these larger fragments can be improved by depurination (fragmentation).
8. After blotting, carefully dismantle the transfer apparatus. Before separating	**8.** Rinsing the membrane following transfer is not advised. Extensive

Protocol	Notes
8. *Continued.* the gel and membrane, mark the membrane to allow identification of the tracks with a pencil or chinagraph pen.	**8.** *Continued.* experimentation at GE Healthcare laboratories has shown that rinsing the membrane before fixation produces blots of variable quality because nucleic acid is removed from the membrane during this step.
9. Fix the nucleic acid to the membrane by baking at 80°C for 2 hours or by using an optimized UV crosslinking procedure.	**9.** Details of an optimization procedure are given on page 32. The UV crosslinker has a pre-set UV exposure (70 000 micro-joules/cm^2) which is optimum for Hybond-N and Hybond-NX.
10. Blots may be used immediately. Blots must be thoroughly dried if storage is required.	**10.** Blots may be rinsed in 2 x SSC before storage or hybridization. Blots should be stored wrapped in SaranWrap desiccated at room temperature under vacuum.

9.2. Southern blotting – gel treatments

Protocol	Notes
1. Separate the DNA samples on a suitable neutral agarose gel.	**1.** Efficient separation of a range of DNA fragments may be achieved by varying the type and concentration of

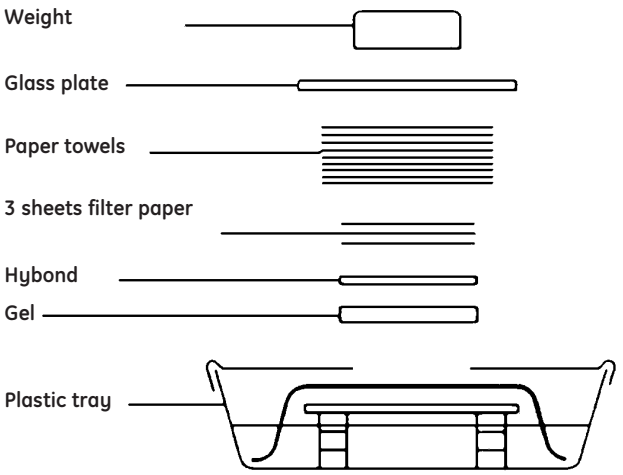

Figure 1. Diagrammatic representation of a capillary blotting apparatus

Protocol

2. Following electrophoresis visualize the DNA samples in the gel with UV light and photograph.

Notes

1. *Continued.*
the agarose in the gel. Ensure the optimum DNA concentration for detection is loaded into each track. 0.1 µg/ml Ethidium Bromide should be included in the gel for visualization.

2. Minimize the exposure of the gel to UV light as this may cause excessive nicking of the nucleic acid.

Protocol

3. Process the gel for blotting, between each step rinse the gel in distilled water.

3a. Depurination
Place in 0.125 M HCl so that the gel is completely covered in the solution. Agitate gently for 10–20 minutes. During this time the bromophenol blue dye present in the samples will change colour.

3b. Denaturation
Submerge the gel in sufficient denaturation buffer. Incubate for 30 minutes with gentle agitation. During this time the bromophenol blue dye will return to its original colour.

3c. Neutralization
Place the gel in sufficient neutralization buffer to submerge the gel. Incubate for 30 minutes with gentle agitation.

4. Set up the capillary blot as described on page 15.

Notes

3a. Depurination is not required for DNA fragments <10 Kb in size. Do not over depurinate, 10 minutes (or until the bromophenol blue turns yellow) is sufficient for most samples.

9.3. Northern blotting – gel preparation and treatment

Successful Northern analysis (7, 8) depends on the quality of the reagents used as well as having pure undegraded RNA samples. The principle systems currently in use are the Glyoxal/ Dimethlysulphoxide (DMSO) and the Formaldehyde/Formamide procedures are described.

Avoid any contamination with RNases, use sterile disposable plastics wherever possible. Glassware may be treated by baking at 180°C overnight or incubating in 0.2% (v/v) Diethylpyrocarbonate (DEPC) followed by autoclaving or baking. Some plastics are also suitable for DEPC treatment.

9.3.1. Formaldehyde/Formamide protocol

Protocol	Notes
1. Prepare the MOPS/ Formaldehyde gel as follows: Preheat 17.5 ml of Formaldehyde and 30 ml 10 x MOPS buffer at 55°C. Dissolve 3–4.5 g of Agarose in 250 ml of nuclease free water. Cool to 55°C. Add the 10 x MOPS buffer and Formaldehyde, mix and pour immediately. Allow the gel to set.	**1.** The Agarose gel is ≃ 0.7M with respect to Formaldehyde and 1 x with respect to the MOPS buffer. This formulation can be scaled up or down as appropriate for the size of gel required. SybrGreen™ or Ethidium Bromide (0.01 µg/ml) may be included in the gel for visualization. RNA does not stain as well with Ethidium Bromide as the same amount of DNA. Excessive amounts of Ethidium Bromide will also

Protocol

Notes

1. *Continued.*
inhibit RNA transfer(9). Other staining procedures post electrophoresis for example Ethidium Bromide (10) or Acridine Orange (11), or methods which stain the blot, for example Methylene Blue (12) may also be used.

2. Prepare the RNA sample(s), using the table below:

	Volume (ml)	final conc
RNA	V	
Formaldehyde	5.5	2.2 M
Formamide	15	50%
10 x MOPS buffer	1.5	
0.5 x Water	8-V	
TOTAL	30	

Place the sample(s) at 55°C for 15 minutes to denature. After denaturation add 3 µl of 10 x nucleic acid dye loading buffer. Mix and load onto the Agarose gel.

2. Sample must be deproteinized.

3. Separate the RNA samples using 1 x MOPS buffer as the electrophoresis buffer.

3. Ethidium Bromide at 0.01 µg/ml should be included in the electrophoresis buffer if

Protocol	Notes
	3. *Continued.* visualization is required.
4. Following electrophoresis, visualize the RNA within the gel with UV light and photograph.	**4.** The integrity of the RNA may be assessed by the absence of smearing and the fluorescent signal, the ratio of 28 S to 18 S RNA should be 2:1.
5. Place the gel in a suitable tray or dish and cover with distilled water. Incubate the gel with gentle agitation for 15 minutes.	
6. Discard the water and replace with sterile 10 x SSC. Agitate for 15 minutes. Repeat this step once more.	
7. Set up the capillary blot as described on page 15.	**7.** 10 x SSC should be used as the transfer buffer.

9.3.2. Glyoxal/DMSO protocol

Protocol	Notes
1. Prepare the Agarose gel as follows: Dissolve 3–4.5 g of agarose in 270 ml of nuclease free water. Cool to 55°C. Add the 10 x MOPS buffer (30 ml). Cast the gel in an	**1.** Deionize the Glyoxal to pH 7.0 using a suitable mixed bed resin prior to use. Dispense into suitable aliquots and store at -15°C to -30°C until required. This formulation can be

Protocol	Notes
1. *Continued.* appropriate enclosure and allow the gel to set.	**1.** *Continued.* scaled up or down as appropriate for the size of gel required. SybrGreen may be included in the gel for visualization. **Do not** prepare the gel using Ethidium Bromide as it reacts with the glyoxal during electrophoresis (10). RNA does not stain as well as the same amount of DNA with Ethidium Bromide. Excessive amounts of Ethidium Bromide will also inhibit RNA transfer (9). Other staining procedures post electrophoresis for example Acridine Orange (11), or methods which stain the blot, for example Methylene Blue (12) may also be used.
2. Prepare the RNA sample(s), using the table on the next page	**2.** Sample must be deproteinized. Samples may be stored at -15°C to -30°C for short periods.

Protocol

2. *Continued.*

	Volume (ml)	final conc
RNA	V	
Glyoxal	5	1 M
DMSO	15	50% (v/v)
10 x MOPS buffer	1.5	
0.5 x Water	5.5-V	
TOTAL	27	

Place the sample(s) at 50°C for 60 minutes to denature. After denaturation add 3 µl of 10 x nucleic acid dye loading buffer. Mix and load onto the agarose gel.

3. Separate the RNA samples using 1 x MOPS buffer as the electrophoresis buffer.

4. Following electrophoresis, if appropriate, visualize the RNA within the gel with UV light and photograph.

5. Place the gel in a suitable tray or dish and cover with

Notes

3. SybrGreen is recommended for visualization. Nucleic acid loading buffer must be prepared using RNase free reagents/solutions.

4. The integrity of the RNA may be assessed by the absence of smearing and the fluorescent signal, the ratio of 28 S to 18 S RNA should be 2:1.

Protocol	Notes
5. *Continued.* distilled water. Incubate the gel with gentle agitation for 15 minutes.	
6. Discard the water and replace with sterile 10 x SSC. Agitate for 15 minutes. Repeat this step once more.	
7. Set up the capillary blot as described on page 15 using a neutral transfer buffer.	**7.** 10 x SSC or 20 x SCC can be used as the transfer buffer.

9.4. Colony and plaque lifts (10)

Protocol	Notes
1. Plate out the cells or bacteriophage in the usual way. Incubate overnight at the required temperature.	**1.** Do not allow the colonies to grow too large. A colony/plaque density of up to 200 per 83 mm plate is optimal for accurate selection of positive clones.
2. Pre-cool the petri-dishes for at least 30 minutes at 2–8°C before taking the lift.	**2.** Pre-cooling prevents smearing of the colonies and separation of the top Agar layer. Plates must be free of excess moisture.
3. Select the correct size of membrane disc.	**3.** The hydrophilic nature of nylon ensures accurate colony/plaque lifts. The membrane may be pre-

Protocol

4. Bend the membrane and place the resulting trough across the centre of the petri-dish. Release the trough and allow the membrane to sit on the surface. Mark the disc position on the plate at several positions using a pin to ensure correct orientation of the colonies/plaques in subsequent manipulations.

5. After 30–60 seconds remove the membrane from the petri-dish in one continuous movement using blunt ended forceps. Place colony/plaque side uppermost on a sheet of Hybond blotting paper.

Notes

3. *Continued.*
wet, for example on an unused Agar plate. Excess liquid must be removed from the membrane before proceeding, this is achieved by placing the disc on a sheet of Hybond blotting paper.

4. This procedure will prevent air being trapped under the membrane. Do not force the membrane down, as it unrolls, the membrane disc will flatten. Do not attempt to move the membrane disc once it has touched the Agar surface.

5. Extending the time the membrane remains on the surface of the agar will cause diffusion of the colonies/plaques. Replicate filters can be prepared by placing a fresh membrane disc on top of this template membrane. Press the membrane firmly together using a replica

Protocol	Notes
	5. *Continued.* plating tool, avoid any lateral movement. Mark the replica membrane. Replica filters should then be incubated on fresh Agar plates under appropriate conditions until colonies of 0.5–1 mm diameter are obtained.
6. The DNA must be liberated from the bacteria or bacteriophage, denatured and then fixed to the membrane following a neutralization step. This is achieved by placing the membrane discs colony/ plaque uppermost on a series of solution saturated Hybond blotting paper filters:- a) Optional lysis step, 10% (w/v) SDS for 1–3 minutes b) Denaturation step, denaturation buffer for 2–5 minutes c) Neutralization step, neutralization buffer for 3 minutes. Repeat this step once more.	**6.** The Hybond blotting paper should be moist, though not too wet as this will cause diffusion of the colonies/ plaques. Timings should be optimized, prolonged incubations will cause diffusion of the target DNA making accurate selection of positive clones difficult. Avoid fluid reaching the upper surface of the membrane. When transferring membrane, remove as much fluid as possible from the underside of the membrane. This may be achieved by transferring briefly to dry Hybond blotting paper between treatments.

Protocol	Notes
7. Finally, vigorously wash the membrane disc in 2 x SSC to remove the proteinous debris.	**7.** Adequate removal of cell debris may be essential for some non-radioactive labelling and detection procedures. Manufacturer's procedure should be followed under these circumstances.
8. Transfer the disc, DNA side uppermost, to a pad of dry Hybond blotting paper, air dry.	
9. Fix the DNA to the membrane by baking for 2 hours at 80°C or by using an optimized UV crosslinking procedure.	**9.** Details of an optimization procedure are given on page 32. The UV crosslinker has a pre-set UV exposure (70 000 micro-joules/cm^2) which is optimum for Hybond-N and Hybond-NX.
10. Membranes may be used immediately or stored, once dry.	**10.** Membranes should be stored wrapped in SaranWrap desiccated at room temperature under vacuum.

9.5. Protocol for dot blotting (manual)

The following is a general protocol for dot blotting target nucleic acids. A number of commercially available devices are also available. These provide for a more consistent and even application of the sample than the manual procedure described below. This parameter is particularly important in those experiments requiring quantification.

Protocol	Notes
1. Cut the membrane to an appropriate size.	**1.** The membrane should be cut with clean scissors.
2. Using a pencil, mark the membrane lightly with a grid or dots to guide subsequent sample application. There should be a minimum distance of 1 cm between samples applied in a volume 5 µl or less.	
3. Pre-wetting the membrane is not required.	**3.** Membranes may be pre-wet if desired, see critical parameters page 9.
4. Dilute the samples in an appropriate buffer to the required concentration. TE buffer or 2 x SSC may be used for DNA samples. RNA samples should be prepared using the information in the table on page 24. A sample size of 1–2 µl is ideal for manual dot blotting.	**4.** Carrier substance may be included in the diluent buffer to improve retention of very small amounts of target on the membrane. These include: • sonicated herring sperm DNA for DNA samples • tRNA for use with RNA samples.

Protocol	Notes
	4. *Continued.* Larger sample volumes of 50–200 µl are common for commercial apparatus. This ensures an even application of the sample over the whole dot or slot.
5. Nucleic acid samples must be denatured to provide a suitable single-stranded target molecule for subsequent hybridizations. Denature the samples by heating in a boiling water bath for 5 minutes. Chill rapidly on ice, then centrifuge briefly to collect sample at the bottom of the tube.	**5.** Samples may also be prepared in 0.2 M NaOH incubated at 37°C for 15 minutes and dotted directly onto the membrane, in a denatured condition. Alternatively RNA samples may be preheated to 55°C for 15 minutes, see page 21.
6. Carefully apply the sample to the membrane, avoiding touching the membrane with the pipette tip. Leave the membrane to air dry.	**6.** If the sample volume is greater than 2 µl, then apply in successive 2 µl aliquots to the same position on the membrane, allow the aliquot to dry between each application. This will prevent the sample spreading.
7. Fix the nucleic acid sample to the membrane by UV crosslinking or baking at	**7.** Details of an optimization procedure are given on page 32. The UV crosslinker has a

Protocol

7. *Continued.*
80°C for 2 hours.

8. Blots may be used immediately or stored wrapped in SaranWrap desiccated at room temperature under vacuum.

Notes

7. *Continued.*
pre-set UV exposure (70 000 micro-joules/cm^2) which is optimum for Hybond-N and Hybond-NX.

10. Additional information

10.1. Determination of the optimum UV crosslinking conditions using a UV transilluminator

Protocol

1. Produce five or six identical control blots, for example Lambda H*ind* III on the membrane of choice.

2. Protect the surface of the membrane by covering the transilluminator with SaranWrap. Expose each blot DNA side down on the transilluminator for a different length of time, for example 30 seconds to 5 minutes.

Notes

1. The type of blot should reflect the technique for which the calibration is being used.

2. The length of exposure required for optimum fixation will vary depending on the wavelength of the UV bulb and its age. The energy emitted from a UV bulb is reduced with use. Regular recalibration is advised if the apparatus is extensively used. This inconvenience may be overcome with the use of UV crosslinkers which are able to compensate for this effect, when used on the constant energy setting. A UV crosslinker with pre-set or manual energy and time settings is available from GE Healthcare.

Protocol	Notes
3. Hybridize all the blots together with a suitably labelled probe.	
4. Following autoradiography, the optimum UV exposure time will be indicated by selecting the blot showing the strongest signal.	

10.2. Hybridization protocols - hybridizations in bags and boxes

Protocol	Notes
1. Prepare the hybridization buffer, for example 5 x SSC 5 x Denhardt's solution 0.5% (w/v) SDS Combine all the components, make up to the required volume.	**1.** There are a wide variety of hybridization buffers used by researchers. This Denhardt's based buffer is that used in the quality control of all nylon membranes from GE Healthcare. The hybridization buffer may be stored at -15°C to -30°C if required. A reduced concentration of SDS may lead to elevated backgrounds following hybridization.
2. Preheat the required volume of hybridization buffer to an appropriate temperature. Pre-wet the blot in a suitable buffer. Place the blots in the	**2.** Pre-wetting in a suitable buffer is essential for large blots (>100 cm^2) or multiple blots. Details of the pre-wetting procedures are

Protocol

2. *Continued.*
hybridization buffer, at least 125 µl/cm^2 of membrane is required. Prehybridize for at least 30 minutes with constant agitation, at the desired hybridization temperature.

3. Pipette the required amount

Notes

2. *Continued.*
given on page 9, critical parameters. Hybridization may be carried out in bags, or boxes, provided there is sufficient buffer for the container. Adequate circulation of the buffer is essential. When hybridizing several blots together, the blot should move freely within the buffer.
For radioactive applications use a probe concentration of 0.5–2 x 10^6 incorporated counts per ml of hybridization buffer for single copy gene detection, ie. high sensitivity application) or 0.125–0.5 x 10^6 incorporated counts per ml of hybridization buffer for high target work, for example colonies/plaques, PCR products etc. Probe purification, to remove unincorporated radioactive nucleotides, is strongly recommended.

Protocol	Notes
3. *Continued.* of labelled probe into a clean microcentrifuge tube. If the volume is less than 20 µl, make up to this volume with water or TE buffer. Denature the probe by boiling for 5 minutes and snap cool on ice.	
4. Briefly centrifuge the denatured probe and then add to the pre-hybridization buffer.	**4.** Avoid placing the probe directly on the blots, as this will cause excessive background.
5. Hybridize overnight with gentle agitation.	**5.** Hybridization temperatures may vary with the probe. Lower temperatures achieve lower stringency. The temperature of hybridization used will depend on the degree of homology between the probe and the target. Hybridization time can also vary. Short hybridization times may be suitable for high target applications.
6. Prepare the stringency wash solutions. The wash solution should be used in excess, at least 1–5 ml/cm^2 of membrane.	**6.** Stringency washes will depend on the nature of the probe and target to be hybridized. Salt concentration and temperature should be

Protocol	Notes
6. *Continued.* 2 x SSC, 0.1% (w/v) SDS 1 x SSC, 0.1% (w/v) SDS 0.1 x SSC, 0.1% (w/v) SDS	**6.** *Continued.* taken into consideration. The lower the salt concentration, the greater the stringency. The higher the washing temperature, the greater the stringency. Most commonly, stringency washes proceed from 'high salt'/'low temperature', for example 5 x SSC, 0.1% SDS at room temperature, to 'low salt'/'high temperature', for example 0.1 x SSC, 0.1% at 65°C (nominal hybridization temperature).
7. After the hybridization, wash the blots by incubating twice, 5 minutes each, in 2 x SSC, 0.1% SDS, followed by 1 x SSC, 0.1% SDS for 15 minutes, and finally 0.1 x SSC, 0.1% SDS for 2 x 10 minutes, at 65°C.	**7.** Some procedures include room temperature washes under low stringency conditions. Do not allow the SDS to come out of solution during these washes, significant levels of background may result. Adequate circulation of the stringency buffer is essential when washing. Washing in boxes is advised.
8. Remove the blot from the last stringency wash, drain, wrap	**8.** The use of SaranWrap with ^{35}S labelled probes

Protocol	Notes
8. *Continued.* in SaranWrap and expose to X-ray film, for example Hyperfilm MP. Keep the blot moist if it is to be reprobed. If reprobing is desired, it may be more suitable to seal the blot in a plastic bag.	**8.** *Continued.* will significantly increase exposure times. In this case the blot should be air dried before autoradiography, if reprobing is not required.

10.3. Hybridization in tubes

There are numerous commercially available rotisserie devices suitable for use as hybridization ovens for example GE Healthcare hybridization oven/shaker RPN2510E/2511E. These can accommodate 2 to 24 hybridization tubes. The major advantage of this approach to hybridization is the use of low volumes of hybridization buffer, and therefore minimal probe volumes. This is achieved because fluid is able to move continually over the membrane. Hybond-NX has been specifically designed for use with low volumes of hybridization buffer.

Protocol	Notes
1. Prepare the hybridization buffer, for example 0.5 M phosphate buffer, pH 7.2 7% (w/v) SDS 10 mM EDTA(13) Ensure the SDS is in solution before use. Gentle heating may be necessary.	**1.** This is a modification of the Church and Gilbert buffer, routinely used at GE Healthcare laboratories. It has been shown to be suitable for Southerns, Northerns, dot blots and library screening applications. The hybridization buffer may be stored at room temperature.

Protocol	Notes
2. Preheat the required volume of hybridization buffer to an appropriate temperature.	**2.** Hybond-NX has been specifically designed for use with very low volumes of hybridization buffer (30–70 µl/cm^2). A minimum of 70 µl/cm^2 is recommended for other Hybond nylon membranes. High backgrounds will result if sub optimum volumes are used for the membrane and hybridization conditions.
3. Pre-wet the blot in a suitable dish, first in water then in an appropriate buffer. Ensure that the nucleic acid side is uppermost. Roll the blot along its length in such a way as to minimize overlap in the tube. Place inside the hybridization tube.	**3.** If there is significant overlap of the blot use of a nylon mesh should be considered. The mesh achieves separation of the blot layers allowing better probe access to these areas. It is strongly advised that minimum hybridization volume should not be used under these conditions. The nylon mesh should be at least 0.5 cm larger than the blot. Place the mesh in the pre-wetting solution before the blot, in subsequent manipulations treat as 'one'. The nylon mesh may be reused after washing in 10% (w/v) SDS

Protocol	Notes
	3. *Continued.* and extensive rinsing in distilled water.
4. Add a small volume of appropriate buffer to the hybridization tube, cap the tube. Unroll the blot by rotating the tube in the opposite direction to the 'rolled' blot.	**4.** It is important not to allow air to become trapped between the inner surface of the tube and the membrane. This can cause areas of no signal or background following hybridization.
5. Drain the tube of excess liquid and replace with the appropriate volume of hybridization buffer.	**5.** Hybond-NX has been specifically designed for use with very low volumes of hybridization buffer. A minimum of 70 µl/cm^2 is recommended for other Hybond nylon membranes.
6. Prehybridize for 30 minutes at the appropriate temperature. Ensure that the tube is placed in the correct orientation within the oven to avoid 'rolling' up of the blot.	
7. Pipette the required amount of labelled probe into a clean microcentrifuge tube. If the volume is less than 20 µl, make up to this volume with water or TE buffer. Denature the probe by boiling for 5	**7.** For radioactive applications use a probe concentration of 0.5–2 x 10^6 incorporated counts per ml of hybridization buffer for single copy gene detection, ie. high sensitivity application) or

Protocol	Notes
7. *Continued.* minutes and snap cool on ice.	**7.** *Continued.* $0.125–0.5 \times 10^6$ incorporated counts per ml of hybridization buffer or high target work, for example colonies/plaques, PCR products etc. Probe purification, to remove unincorporated radioactive nucleotides, is strongly recommended.
8. Briefly centrifuge the denatured probe and then add to the pre-hybridization buffer.	**8.** Avoid placing the probe directly on the blot. Probe may be added to the hybridization while the tube is in a vertical position. If necessary probe may be mixed with a portion of the hybridization buffer and added to the tube in a larger volume.
9. Hybridize overnight at the appropriate temperature.	
10. Prepare the stringency wash solutions. The wash solution should be used in excess. Use a volume that occupies 33–50% of the tube.	**10.** See step 6 page 35.

Protocol

11. After the hybridization wash the blot as follows:
a) rinse briefly in 2 x SSC, 0.1% (w/v) SDS
b) twice, 5 minutes each in 2 x SSC, 0.1% (w/v) SDS
c) twice, 10 minutes each in 1 x SSC, 0.1% (w/v) SDS
d) four times, 5 minutes each in 0.1 x SSC, 0.1% (w/v) SDS

12. Remove the blot from the last stringency wash, drain and wrap in SaranWrap and expose to X-ray film, for example Hyperfilm MP. Keep the blot moist if it is to be reprobed.

Notes

11. Washing in boxes is much more effective and is recommended if feasible. The inefficiencies of washing in tubes may be overcome by increasing the number of stringency washes while maintaining the same total wash time.

12. See step 8 page 36.

10.4. Stripping protocol for nucleic acid blots

For the successful removal of probes, membranes must never be allowed to dry out after hybridization and washing, since this process has the effect of 'fixing' the hybrid.

The two most common stripping procedures are described below. Some users have experienced variability in the alkali method, and in these cases a combination of both of these procedures has proved more suitable. For non-radioactive probes, special conditions are often required. Please refer to manufacturer's protocols for reprobing non-radioactive systems.

Hot SDS procedure

Protocol	Notes
1. Place the moist membrane in an appropriate sized tray.	
2. Prepare a boiling solution of 0.1% (w/v) SDS, pour the solution onto the blot and allow to cool to room temperature.	**2.** This step may be repeated if the probe is particularly difficult to remove. Some non radioactive labelling and detection systems have specific requirements for reprobing, the manufacturer's instructions should always be followed.
3. Rinse the blot briefly in 2 x SSC.	
4. Check the removal of the probe using the appropriate procedure for the labelling and detection system used.	
5. Hybridize overnight using the appropriate conditions.	

Alkali procedure

Protocol	Notes
1. Place the moist membrane in an appropriate sized box.	
2. Pre-heat 0.2 M NaOH to 42°C	**2.** Higher concentrations of NaOH alone and/or in combination with NaCl may also be used.

Protocol

3. Add the alkali to the blot and incubate at 42°C for 10 minutes with constant agitation.

4. Repeat step 3 with fresh alkali solution.

5. Wash the membrane in 2 x SSC for 15 minutes.

6. Check the removal of the probe using the appropriate procedure for the labelling and detection system used.

7. Hybridize overnight using the appropriate conditions.

Notes

3. Incubation times may also vary. Optimization of the procedure for the probe and target in use is strongly advised.

10.5. Recommended applications for blotting membranes

10.5.1. Hybond membranes for binding nucleic acid

Applications	Hybond-NX (nylon)	Hybond-XL (positively charged nylon)	Hybond-N+ (positively charged nylon)	Hybond-N (neutral nylon)
Southern blotting				
DNA fingerprinting	+	+	+	+
Radioactive	++	+++	++	++
ECL	-	-	+++	-
AlkPhos Direct™	-	-	+++	-
Gene Images™	-	-	+++	-
Alkali blotting/fixation	--	+++	--	--
Low volume hybridizations	+++	+++	+	--
Rapid-hyb™ buffer	+	+++	++	+
Northern blotting				
Radioactive detection	++	+++	+	++
Non-radioactive detection	-	-	++	-
Dot/slot blots				
Radioactive detection	++	+++	++	++
Non-radioactive detection	-	-	++	-
Colony/plaque lifts				
Radioactive detection	+++	++	+	++
Non-radioactive detection	+	-	++	+

10.5.2. Hybond membranes for binding protein

Applications	Hybond-P	Hybond-ECL	Hybond-C Extra
Western blotting			
ECL detection	+++	+++	+
ECL Plus™ detection	+++	++	+
Chromogenic detection	++	++	+
Colloidal gold detection	++	++	-

10.5.2. Hybond membranes for binding protein

Continued

Applications	Hybond-P	Hybond-ECL	Hybond-C Extra
Western blotting *Continued*			
ECF™′ detection	+++	+	-
Radioactive detection	+	+	++
Glycoprotein detection	+++	+	+
Reprobing Westerns	+++	-	+
Expression screening	+	-	+++

Key: Suitable = +, Recommended = ++, Highly recommended = +++
Not recommended = -, Unsuitable = --

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12. Related products

Hybond membranes for for nucleic acid transfer

Size	Pack size	Code Hybond-N+ (positively charged nylon)	Hybond- XL (positively charged nylon)
82 mm diam	50 discs	RPN82B	RPN82S
87 mm diam	50 discs	RPN87B	RPN87S
132 mm diam	50 discs	RPN132B	RPN132S
137 mm diam	50 discs	RPN137B	RPN137S
11.9 x 7.8 cm	50 sheets	RPN119B	RPN119S
22.2 x 22.2 cm	50 sheets	RPN2250B	RPN2222S
22.5 x 22.5 cm	50 sheets	RPN225B	
15 x 73 mm	50 sheets	RPN1576B	
12 x 10 cm	20 sheets	RPN1210B	RPN1210S
15 x 10 cm	20 sheets	RPN1510B	RPN1510S
15 x 20 cm	10 sheets	RPN1520B	RPN1520S
20 x 20 cm	10 sheets	RPN2020B	RPN2020S
22 x 22 cm	10 sheets	RPN2222B	
30 x 50 cm	5 sheets	RPN3050B	RPN3050S
20 cm x 3 m	1 roll	RPN203B	RPN203S
30 cm x 3 m	1 roll	RPN303B	RPN303S
82 mm	50 gridded discs	RPN1782B	
87 mm	50 gridded discs	RPN1787B	
132 mm	50 gridded discs	RPN1732B	
137 mm	50 gridded discs	RPN1737B	

Hybond blotting paper		
20 x 20 cm	100 sheets	RPN6101M
DNA labelling kits		
Megaprime DNA Labelling System dNTP	30 reactions	RPN1604
AlkPhos Direct Labelling and Detection System with ECF		RPN3692
Rediprime™II DNA Labelling System	30 reactions	RPN1633
Ready-To-Go DNA labeling beads (-dCTP)	20 reactions	27-9240-01
AlkPhos Direct Labeling and Detection System with CDP-Star for 2500cm^2 membrane		RPN3690
ECL Direct Nucleic Acid Labeling and Detction System to label 5ug		RPN3000
Gene Images 3' Oligolabeling Kit For 1000 pmol oligo		RPN5770
Gene Images Random-Prime DNA Lableing kit	30/60 reactions	RPN3520/3525
Gene Images ECL Detection kit For 2000/4000cm^2 membrane		RPN3135/3130
Gene Images CDP-Star Detection Kit For 2500/5000cm^2 membrane		RPN3550/3555
Gene Images ECF Detection Kit For 2500cm^2 membrane		RPN3580

Nucleotides

Redivue formulation ^{32}P- and ^{33}P-labelled radionucleotides

Standard formulation ^{32}P- and ^{33}P-labelled radionucleotides

See GE Healthcare catalogue for full listings

Additional products

Rapid-hyb Buffer	125 ml	RPN1635
Rapid-hyb Buffer	500 ml	RPN1636

Scanning instrumentation

Typhoon™ 9200 Variable Mode Imager
Storm™ Gel and Blot Imaging System

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