Amersham Hybond-N+

Product Booklet

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1 Introduction

Product codes

RPN82B	82 mm diam, 50 discs
RPN87B	87 mm diam, 50 discs
RPN132B	132 mm diam, 50 discs
RPN137B	137 mm diam, 50 discs
RPN1576B	11.5 × 7.3 cm, 50 sheets
RPN119B	11.9 × 7.8 cm, 50 sheets
RPN225B	22.5 × 22.5 cm, 50 sheets
RPN1210B	12 × 10 cm, 20 sheets
RPN1510B	15 × 10 cm, 20 sheets
RPN1520B	15 × 20 cm, 10 sheets
RPN2020B	20 × 20 cm, 10 sheets
RPN2222B	22.2 × 22.2 cm, 10 sheets
RPN3050B	30 × 50 cm, 5 sheets
RPN2250B	22.2 × 22.2 cm, 50 sheets
RPN203B	20 cm × 3 m, 1 roll
RPN303B	30 cm × 3 m, 1 roll
RPN1782B	82 mm diam, 50 gridded discs
RPN1787B	87 mm diam, 50 gridded discs
RPN1732B	132 mm diam, 50 gridded discs
RPN1737B	137 mm diam, 50 gridded discs

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety warnings and precautions

For use and handling of the products in a safe way, refer to the Safety Data Sheets. This product may also be used with radioactive materials.

CAUTION

Sodium Dodecyl Sulfate: irritant

Formaldehyde: toxic substance

Formamide: toxic substance

Ethidium Bromide: mutagenic substance

Sodium Hydroxide: corrosive

Hydrochloric Acid: corrosive

Diethylpyrocarbonate: explosive, toxic substance

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.

Stability

When stored under appropriate conditions 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 Components

Main components

RPN82B	82 mm diam, 50 discs
RPN87B	87 mm diam, 50 discs
RPN132B	132 mm diam, 50 discs
RPN137B	137 mm diam, 50 discs
RPN1576B[1]	11.5 × 7.3 cm, 50 sheets
RPN119B	11.9 × 7.8 cm, 50 sheets
RPN225B	22.5 × 22.5 cm, 50 sheets
RPN1210B	12 × 10 cm, 20 sheets
RPN1510B	15 × 10 cm, 20 sheets
RPN1520B	15 × 20 cm, 10 sheets
RPN2020B	20 × 20 cm, 10 sheets
RPN2222B	22.2 × 22.2 cm, 10 sheets
RPN3050B	30 × 50 cm, 5 sheets
RPN2250B	22.2 × 22.2 cm, 50 sheets
RPN203B	20 cm × 3 m, 1 roll
RPN303B	30 cm × 3 m, 1 roll
RPN1782B	82 mm diam, 50 gridded discs
RPN1787B	87 mm diam, 50 gridded discs
RPN1732B	132 mm diam, 50 gridded discs

RPN1737B	137 mm diam, 50 gridded discs

[1] Designed to fit Omni Tray from Nalge Nunc International

Critical parameters

Handling:

The membranes should be handled wearing gloves or using blunt ended forceps to prevent contamination. All membranes should be cut using 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 prewetting before use in blotting procedures. Wetting is however advised for large blots (>100cm^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, energy settings (where appropriate) and concentration (where appropriate) are optimized.

Other materials required

Equipment

- Agarose gel electrophoresis apparatus, for example 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
- Saran™ Wrap or similar cling film

3 Description

All Hybond membranes are identical on both sides.

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

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

2. Supported, where the active substrate is cast onto an inert 'web' or support. Hybond-C extra, Hybond-NX, Hybond-N 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.

The membrane, which is identical on both sides, is manufactured in long rolls known as 'master rolls'. Production runs are carefully controlled and constantly monitored to ensure the most consistent product reaches the user. Samples are taken from the beginning, middle and end of each master roll and used in single copy gene detection (see *Quality control, on page 8*).

Quality control

Every lot of Hybond-N+ is tested using related Cytiva products and protocols to ensure maximum compatibility and optimum performance.

Description:	Nylon hybridization transfer membrane
Application test:	*Hind* III restricted human genomic DNA, separated using neutral agarose gel electrophoresis, is Southern(1) blotted onto Hybond-N+ and hybridized with N-ras protooncogene probe.
Specifications:	Detection of 0.5 pg of target DNA. Hybridization volume 125 µL/cm^2.

Labelling and detection:	Performed using the appropriate system:
	appropriate system:
	Megaprime random prime labelling kit with Redivue [a-^{32}P]-dCTP label, radioactive signal detected using Hyperfilm™MP
Storage:	Store in a clean dry environment.

Solutions

All reagents should be of AnalaR grade where possible.

10× 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× TAE (DNA electrophoresis buffer)	242 g Trizma™ base
	18.6 g Ethylenediaminetetraacetic 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× 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× MOPS buffer	41.2 g 3-(N-morpholino) propanesulfonic 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. Do not use if the solution appears yellow in color.

100× Denhardt's solution	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.

4 Protocols

This pack leaflet is limited to the classical capillary blotting technique (*Protocol for capillary blotting, on page 11* – *Colony and plaque lifts (10), on page 19*) used for the transfer of separated nucleic acid fragments from an agarose gel to a solid support, and is representative of the procedures used in Cytiva laboratories.

Fig. 1, on page 14 is a diagrammatic representation of the transfer apparatus used in this technique.

Protocol for capillary blotting

Step	Action
1	Prepare the gel for transfer. ***Note:*** *Details of gel treatments may be found on Southern blotting – Neutral transfer gel treatment protocol, on page 15 or Northern blotting – gel preparation and treatment, on page 16.*

Step	Action
2	Cut a sheet of membrane to an appropriate size. ***Note:*** *The membrane should be cut with clean scissors.*
3	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. ***Note:*** *At least 800 mL of buffer is required for a 20 × 20 cm gel and a dish 24 × 24 cm. Ensure the wick ends are immersed in the transfer buffer.*
4	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 prevent the transfer buffer being absorbed directly into the paper towels. ***Note:*** *Air bubbles block the transfer of nucleic acid to the membrane. They may be removed at any stage by rolling a clean pipette or glass rod over the surface.*
5	Place the membrane on top of the gel. Avoid trapping any air bubbles. ***Note:*** *Do not attempt to move the membrane once it has touched the gel surface.*

Step	Action
6	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.
7	Place a stack of absorbent towels on top of the Hybond Blotting paper at least 5 cm high.
8	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 × 20 cm gel. ***Note:*** *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.*
9	After blotting, carefully dismantle the transfer apparatus. Before separating the gel and membrane, mark the membrane to allow identification of the tracks with a pencil or chinagraph pen. ***Note:*** *Rinsing the membrane following transfer is not advised. Extensive experimentation at Cytiva has shown that rinsing the membrane before fixation produces blots of variable quality because nucleic acid is removed from the membrane during this step.*

Step	Action
10	Fix the nucleic acid to the membrane by baking at 80°C for 2 hours or by using an optimized UV crosslinking procedure. ***Note:*** *Details of an optimization procedure are given in* *Determination of the optimum UV crosslinking conditions using a UV transilluminator, on page 37**.* *The (UVC 500 crosslinker, available from Cytiva, has a pre-set UV exposure (70 000 microjoules/ cm^2) which is suitable for Hybond-N+.*
11	Blots may be used immediately. Blots must be thoroughly dried if storage is required. ***Note:*** *Blots may be rinsed in 2 x SSC before storage or hybridization. Blots should be stored wrapped in Saran Wrap desiccated at room temperature under vacuum.*

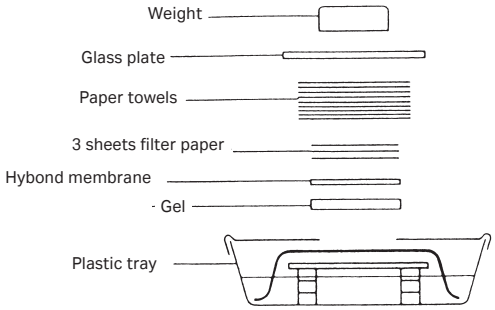

Fig 1. Diagrammatic representation of a capillary blotting apparatus.

Southern blotting – Neutral transfer gel treatment protocol

Step	Action
1	Separate the DNA samples on a suitable neutral agarose gel. ***Note:*** *Efficient separation of a range of DNA fragments may be achieved by varying the type and concentration of 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	Following electrophoresis visualize the DNA samples in the gel with UV light and photograph. ***Note:*** *Minimize the exposure of the gel to UV light as this may cause excessive nicking of the nucleic acid.*
3	Process the gel for blotting, between each step rinse the gel in distilled water. **a.** Depurination Place in 0.125 M HCl so that the gel is completely covered in the solution. Agitate gently for approximately 10 minutes. During this time the bromophenol blue dye present in the samples will change color. ***Note:*** *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 usually sufficient for most samples.*

Step	Action
	b. 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 color.
	c. 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 in *Protocol for capillary blotting, on page 11*.

Northern blotting – gel preparation and treatment

RNA is separated under denaturing conditions, the principle systems currently in use are the glyoxal/dimethylsulphoxide and the formaldehyde/formamide procedures. This booklet restricts itself to the latter. Successful Northern analysis(7,8) depends on the quality of the reagents used as well as having pure undegraded RNA samples.

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.

Step	Action
1	Prepare the MOPS/formaldehyde gel as follows: Preheat 17.5 mL of formaldehyde and 30 mL 10 × MOPS buffer at 55°C.

Step	Action
	Dissolve 3–4.5 g of agarose in 250 mL of nuclease free water. Cool to 55°C. Add the 10 × MOPS buffer and formaldehyde. Cast the gel in an appropriate enclosure and allow the gel to set. ***Note:*** *The agarose gel is 0.7 M with respect to formaldehyde and lx with respect to the MOPS buffer. This formulation can be scaled up or down as appropriate for the size of gel required. SYBR™ Green or Ethidium Bromide (0.01 µg/mL) may be included in the gel for visualization. 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).*
2	Prepare the RNA sample(s), using the table below. Place the sample(s) at 55°C for 15 minutes to denature. After denaturation add 3 µL of 10× nucleic acid dye loading buffer. Mix and load onto the agarose gel. ***Note:***

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

Step	Action
	Sample must be deproteinized. Samples may be stored at -15°C to -30°C for short periods. Nucleic acid loading buffer must be prepared using RNase free reagents/solutions.
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. ***Note:*** *The integrity of the RNA may be assessed by the absence of smearing and the fluorescent signal, the ratio of 28S to 18S 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 using a neutral transfer buffer as described under *Solutions, on page 9*. ***Note:*** *10 × SSC or 20 × SCC can be used as the transfer buffer.*

Colony and plaque lifts (10)

Step	Action
1	Plate out the cells or bacteriophage in the usual way. Incubate overnight at the required temperature. ***Note:*** *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. ***Note:*** *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. ***Note:*** *The hydrophilic nature of nylon ensures accurate colony/plaque lifts. If desired the membrane may be pre-wet before use, for example on an unused agar plate or on a TE buffer saturated Hybond Blotting paper pad. Excess liquid must be removed from the membrane before proceeding, this is achieved by placing the disc on a dry sheet of Hybond Blotting paper, see* *Protocol for capillary blotting, on page 11**.*

Step	Action
4	Bend the membrane and place the resulting trough across the centre of the petridish. 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. ***Note:*** *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	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. ***Note:*** *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 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.*

Step	Action
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 pads:-

Note:
An initial (optional) lysis step, 10% (w/v) SDS for 1-3 minutes may be included in the colony lift procedure. 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.

a. **Denaturation step**, denaturation buffer for 2–5 minutes

b. **Neutralization step**, neutralization buffer for 3 minutes. Repeat this step once more.

Step	Action
7	Finally, vigorously wash the membrane disc in 2x SSC to remove the proteinous debris. ***Note:*** *Adequate removal of cell debris from colony lifts is essential.*
8	Transfer the disc, DNA side 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. ***Note:*** *Details of an optimization procedure using a UV transilluminator are given on page 36. The UVC 500 UV crosslinker, available from Cytiva, has a pre-set UV exposure (70 000 microjoules/ cm^2) which is suitable for Hybond-N+.*
10	Membranes may be used immediately or stored, once dry. ***Note:*** *Membranes should be stored wrapped in Saran Wrap desiccated at room temperature under vacuum.*

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, for example the PR648 Slot blot manifold available from Cytiva. 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.

Step	Action
1	Cut the membrane to an appropriate size. ***Note:*** *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. ***Note:*** *Membranes may be prewet if desired, see* *Critical parameters, on page 6**.*

Step	Action
4	Dilute the samples in an appropriate buffer to the required concentration. TE buffer or 2x SSC may be used for DNA samples. RNA samples should be prepared using the information in *Northern blotting – gel preparation and treatment, on page 16*. A sample size of 1–2 µL is ideal for manual dot blotting. ***Note:*** *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.* *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. ***Note:*** *RNA samples may be preheated to 55°C for 15 minutes, see* *Northern blotting – gel preparation and treatment, on page 16* *Step 2.*

Step	Action
6	Carefully apply the sample to the membrane, avoiding touching the membrane with the pipette tip. Leave the membrane to air dry. ***Note:*** *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 80°C for 2 hours. ***Note:*** *Details of an optimization procedure are given on page 32. The UVC 500 UV crosslinker , available from Cytiva, has a pre-set UV exposure (70 000 micro-joules/cm2) which is optimum for Hybond- N+.*
8	Blots may be used immediately or stored wrapped in Saran Wrap desiccated at room temperature under vacuum.

Hybridizations in bags and boxes (5,6)

Step	Action
1	Prepare the hybridization buffer, for example **Denhardt's buffer** 5 × SSC 5 × Denhardt's solution 0.5% (w/v) SDS

Step	Action

Modified Church and Gilbert buffer(11)

7% (w/v) SDS

0.5 M phosphate buffer, pH 7.2

10 mM EDTA

Note:

There are a wide variety of hybridization buffers used by researchers. This Denhardt's based buffer is used in the testing of Hybond nylon membranes. A reduced concentration of SDS has been found to elevate backgrounds following hybridization. The Denhardt's hybridization buffer may be stored at -15°C to -30°C if required. This modification of the Church and Gilbert buffer, is routinely used in Cytiva 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. Ensure the SDS is fully dissolved before use. This may be achieved with gentle heating.

Step	Action
2	Prepare the radiolabelled probe using the appropriate procedure. ***Note:*** *For radioactive applications use a probe concentration of 0.5–2 × 10^6 incorporated counts per mL of hybridization buffer for single copy gene detection, i.e. high sensitivity application or 0.125–0.5 × 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.*
3	Preheat the required volume of hybridization buffer to the appropriate temperature. ***Note:*** *Pre-wetting in a suitable buffer is essential for large blots (>100cm^2) or multiple blots. Details of the pre-wetting procedures are given on page 4, 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.*

Step	Action
4	Pre-wet the blot in a suitable buffer for example 5 × SSC or 0.5 M phosphate buffer. Place the blot(s) in the hybridization buffer. 125 µL of hybridization buffer per cm^2 is a suitable volume. Prehybridize for at least 30 minutes with constant agitation, at the desired hybridization temperature (see step *step 7*).
5	When using labelled double stranded probes, pipette the required amount 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. Briefly centrifuge to draw the contents to the bottom of the tube.
6	Add the probe to the prehybridization buffer. ***Note:*** *Avoid placing the probe directly on the blots, as this will cause excessive background.*
7	Hybridize overnight with gentle agitation at the required temperature. ***Note:*** *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. 65–68°C is suitable for most long probes(>100 bases). With short/oligo probes (<50 bases) hybidization*

Step	Action
	temperature are usually defined as Tm-5°C: Tm (melting temperature) = (4 x number of G+C bases) + (2 x number of A+T bases) (12) Hybridization time can also vary. Short hybridization times may be suitable for high target applications.
8	Prepare the stringency wash solutions. The wash solution should be used in excess, at least 1-5 mL/cm^2 of membrane. Low stringency wash: 2 × SSC, 0.1% (w/v) SDS Medium stringency wash: 1 × SSC, 0.1% (w/v) SDS High stringency wash: 1 × SSC, 0.1% (w/v) SDS ***Note:*** *Stringency washes will depend on the nature of the probe and target to be hybridized. Salt concentration and temperature should be 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). Some procedures include room temperature washes under*

Step	Action
	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.
9	After the hybridization, wash the blots by incubating twice, 5 minutes each, in 2 × SSC, 0.1% SDS, followed by 1 × SSC, 0.1% SDS for 15 minutes, and finally 0.1 × SSC, 0.1% SDS for 2 × 10 minutes, at the hybridization temperature.
10	Remove the blot from the last stringency wash, drain, wrap in Saran Wrap 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. ***Note:*** *The use of Saran Wrap with 35S labelled probes will significantly increase exposure times. In this case the blot should be air dried before autoradiography, if reprobing is not required.*

Hybridization in tubes

There are numerous commercially available rotisserie devices suitable for use as hybridization ovens (for example Cytiva hybridization oven/shaker RPN2510E/251E). These can accommodate 2–7 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.

Step	Action
1	Prepare the hybridization buffer, for example: **Denhardt's buffer** 5 × SSC 5 × Denhardt's solution 0.5% (w/v) SDS **Modified Church and Gilbert buffer(13)** 0.5 M phosphate buffer, pH 7.2 7% (w/v) SDS l0 mM EDTA ***Note:*** *There are a wide variety of hybridization buffers used by researchers. This Denhardt's based buffer is used in the quality control of all Hybond nylon membranes. A reduced concentration of SDS has been found to elevate backgrounds following hybridization. The Denhardt's hybridization buffer may be stored at -15°C to -30°C if required.*

Step	Action
	This modification of the Church and Gilbert buffer, is routinely used in Cytiva 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. Ensure the SDS is fully dissolved before use. This may be achieved with gentle heating.
2	Prepare the radiolabelled probe using the appropriate procedure.
3	Preheat the required volume of hybridization buffer to an appropriate temperature. ***Note:*** *High backgrounds will result if sub optimum volumes are used for the membrane and hybridization conditions.*
4	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. ***Note:*** *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 hybridization volume should be increased (70–125 µL/cm^2). The nylon mesh should be at least 0.5 cm*

Step	Action
	larger than the blot. Place the mesh in the prewetting solution before the blot, in subsequent manipulations treat as 'one'. The nylon mesh may be reused after washing in 10% (w/v) SDS and extensive rinsing in distilled water.
5	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. ***Note:*** *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.*
6	Drain the tube of excess liquid and replace with the appropriate volume of hybridization buffer. ***Note:*** *High backgrounds will result if sub-optimum volumes are used for the membrane and hybridization conditions.*
7	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.

Step	Action
8	When using labelled double stranded probes, pipette the required amount 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. Briefly centrifuge to draw the contents to the bottom of the tube ***Note:*** *For radioactive applications, use a probe concentration of 0.5–2 × 10^6 incorporated counts per mL of hybridization buffer for single copy gene detection, i.e. high sensitivity application or 0.125–0.5 × 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.*
9	Add the probe to the prehybridization buffer. ***Note:*** *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.*

Step	Action
10	Hybridize overnight at the required hybridization temperature. Prepare the stringency wash solutions. The wash solution should be used in excess. Use a volume that occupies 33–50% of the tube. Low stringency wash; 2 × SSC, 0.1% (w/v) SDS Medium stringency wash: 1 × SSC, 0.1% (w/v) SDS High stringency wash: 0.1 × SSC, 0.1% (w/v) SDS ***Note:*** *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. 65–68°C is suitable for most long probes(>100 bases). With short/oligo probes (<50 bases) hybidization temperature are usually defined as Tm-5°C: Tm (melting temperature) = (4 × number of G+C bases) + (2 × number of A+T bases) (14) Hybridization time can also vary. Short hybridization times may be suitable for high target applications.*
11	After the hybridization wash the blot as follows: a. rinse briefly in 2 × SSC, 0.1% (w/v) SDS b. wash twice, 5 minutes each in 2 × SSC,0.1% (w/v) SDS

Step	Action
	c. wash twice, 10 minutes each in 1 × SSC,0.1% (w/v) SDS
	d. wash four times, 5 minutes each in 0.1 × SSC, 0.1% (w/v) SDS
	Note: *Washing in boxes is much more effective and is recommended if feasible. The inefficiency of washing in tubes may be overcome by increasing the number of stringency washes while maintaining the same total wash time.*
12	Remove the blot from the last stringency wash, drain and wrap in Saran Wrap and expose to X-ray film, for example Hyperfilm MP. Keep the blot moist if it is to be reprobed.
	Note: *The use of Saran Wrap with ^{35}S labelled probes will significantly increase exposure times. In this case the blot should be air dried before autoradiography, if reprobing is not required.*

Stripping protocol - Hot SDS procedure

Step	Action
1	Place the moist membrane in an appropriate sized tray.

Step	Action
2	Prepare a boiling solution of 0.1% (w/v) SDS, pour the solution onto the blot and allow to cool. ***Note:*** *This step may be repeated if the probe is particularly difficult to remove.*
3	Rinse the blot briefly in 2 × 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.

5 Additional information

Determination of the optimum UV crosslinking conditions using a UV transilluminator

Step	Action
1	Produce five or six identical control blots, for example Lambda Hind III on the membrane of choice. ***Note:*** *The type of blot should reflect the technique for which the calibration is being used.*

Step	Action
2	Protect the surface of the membrane by covering the transilluminator with Saran Wrap. Expose each blot DNA side down on the transilluminator for a different length of time, for example 30 seconds to 5 minutes. ***Note:*** *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 Cytiva (the UVC 500 UV crosslinker).*
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.

Recommended applications for blotting membranes

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	+++	+++	+	--

Applications	Hybond-NX (nylon)	Hybond-XL (positively charged nylon)	Hybond-N+ (positively charged nylon)	Hybond-N (neutral nylon)
Rapid-hyb'buffer	+	+++	++	+
Northern blotting				
Radioactive detection	++	+++	+	++
Non-radioactive detection	-	-	++	-
Dot/slot blots				
Radioactive detection	++	+++	++	++
Non-radioactive detection	-	-	++	-

Applications	Hybond-NX (nylon)	Hybond-XL (positively charged nylon)	Hybond-N+ (positively charged nylon)	Hybond-N (neutral nylon)
Colony/ plaque lifts				
Radioactive detection	+++	++	+	++
Non-radioactive detection	+	-	++	+

Hybond membranes for binding protein

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

Applications	Hybond-P	Hybond ECL	Hybond-C Extra
Radioactive detection	+	+	++
Glycoprotein detection	+++	+	+
Reprobing Westerns	+++	-	+
Expression screening	+	-	+++

Key: Suitable = +, Recommended = ++, Highly recommended = +++

Not recommended = -, Unsuitable = --

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7 Related products

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™ You-Prime First-Strand Beads		27926401
First-Strand cDNA Synthesis Kit		27926101
Gene Images DNA Random-Prime Lableing kit	30 reactions	RPN3520
Gene Images 3' Oligo labeling Kit	30 reactions	RPN5770

Nucleotides

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

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

Additional products

Rapid-hyb Buffer	125 mL	RPN1635
Rapid-hyb Buffer	500 mL	RPN1636
Liquid Blocking Reagent	100 mL	RPN3601

Scanning instrumentation

Typhoon™ 8600 Variable Mode Imager

Storm' Gel and Blot Imaging System

Contact your Cytiva representative for the most current information.

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