instructions

product codes

See list to right

Hybond-XL

a nylon membrane designed for nucleic acid transfer

RPN82S	82 mm
RPN87S	87 mm
RPN132S	132 mm
RPN137S	137 mm
RPN1210S	12 x 10 cm
RPN1510S	15 x 10 cm
RPN1520S	15 x 20 cm
RPN2020S	20 x 20 cm
RPN2222S	22 x 22 cm
RPN3050S	30 x 50 cm
RPN203S	20 cm x 3 m
RPN303S	30 cm x 3 m

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.

ⓘ RPN2020SPL Rev-A, 2003

Amersham Biosciences

Handling

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
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.

Expiry
Expiry information can be found on the external packaging.

Components

Hybond-XL membranes

RPN82S 82 mm diam. 50 discs
RPN87S 87 mm diam. 50 discs
RPN132S 132 mm diam. 50 discs
RPN137S 137 mm diam. 50 discs
RPM1210S 12 x 10 cm

Page finder

20 sheets
RPN1510S 15 x 10 cm 20 sheets
RPN152S 15 x 20 cm 10 sheets
RPN2020S 20 x 20 cm 10 sheets
RPN2222S 22 x 22 cm 10 sheets
RPN3050S 30 x 50 cm 5 sheets
RPN203S 20 cmx x 3 m 1 roll
RPN303S 30 cmx x 3 m 1 roll

Other materials required

Equipment

- Agarose gel electrophoresis apparatus, for example Amersham Biosciences. GNA 200 submarine gel electrophoresis system
- Microwave
- Whatman™ 3 MM paper
- Absorbent paper towels
- Trays/dishes
- Glass plates
- 750g weight

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 Sulphate: irritant

Formaldehyde: toxic substance

Formamide: toxic substance

Ethidium Bromide: mutagenic substance

Sodium Hydroxide: corrosive

Hydrochloric Acid: corrosive

Diethylpyrocarbonate: explosive, 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.

- 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

Description

Hybond-XL™ is a novel charged nylon membrane from Amersham Biosciences. It has been designed exclusively for nucleic acid transfer applications using radioactive detection to achieve an improved signal to noise ratio. The design process used ensures a robust product capable of producing excellent results with the wide variety of procedures, applications and target/probe combinations used in life science laboratories world-wide. This new membrane retains all of the advantages of other nylon membranes:

- high binding capacity
- superior binding of small fragments
- excellent handling properties
- effective probe removal
- efficiency of reprobing

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 section page 7).

Solutions required for nucleic acid electrophoresis and blotting

All reagents should be of AnalaR™ grade where possible.

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.

10x nucleic acid loading dye mix

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.

50x TAE (DNA electrophoresis buffer)

11 ml Hydrochloric acid, concentrated (HCl)
989 ml Distilled water
Mix. Store at room temperature for up to 1 month.

Depurination solution

87.66 g Sodium chloride (NaCl)
20 g Sodium hydroxide (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.

Denaturation buffer

87.66 g Sodium chloride (NaCl)
60.5 g Trizma base
Add approximately 800 ml of distilled water.

Neutralization buffer

	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 (20x SSC)	88.23 g Tri-sodium Citrate 175.32 g Sodium chloride (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 Ethylenediamine tetra-acetic acid (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.
10x MOPS buffer	41.2 g 3-(N-morpholino)propanesuphonic Acid (MOPS) 10.9 g Sodium Acetate, 3-hydrate 3.7 g Ethylenediamine tetra-acetic acid (EDTA), sodium salt Add approximately 800 ml of nuclease free distilled water. Mix to dissolve. Adjust to pH 7 with Sodium hydroxide (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 colour.

100x 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.

Quality control

Hybond-XL is QC tested using related Amersham Biosciences products and protocols to ensure maximum compatibility and optimum performance.

Date for individual batches and detailed QC protocols are always available on request.

Description:
Nylon hybridization transfer membrane

Quality control assay:
*Hin*d III restricted human genomic DNA, separated using SepRate™ -DNA agarose, is Southern blotted onto Hybond-XL and hybridized with N-*ras* proto-oncogene probe.

Specifications:
Detection of 0.1 pg of target DNA. Background below 0.15 OD. Hybridization volume 35 $\mu l/cm^2$

Labelling and detection:
Performed using : 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.

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
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, or alkali. It is essential that fixation times, energy settings (UV) and concentration (alkali) are optimized.

Blotting protocols

This instruction for use limits itself to the classical capillary blotting technique(1) used for the transfer of separated nucleic acid fragments from an agarose gel to a solid support (2,3,4,5,6), and is representative of the procedures used in Amersham Biosciences laboratories.

Details of the gel treatments required before the transfer of DNA or RNA may be found on pages 11 and 15. Figure 1 (page 12) is a diagrammatic representation of the transfer apparatus used in this technique.

❶ Protocol for capillary blotting

1) Prepare the gel for transfer.	**1) Details of gel treatments may be found on page 11 (Southern blotting) or page 15 (Northern blotting)**
2) Cut a sheet of membrane to an appropriate size.	**2**) The membrane should be cut with clean sharp 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 3 MM paper saturated in transfer buffer.	**3**) 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	**4**) Air bubbles block the transfer of nucleic acid to the membrane. They may be removed at any stage by rolling a clean pipette or

film to prevent the transfer buffer being absorbed directly into the paper towels.	glass rod over the surface.
5) Place the membrane on top of the gel. Avoid trapping any air bubbles.	5) Do not attempt to move the membrane once it has touched the gel surface.
6) Place three sheets of 3MM 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 3MM 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.	8) 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).
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.	9) Rinsing the membrane following transfer is not advised. Extensive experimentation has shown that rinsing the membrane before fixation produces blots of variable quality because nucleic acid is removed from the membrane during this step.

10) Fix the nucleic acid to the membrane by baking at 80 °C for 2 hours or by using an optimized UV crosslinking procedure.

10) Details of an optimization procedure are given on page 41. The Hoefer UV crosslinker from Amersham Biosciences has a pre-set UV exposure (70 000 micro-joules/cm^2) which is suitable for Hybond-XL.

11) Blots may be used immediately. Blots must be thoroughly dried if storage is required.

11) Blots may be rinsed in 2× SSC before storage or hybridization. Blots should be stored wrapped in SaranWrap desiccated at room temperature under vacuum.

❷ Southern blotting – gel treatments Neutral transfer protocol

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 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.

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

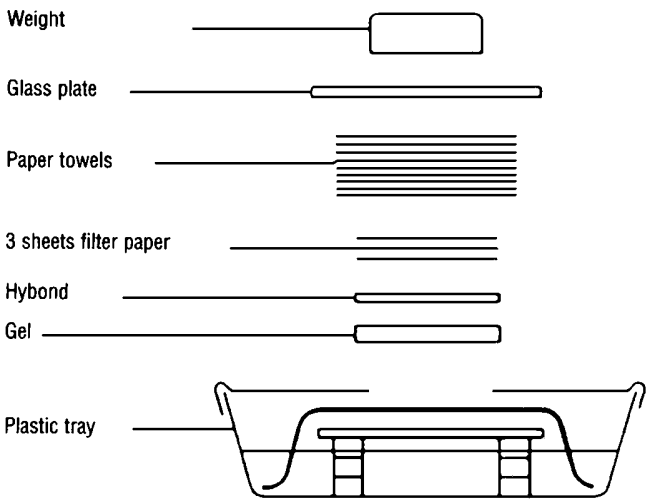

Figure 1. Diagrammatic representation of a capillary blotting apparatus

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

3a) Depurination
Place in 0.125 M HCl so that the gel is completely covered in the solution. Agitate gently for 10 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 9.

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.

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 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 visulization.

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

2) 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, rinsing the gel in distilled water between each step.

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

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.

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.
4) Set up the capillary blot as described on page 9, using Denaturation solution as the transfer buffer.

❹ 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.

Formaldehyde/Formamide protocol

1) Prepare the MOPS/Formaldehyde gel as follows:
Preheat 17.5 ml of Formaldehyde and 30 ml 10× MOPS buffer at 55 °C.

1) The Agarose gel is ≈ 0.7 M with respect to Formaldehyde and 1× 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

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.

(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 with Ethidium bromide. Excessive amounts of Ethidium Bromide will also 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 (µl)	final conc
RNA	V	
Formaldehyde	5.5	2.2 M
Formamide	15	50%
10× MOPS buffer	1.5	0.5×
Water	8-V	
TOTAL	30	

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

2) Sample must be deproteinized.

Samples may be stored at -15 °C to -30 °C for short periods.

gel.

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

3) SybrGreen is recommended for visualization. When Ethidium Bromide is used for visualization the addition of Ethidium Bromide to the electrophoresis buffer (0.01 µg/ml) improves results. Nucleic acid loading buffer must be prepared using RNase free reagents/solutions .

4) Following electrophoresis, if appropriate, 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 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× SSC. Agitate for 15 minutes. Repeat this step once more.

7) Set up the capillary blot as described on page 9 using the neutral transfer buffer.

7) 10× SSC or 20x SCC can be used as the transfer buffer.

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 4 °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. If desired the membrane may be pre-wet before use, for example on an unused agar plate or on a TE buffer saturated 3MM pad. Excess liquid must be removed from the membrane before proceeding, this is achieved by placing the disc on a dry sheet of 3MM paper.
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	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.

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 3MM paper.

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 planting 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 3MM paper pads:-

a) Denaturation step, denaturation buffer for 2-5 minutes.

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

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

When transferring membrane, remove as much fluid as possible from the underside of the membrane. This may be achieved by transferring briefly to dry 3MM paper between treatments.

7)Adequate removal of cell debris from colony lifts is essential

7) Finally, vigorously wash the membrane disc in 2x SSC to remove the proteinous debris.

8) Transfer the disc, DNA side uppermost, to a pad of dry 3MM 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 using a UV transilluminator are given on page 33. The UV crosslinker has a pre-set UV exposure (70 000 micro-joules/cm^2) which is suitable for Hybond-XL.

10) Membranes may be used immediately or stored, once dry.

10) Membranes should be stored wrapped in Saran Wrap desiccated at room temperature under vacuum.

6 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 Hoefer™ PR 600 apparatus. 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.

1) Cut the membrane to an appropriate size.

1) The membrane should be cut with clean sharp 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 8.

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 the table on page 19. A sample size of 1-2µl 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.

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 19

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 from spreading.

7) Fix the nucleic acid sample to the membrane by UV crosslinking or baking at 80 °C for 2 hours.

7) Details of an optimization procedure are given on page 23. The Amersham UV crosslinker has a pre-set UV exposure (70 000 micro-joules/cm^2) which is optimum for Hybond-XL.

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

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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]
0.5M 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.

Combine all the components, make up to the required volume.

1) This Denhardt's based buffer is that used in the quality control of all Hybond nylon membranes. A reduced concentration of SDS may lead to elevated 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 Amersham /biosciense's Laboratories. It has been shown to be suitable for S 2outherns, 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.

2) 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 (i.e. high sensitivity application) or 0.125 - 0.5 x 10^6 incorporated counts per ml of hybridization

3) Preheat the required volume of hybridization buffer to the appropriate temperature.

buffer for high target work (for example colonies/ plaques, PCR products etc). Probe purification, to remove unincorporated radioactive nucleotides, is strongly recommended

4) Pre-wet the blot in a suitable buffer for example 5x 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 Note 7).

4) Details of the pre-wetting procedures are given on page 6, 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 blots should move freely within the buffer.

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 pre-hybridization buffer.	6) Avoid placing the probe directly on the blots, as this will cause excessive background.
7) Hybridize overnight with gentle agitation at the required temperature.	7) Hybridization temperature 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) hybridization temperature is usually defined as Tm-5 °C: Tm (melting temperature) = (4x number of G+C bases) + (2x number of A+T bases) ([14]) 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. For example; Low stringency wash: 2x SSC, 0.1% (w/v) SDS	8) 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 stingency. The higher the washing temperature, the greater the stringency.

Medium stringcncy wash:
1x SSC, 0.1% (w/v) SDS
High stringency wash:
0.1x SSC, 0.1% (w/v) SDS

Most commonly, stringency washes proceed from “high salt”/ “low temperature”, for example 5x SSC, 0.1% (w/v) SDS at room temperature, to “low salt/high temperature”, for example0.1x SSC, 0.1% (w/v) SDS at 65 °C (nominal hybridization temperature).

9) After the hybridization, wash the blots by incubating twice, 5 minutes each, in 2x SSC, 0.1% (w/v) SDS, followed by 1x SSC, 0.1% (w/v) SDS for 15 minutes, and finally 0.1x SSC, 0.1% (w/v) SDS for 2 x 10 minutes, at the hybridization temperature.

9) 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.

10) Remove the blot from the last stringency wash, drain, wrap 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.

10) The use of SaranWrap ^{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.

Hybridization in tubes

There are numerous commercially available rotisserie devices suitable for use as hybridization ovens. 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.

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]
0.5M 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.

Combine all the components, make up to the required volume.

2) Prepare the radiolabelled probe using the appropriate procedure.

3) Preheat the required volume of hybridization buffer to the appropriate temperature.

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 Hybond nylon membranes. A reduced concentration of SDS may lead to elevated 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 Amersham Bioscience's 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.

3) Hybond-XL has been designed for use with very low volumes of hybridization buffer (30-70 µl/cm^2). 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.

4) 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 to 70-125 µl/cm^2.

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 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

5) 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

tube in the opposite direction to the "rolled" blot.

no signal or background following hybridization.

6) Drain the tube of excess liquid and replace with the appropriate volume of hybridization buffer.
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.

6) Hybond-XL has been designed for use with low volumes of hybridization buffer (30-70 µl/cm2). High backgrounds will result if sub optimum volumes are used for the membrane and hybridization conditions.

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.

8) 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 (i.e. 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

9) Add the probe to the pre-hybridization buffer.

9) 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.

10) Hybridize overnight at the required hybridization temperature.

10) Hybridization temperatures may vary with the probe. Lower temperatures achieve lower stringency. The temperature of hybridization used will depend on the degree of homolgy between the probe and the target.

65-68 °C is suitable for most long probes (>100 bases). With short/oligo probes (<50 bases) hybridization temperature is usually defined as Tm-5 °C:

Tm (melting temperature) =

(4x number of G+C bases) + (2x number of A+T bases) [14]

Hybridization time can also vary. Short hybridization times may be suitable for high target applications .

11) Prepare the stringency wash solutions. The wash solution should be used in excess. Use a volume that occupies 30-50% of the tube.

For example;
Low stringency wash:
2x SSC, 0.1% (w/v) SDS
Medium stringency wash:
1x SSC, 0.1% (w/v) SDS
High stringency wash:
0.1x SSC, 0.1% (w/v) SDS

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) After the hybridization wash the blot as follows:

a) rinse briefly in 2x SSC, 0.1% (w/v) SDS

b) twice, 5 minutes each in 2x SSC, 0.1% (w/v) SDS

c) twice, 10 minutes each in 1x SSC, 0.1% (w/v) SDS

d) four times, 5 minutes each in 0.1x SSC, 0.1% (w/v) SDS

13) 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.

13) The use of SaranWrap 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 protocols

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 commonly used stripping procedures are described below. Some users have experienced variability in these methods. In these cases a combination of both of these procedures has proved suitable.

Hot SDS procedure

1) Place the moist membrane in an appropriate sized tray.

2) Prepare a boiling solution 0f 0.1% (w/v) SDS, pour the solution onto the blot and allow to cool (15-30 minutes)

2) This step may be repeated if the probe is particularly difficult to remove.

3) Rinse the blot briefly in 2x 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

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.

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

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

4) Repeat step 3 with fresh alkali solution.

Additional information

Determination of the optimum UV crosslinking conditions using a UV transilluminator

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

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

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.

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 Amersham Biosciences.(RPN2500/2501).

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.

Determination of the optimum alkali fixation

1) Produce five or six identical control blots, for example Lambda *Hin*d III on the membrane of choice.	1) The type of blot should reflect the technique for which the calibration is being used.
2) Prepare a fresh solution of 0.4M NaOH. Prepare and alkali soaked pad of 3MM paper in an appropriate container.	2) Fresh alkali solution must be used. The 3MM soaked pad must be drained of excess liquid.
3) Expose each blot DNA side uppermost on the alkali soaked 3MM pad for a different length of time, for example 60 seconds to 10 minutes.	3) For storage, blots must be neutralized. Following air drying rinse briefly in 2x SSC, air dry again and store desiccated under vacuum.
4) Hybridize all the blots together with a suitably labelled probe.	
5) Following autoradiography, the optimum alkali incubation time will be indicated by selecting the bolt showing the strongest signal.	

Related products

Nucleotides

Application areas	Compound	Specific Activity Ci/mmol	Redivue code	Standard code
DNA labelling	[α–^{32}P]dATP	~3000	AA0004	PB10204
	[α–^{32}P]dCTP	~3000	AA0005	PB10205
	[α–^{32}P]dGTP	~3000	AA0006	PB10206
	[α–^{32}P]dTTP	~3000	AA0007	PB10207
RNA labelling	[α–^{32}P]ATP	~3000		PB10200
	[α–^{32}P]CTP	~3000		PB10202
	[α–^{32}P]GTP	~3000		PB10201
	[α–^{32}P]UTP	~3000		PB10203
End labelling of DNA	[γ–^{32}P]ATP	~3000	AA0068	PB10168
		>5000	AA0018	PB10218
	[γ–^{32}P]GTP	>5000		PB10244
	[α–^{32}P]ddATP	~3000		PB10233

Only some of the wide variety of nucleotides codes available are shown, please refer to the catalogue for a comprehensive listing.

Labelling Kits

Megaprime DNA labelling system	
Suitable for use with labelled dCTP	RPN1606/1607
Suitable for use with any labelled dNTP	RPN1604/1605
Ready to go labelling beads	
Ambient stable single dose reaction spheres for use with dCTP	27-9240-01
Rediprime DNA labelling system premixed individually dispensed freeze dried Megaprime reactions for use with dCTP only	RPN1633/1634
End labelling kits	
3' end labelling	N4020
3' end labelling	RPN1509
RNA labelling kits	RPN3100 27-9276-01

Please contact your local customer service office for ordering details associated with Amersham Biosciences products.

Companion products

ProbeQuant™ G-50 Micro Column	27-5335-01
SeeDNA™, co-precipitant	RPN5200

A range of branded a autoradiography products

Hyperfilm
Hypercassettes
Hyperscreens
Kodak™ BioMax™
Kodak™ X-Omat

Please refer to the catalogue for a comprehensive listing.

Please contact your local customer service office for ordering details associated with Amersham Biosciences products.

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Legal

Product information

Product name	code
Hybond-XL FA nylon membrane designed exclusively for nucleic acid transfer	RPN2020S

Related products

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