instructions

product codes

RPN4800/1/2/3/4

Hybond Northern Blot

for examination of gene expression profiles from human or mouse tissues

RPN4800 Human d 12 poly A^+ RNAs
Tissues represented; (in order): brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocyte.

RPN4801 Human a 8 poly A^+ RNAs
Tissues represented; (in order): heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas.

RPN4802 Human b 7 poly A^+ RNAs
Tissues represented; (in order): stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland bone marrow.

RPN4803 Human c 8 poly A^+ RNAs
Tissues represented; (in order): spleen, thymus, prostate, testis, uterus, small intestine, colon, peripheral blood leukocytes.

RPN4804 Mouse a 8 poly A^+ RNAs
Tissues represented; (in order): heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis.

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.

RPN4800PL Rev-A, 2001

Amersham Biosciences

Handling

Storage: The kit components should be stored as follows

- Store unused Hybond™ Northern Blots at room temperature in a sealed plastic bag protected from light
- Store used Hybond Northern Blots at -15 °C to -30 °C in a sealed plastic bag until required
- Store Control probe at -15 °C to -30 °C
- Store Rapid-hyb™ hybridization buffer at room temperature. A precipitate may form at lower temperatures but can easily be dissolved by gently warming at 65 °C and stirring thoroughly with a magnetic stirrer.

Expiry: For expiry details see outer packaging

Page finder

Components

Each kit contains the following

Hybond Northern Blot: containing 2 µg of poly A^+ RNA isolated from different tissues (see front cover for specific blot details).

Human β-actin cDNA control probe: (2.0kb) in 20 µl of TE buffer, 100 ng

Rapid-hyb hybridization buffer: 25 ml

Sufficient reagents for 2 labelling and hybridization experiments

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 and components are 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.

Human blood products provided as a component of this pack have been obtained from donors who were tested individually and were found to be negative for the presence of Human Immunodeficiency Virus antibody (HIV-Ab)* as well as for Hepatitis B surface antigen (HBsAg) using EIA.

As no test method can offer complete assurance that Hepatitis B virus, Human

Other materials required

Labelling and hybridization with radioactive probes

- Megaprime™ DNA Labelling Kit, RPN1604
- Redivue™ [α-^{32}P] dCTP, 3000 Ci/mmol, AA0005
  or
- [α-^{32}P] dCTP, 3000 Ci/mmol, PB10205
- Sterile RNase free water, US70783-500 ml
- 0.5 M Sodium EDTA, US15694-100 ml
  Prepare 10 ml of 0.2 M Sodium EDTA by diluting 4 ml of 0.5 M solution with 6 ml of sterile nuclease free water
- ProbeQuant™ G-50 Micro Columns, 27-5335-01
- 20×SSC (3 M NaCl, 0.3 M Sodium Citrate pH 7.0), US19629-500 ml
- 20% SDS US75832-500 ml
- Prepare 500 ml of the following wash buffers as follows:
  2× SSC, 0.1% SDS
  50 ml of 20× SSC

Immunodeficiency Virus antibody (HIV-Ab) or other infectious agents are absent, all human blood products should be considered potentially infectious. Handling, use, storage and disposal should be in accordance with the procedures defined by an appropriate National biohazard safety guideline or regulation, where it exists (for example USA Centre for Disease Control/National Institutes of Health manual 'Biosafety in Microbiological and Biomedical Laboratories').

* HIV is the abbreviation used for HTLV-III and LAV.

Instructions relating to handling and use of test materials.

1) All operations should be carried out in restricted areas by trained and authorized persons. Storage of test materials should be in specially designated areas accessible only to authorized personnel.
2) Wear appropriate protective laboratory clothing including disposable gloves. Avoid 'sharps' (for example sharp cutting edges, needles, scissors) which may puncture the skin or damage protective clothing. Do not spill or splash reagents or form an aerosol. No smoking, eating or drinking should be allowed in areas where specimens or test materials are handled. Do not pipette by mouth. Wash hands before leaving the

2.5 ml of 20% SDS
1× SSC, 0.1% SDS
25 ml of 20× SSC
2.5 ml of 20% SDS
0.1× SSC, 0.1% SDS
2.5 ml of 20× SSC
2.5 ml of 20% SDS
made up to 500 ml with water

- 50× TE buffer, US75834-100 ml
  Prepare 10 ml of 1× TE buffer by diluting 200 µl of 50× stock with 9.8 ml of sterile nuclease free water

The preceding materials are all available from Amersham Biosciences.

Analysis of incorporation of radioactivity into probes

- PEI cellulose chromatography plates with glass support, Merck 5725
- 1.25 M KH_2PO_4, 170.11 g per litre
- Carrier DNA 50 mg/ml
  Dissolve 500 mg fish sperm DNA (Amersham Biosciences US14405-10 g) in 10 ml of sterile nuclease free water. Shear with

laboratory.

Decontamination and disposal of waste

All specimens and test materials should be decontaminated before disposal. An effective way of decontaminating waste is subjecting it to autoclaving for a minimum of 15 minutes at 121 ºC or higher. Decontaminated materials may be disposed of as laboratory waste.

Spills and breakages

Cover any spills with absorbent material and saturate with a disinfectant appropriate to the surface and material spilt and use according to the manufacturer's instructions. Carefully gather up all of the materials and decontaminate the area with fresh disinfectant. 'Sharps' should be disposed of in a secure and safe container. Dispose of all materials used to wipe up the spills as though potentially infectious.

repetitive pipetting with a small gauge needle. Store at -15 °C to -30 °C in small aliquots.
- 10% Trichloroacetic Acid (TCA), Sigma T 9159. Dissolve 10 g TCA in sterile water

Labelling and hybridization with non-radioactive probes

- AlkPhos Direct™ Labelling and Detection System with ECF™, Amersham Biosciences RPN3692
- Primary wash buffer:

Urea	120 g
SDS	1 g
0.5 M Sodium Phosphate buffer pH 7.0	100 ml
NaCl	8.7 g
1 M $MgCl_2$	1 ml
Blocking reagent from kit	2 g
Water to 1 litre	

  Store at 2-8 °C for up to one week.
- Secondary wash buffer: Prepare 20× stock buffer by dissolving 121 g Tris base and 112 g of NaCl in water. Adjust pH to 10 with HCl. Make up to 1 litre with water. Store at

Description

Hybond Northern Blots are pre-made, ready to hybridize RNA blots prepared using the highest quality mRNA from different tissue types, containing full-length poly A^+ transcripts with virtually no genomic DNA contamination. Hybond Northern Blots allow you to examine gene expression profiles from human or mouse tissues, providing easy access to mRNA from tissues that are difficult and costly to obtain. See Table 1 for list of Hybond Northern Blots and details of the mRNA samples they contain.

Each lane in Hybond Northern Blots contains approximately 2 µg of purified poly A^+ RNA. The amount of poly A^+ RNA has been adjusted to obtain a consistent β-actin signal in each lane. Therefore, the actual amount of RNA loaded may vary slightly between samples. The RNA has been separated on a denaturing formaldehyde/1.0% agarose gel and blotted onto positively charged nylon membrane.

100 ng of a 2.0 kb human β-actin cDNA control probe is included in each kit. This probe efficiently cross-hybridizes with mouse β-actin and is therefore a suitable control for all Hybond Northern Blots. Because of the abundance of β-actin in most tissues, the control probe produces a

2-8 °C for up to four months.
Dilulute 50 ml of 20× stock to 1 litre with water and add 2 ml of 1 M $MgCl_2$. Do not store.

- Glass fibre filters, Whatman GF/F filter
- Hyperfilm™ MP Amersham Biosciences RPN6H or Kodak™ BioMax™ MS Amersham Biosciences V8222648
- SaranWrap™ cling film
- Scintillation counter
- Hybridization oven, Amersham Biosciences

- Typhoon™ 8600 Variable Mode Imager or Storm™ Gel and Blot Imaging System

strong hybridization signal. It is therefore recommended that the control probe is used only after hybridizing with your own probe, or if you are experiencing problems with other probes.

This booklet includes complete protocols for using the Hybond Northern Blots with radioactive and non-radioactive probes. Megaprime DNA Labelling System is recommended for generating radioactively labelled probes. These probes are suitable for use with the Rapid-hyb hybridization buffer supplied with the membrane filters.

Rapid-hyb buffer reduces background and decreases hybridization time.

Table 1. Contents of Hybond Northern Blots

Lane	RPN4800 Human d 12 poly A^+ RNA	RPN4801 Human a 8 poly A^+ RNA	RPN4802 Human b 7 poly A^+ RNA	RPN4803 Human c 8 poly A^+ RNA	RPN4804 Mouse a 8 poly A^+ RNA
1	brain	heart	stomach	spleen	heart
2	heart	brain	thyroid	thymus	brain
3	skeletal muscle	placenta	spinal cord	prostate	spleen
4	colon	lung	lymph node	testis	lung
5	thymus	liver	trachea	uterus	liver
6	spleen	skeletal muscle	adrenal gland	small intestine	skeletal muscle
7	kidney	kidney	bone marrow	colon	kidney
8	liver	pancreas		peripheral blood leukocyte	testis
9	small intestine				
10	placenta				
11	lung				
12	peripheral blood leukocyte				

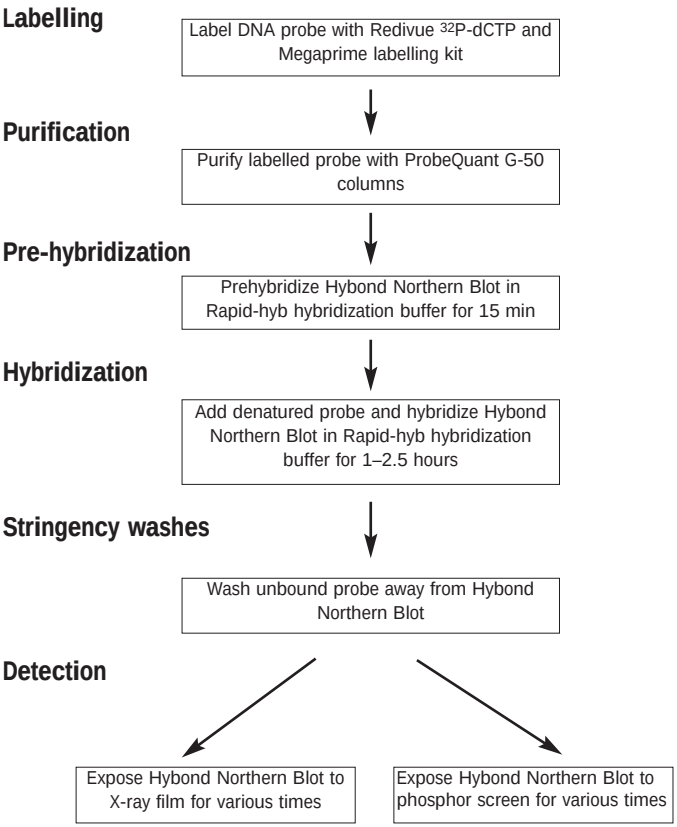

Figure 1. Overview of Hybond Northern Blot protocol with radioactive probes

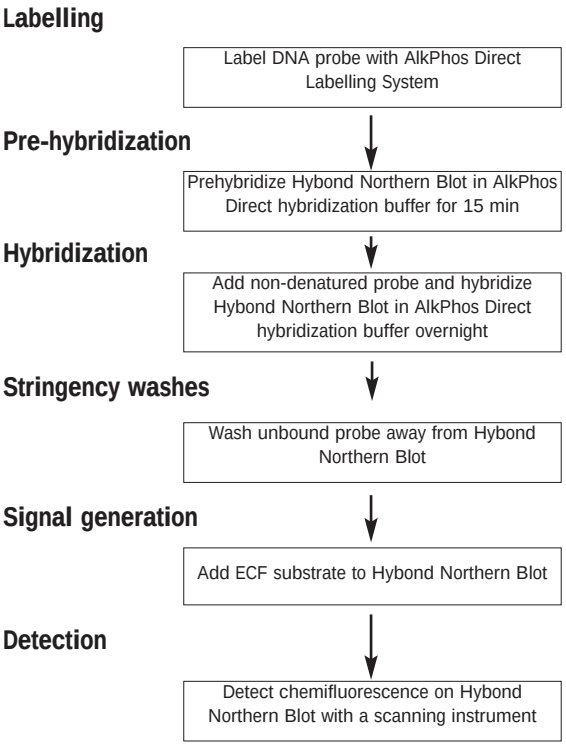

Figure 2. Overview of Hybond Northern Blot protocol with AlkPhos direct labelled probes

Protocol

❶ Introduction

This booklet provides all the information required to use Hybond Northern Blots successfully. It is strongly recommended that the protocol is read thoroughly before starting experiments and is followed precisely. Perform all procedures carefully and follow local safety precautions when working with radioactive materials. Always handle the Hybond Northern Blots carefully with forceps and do not expose the blots to nucleases or chemicals not recommended in the following protocols.

Each lane of Hybond Northern Blots contains approximately 2 µg of poly A^+ RNA purified from seven to twelve different tissues. Refer to Table 1 for the contents of each blot. On the reverse side of the blot, the positions of RNA molecular weight markers are written in ink. The notch on the lower left corner of the membrane shows the position of the first lane of the blot. Figure 3 shows a diagram of the Hybond Northern Blots

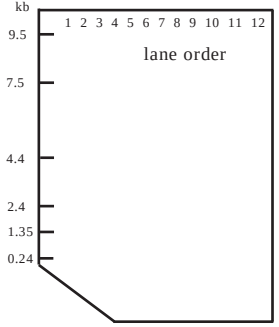

Figure 3. Hybond Northern Blot diagram. Markings are shown on the reverse side of the blot.

The following sections contain complete protocols for radioactive hybridization with the Megaprime DNA Labelling System and non-radioactive hybridization with AlkPhos Direct and ECF detection. These two protocols have been evaluated with Hybond Northern Blots and are recommended for good results. However other probe labelling methods such as oligolabelling, RNA probe labelling and nick translation can also be used.
If you are not experienced with hybridization techniques we recommend that you to perform an initial hybridization with the human β actin control probe to familiarize yourself with the system.

❷ Using Hybond Northern Blots with radioactively-labelled DNA probes

Labelling DNA probes with Redivue nucleotides and Megaprime DNA Labelling System

- Set a waterbath at 37 ºC.
- Place the required tubes from the Megaprime system, excluding the enzyme, at room temperature to thaw. Leave the enzyme at -15 ºC to -30 ºC until required.
- Dilute the DNA to be labelled to a concentration of 5 ng/µl in either sterile nuclease free water or 10 mM TE buffer.
- Place 5 µl of template DNA containing 25 ng of DNA into a clean microcentrifuge tube and add to it 5 µl of primers. If necessary adjust the final volume to 10 µl by adding sterile water to the sample. Denature by heating to 95–100 ºC for 5 minutes in boiling water.
- Spin briefly in a microcentrifuge to collect the contents of the sample to the bottom of the tube.

- Keeping the tube at room temperature, add the following reagents to the denatured template primer mix. Handle radioactivity only in designated areas behind appropriate shielding:

Template-primer mix	10 µl
10× reaction buffer	5 µl
dATP	4 µl
dGTP	4 µl
dTTP	4 µl
Enzyme	2 µl
Sterile water	z µl
Final volume	45 µl

The volume corresponding to z should be adjusted so that the reaction volume is 45 µl after this step. Return the enzyme to a freezer immediately after use.

- Cap the tube and spin for a few seconds in a microcentrifuge to bring the contents of the sample to the bottom of the tube.
- Perform the following steps in an area dedicated to handling radioactive materials. Use appropriate shields and protective clothing. Add 5 µl of Redivue [α-^{32}P]dCTP, specific activity 3000 Ci/mmol, (Amersham Biosciences code AA0005) to the labelling mix. Mix the solution gently by pipetting up and down. Avoid vigorous mixing as it can result in severe loss of enzyme activity. Use [α-^{32}P] dCTP that is less than one week old. Standard formulation of [α-^{32}P] dCTP can also be used.
- Incubate the labelling mix at 37 ºC for 10 minutes. This incubation time is sufficient for labelling the DNA to a high specific activity. If desired, the labelling reaction can be left for up to 1 hour at this temperature.
- Stop the labelling reaction by adding 5 µl of 0.2 M EDTA. Labelled probe can be stored at -15 ºC to -30 ºC in a non-frost free freezer. Prolonged storage of [α-^{32}P]-labelled probes can lead to substantial

probe degradation. High specific activity probes should not be stored longer than 3 days.

Determination of specific activity of the probe

Always calculate the specific activity of your probe. Two alternative protocols for performing this analysis are given below. Following these protocols will ensure that a useful probe has been prepared. Furthermore, this information is necessary for adjusting the amount of probe in the hybridization mix for optimum results.

Method A: Thin layer chromatography

- Prepare a PEI cellulose chromatography plate for use by cutting a thin linear groove into the nitrocellulose layer at 2 cm distance from the top edge of the plate (a pipette tip is ideal for this). Be careful to cut the groove all the way to the edge of the plate, or the buffer will be able to over-run. Mark sample positions along a line that is 2.5 cm from the bottom edge of the plate, about 2 cm apart. This will ensure that there is no interference from neighbouring tracks during evaluation.
- Spot 1 µl samples of labelling reactions along the marked line. Do not damage the PEI-cellulose layer with the tip of the pipette.
- Place the plate in a rectangular chromatography tank so that the bottom of the plate is immersed 2 cm deep in 1.25 M K_2HPO_4. Cover the tank and let sample separation take place. Make sure that the level of the buffer is below the level of the marked sample line. [α-^{32}P]dCTP incorporated into cDNA will not move far from the sample line. Free [α-^{32}P]dCTP will move progressively towards the top of the plate.
- When the buffer has reached the top groove remove the plate from the tank and allow to air-dry.
- Wrap the plate in cling film and expose it to a phosphor screen for up

to one hour. The exact exposure time needs to be chosen so that the phosphor screen is not overexposed. Overexposure will lead to the saturation of signal and erroneous results.

- Scan the phosphor screen on a Storm or Typhoon scanner, or equivalent, with recommended settings for storage phosphor screens.
- Use ImageQuant™ software to calculate the proportion of [α-^{32}P]dCTP that has been incorporated into cDNA. Consult the manual provided with the software for details about performing quantification.

Note: It is also possible to use other methods to estimate the percentage incorporation obtained, such as autoradiography followed by densitometric analysis, or use of a dedicated instrument. In such cases, refer to the manufacturer's handbook for the correct protocol.

Method B: Precipitation with TCA

- Remove 1 or 2 µl aliquot of the reaction mixture to a clean microcentrifuge tube containing 20 µl of water or TE buffer. Mix well by pipetting up and down.
- Transfer 1–10 µl of diluted reaction mixture to two duplicate tubes containing 200 µl of 0.2 M EDTA and 50 µl of carrier DNA solution. Mix well.
- Set aside half of the volume from each tube for the determination of total input radioactivity.
- To the rest of the diluted samples add 2 ml of ice-cold 10% Trichloroacetic Acid (TCA) solution, mix by vortexing and allow to stand in an ice bath for 10–15 minutes. The labelled DNA and carrier DNA will precipitate. Note that TCA is corrosive, and care should be taken when handling it.
- Collect the precipitate by vacuum filtration on a glass fibre or nitrocellulose filter disc.

- Wash the filter discs six times with 2 ml of 10% TCA solution and dry the filter thoroughly, for example using an infra red lamp. Avoid overheating.
- Count the dried filter discs by liquid scintillation or Cerenkov counting and also count the samples set aside for determining the total radioactivity in the sample.
- Determine % incorporation as follows:

$$\% \text{ incorporation} = \frac{\text{mean counts on washed filters}}{\text{mean counts in liquid samples}} \times 100$$

Calculation of probe specific activity

Because labelling with the Megaprime kit will result in net synthesis of cDNA, the amount of cDNA present at the end of reaction (A) must first be calculated.

$$\text{Total cDNA} = \frac{\text{Total no. of } \mu\text{Ci added} \times 13.2^* \times \% \text{ incorporation}}{\text{No. of radioactive dNTPs added} \times \text{av. spec. ac. of dNTPs}} + 25$$

This formula assumes 25% content of each of the four dNTPs in the newly synthesized cDNA.

*13.2 equals four times the average molecular weight of the four dNTPs divided by 100.

The amount of radioactivity incorporated during the reaction in dpm can be calculated as follows:

B = total number of µCi added $\times 2.2 \times 10^4 \times$ % incorporation

Thus the specific activity of the labelled DNA is:

Specific activity = B/A $\times 10^3$ dpm

Using 5 µl of [α-^{32}P]dCTP, specific activity 3000 Ci/mmol (code AA0005), is expected to result in about 1.9 dpm/µg of probe. More detail about the Megaprime kit (RPN1604) can be found in the

corresponding instruction manual supplied with the kit.

Purification of the probe

DNA probes labelled with the Megaprime kit can be used in hybridization without purification. However, removal of unincorporated radioactive nucleotides can reduce background signal. ProbeQuant G-50 columns that contain Sephadex™ G-50 DNA grade F can be used to purify these probes.

- Prepare the columns for nucleotide removal by resuspending the resin in the column by vortexing gently.
- Loosen the cap a quarter of a turn and snap off the bottom closure.
- Place the column in a 1.5 ml screw-cap microcentrifuge tube for support. Alternatively, remove the cap from a standard microcentrifuge tube and use this tube for support. If using a 1.5 ml microcentrifuge tube, 10–20 µl of fluid will remain in the tip of the column after spinning. Blot this fluid from the column using a clean paper towel before applying sample to the column.
- Spin the column for 1 minute at 735 × g. Start the timer and the microcentrifuge simultaneously. Use the column immediately after preparation to avoid drying of the matrix.
- Place the column in a new 1.5 ml tube and slowly apply the sample to the centre of the angled surface of the compacted resin bed, being careful not to disturb the resin. Do not allow any of the liquid to flow around the sides of the bed.
- Spin the column for 2 minutes at 735 × g. Start the timer and the microcentrifuge simultaneously. The purified sample is collected at the bottom of the tube. Discard the column.
- Remove 2 × 1 µl and apply to a PEI cellulose TLC plate to check that purification has been successful. Perform this analysis as detailed on page 14.

Hybridization of Hybond Northern Blots using Rapid-hyb buffer.

Rapid-hyb buffer is a rate enhancing hybridization buffer for rapid hybridization of radiolabelled nucleic acid probes to membrane-bound targets. Its chemical blocking agents ensure low backgrounds, without the need to use carrier DNA. Rapid-hyb buffer should be stored at room temperature.

This protocol is for using radioactive probes only.

- Pre-warm the Rapid-hyb buffer to 65 °C for using with a DNA probe. If an RNA probe is used 70 °C is recommended and for oligonucleotide probes 42 °C.
- Mix the hybridization buffer well to dissolve any precipitate.
- Immerse the blot completely in 5 ml of Rapid-hyb buffer. Make sure that all of the membrane becomes thoroughly wet. Transfer the blot and hybridization buffer into a hybridization bottle, or plastic box. Alternatively, seal the wet blot into a plastic bag with 7 ml of Rapid-hyb buffer (this ensures that over 0.125 ml/cm^2 of Rapid-hyb buffer is used). In any container, the blot should be completely covered by the Rapid-hyb buffer. If necessary add more hybridization solution.
- Perform pre-hybridization for at least 15 minutes at the correct temperature for your probe. Use either a rotary hybridization oven or a shaking water bath.
- Denature the probe prepared by Megaprime Labelling System by heating it at 95–100 °C for 5 minutes. Cool the probe on ice for 5 minutes.
- Add enough of the Megaprime labelled radioactive probe to achieve probe concentration of about 2 ng/ml. If you are performing hybridization in bottles, transfer approximately half of one Megaprime labelled probe into 5 ml of Rapid-hyb buffer. Add this probe mix to the 5 ml of Rapid-hyb buffer used for pre-hybridization,

this will give a final volume of 10 ml for the hybridization. For other containers, add sufficient probe to give the correct final concentration (as in Table 2 below) in the total volume of the hybridization buffer to 1 ml of Rapid-hyb buffer. Mix this with the pre-hybridization solution. Do not add the concentrated probe directly to the membrane, mix it with the remainder of the Rapid-hyb hybridization buffer first.

Rapid-hyb buffer can be used with all types of radioactively labelled nucleic acid probes. The following probe concentrations should not be exceeded as it may lead to increased backgrounds.

Table 2. Concentration of probe in hybridization solution

Random primed DNA probe	2 ng/ml
Nick™ translated DNA probe	2 ng/ml
RNA probe	6 ng/ml
Oligonucleotide probe	10 ng/ml

Perform hybridization with shaking for 1–2.5 hours at 65 °C for a Megaprime probe. Choose the correct hybridization temperature and time for the type of probe you are using.

Table 3. Recommended hybridization conditions

Random primed DNA probe	65 °C	1–2.5 hours
Nick translated DNA probe	65 °C	1–2.5 hours
RNA probe	70 °C	1–2.5 hours
Oligonucleotide probe	42 °C	30–60 minutes

These recommendations are based on an average (G+C) content of 40%. For probes having different composition, hybridization temperature should be determined empirically.

- Remove the hybridization solution and discard it.
- Rinse the blot briefly with 2 × SSC, 0.1% (w/v) SDS
- For DNA or RNA probes, perform the following stringency washes: 20 minutes in 50 ml of 2 × SSC, 0.1% (w/v) SDS at room temperature. 2 × 15 minutes in 50 ml of 1 to 0.1 × SSC, 0.1% (w/v) SDS at 65 °C.

The stringency of the washes can be increased by decreasing the amount of SSC in the wash buffer and also by raising the temperature, especially for RNA probes, up to 70 °C. For high stringency, washes with 0.1 × SSC, 0.1% SDS (w/v) are recommended.

If an oligo probe was used, perform the first wash as for DNA and RNA probes, but use the hybridization temperature for the second and third wash.

- Remove excess wash solution from the blot. Wrap the washed membrane in SaranWrap for detection. Do not let the membrane dry during detection, as it will compromise further use of the blot.

Detection of radioactive hybridization signal

The polyA$^+$ RNA has been immobilized to the unmarked side of the blot. Strongest hybridization signal will be detected from this side. The radioactive hybridization signal on the Hybond Northern blot can be detected with autoradiography using a sensitive X-ray film such as Hyperfilm MP, or Kodak BioMax MS. For maximum sensitivity, perform exposures to film at -70 °C using two intensifying screens and pre-flashed film. It is recommended that varying exposure times are tried to find optimal exposure for the signal derived from each hybridization probe. Alternatively, storage phosphor detection using phosphor screens and scanning imagers such as Typhoon 8600 Variable Mode Imager or Storm Gel and Blot Imaging System can be performed. These detection systems have a large dynamic range and will allow fast detection of varying signal intensities. Furthermore, the use of a

scanning instrument allows easy quantification of the hybridization signals.

Removing radioactive probe from the Hybond Northern Blots

Radioactive probe can be removed from the Hybond Northern Blot with the following protocol:

- Prepare 0.5% (w/v) solution of SDS in sterile water.
- Heat 100 ml of 0.5% (w/v) solution of SDS to 95–100 °C.
- Remove the blot from the plastic wrap and place it in the heated solution. Minimize exposure to air.
- Incubate the blot in the hot solution at room temperature for 10 minutes, shaking frequently.
- Allow the solution to cool for a further 10 minutes.
- Remove the blot from the solution, shake off excess liquid and place the blot in a plastic bag or cover it thoroughly with SaranWrap. Do not let the membrane dry. Store the membrane at -15 °C to -30 °C until further use.
- If radioactivity remains on the blot after the stripping procedure, it can be removed by allowing it to decay for 2-3 half lifes. The half life of ^{32}P is 14 days.
- Stripping success can be monitored with a Geiger counter.

❸ Hybridization of Hybond Northern Blot with AlkPhos Direct labelled probe

The following protocol contains instructions for using AlkPhos Direct Labelling and ECF Detection System (Amersham Biosciences RPN3692) with the Hybond Northern Blots. For more information on using this kit please refer to the instruction booklet distributed with the product.

AlkPhos Direct Labelling System attaches a thermostable alkaline phosphatase to DNA or RNA. The optimized hybridization buffer supplied with the labelling system contains rate enhancers that shorten hybridization time, increase sensitivity of detection and protect the enzyme against denaturation during hybridization. After hybridization, the alkaline phosphatase in the bound probe can be detected with ECF substrate. This substrate produces chemifluorescent signal that can be detected with suitable scanning instruments such as FluorImager™ and Storm. ECF signal can be quantitated from the data obtained from these instruments.

Labelling of DNA probe with AlkPhos Direct

- In a microcentrifuge tube, dilute 100 ng of the DNA probe to be labelled to 10 ng/µl with sterile nuclease free water. The concentration of salt in this sample should be kept as low as possible and should not exceed 50 mM. The DNA to be labelled should be longer than 300 bp.
- Boil the diluted DNA sample for 5 minutes to denature the DNA into single-stranded form.
- Immediately cool the DNA solution on ice for 5 minutes.
- Spin the sample briefly in a microcentrifuge to collect all solution to the bottom of the tube. Return the sample to ice.
- Add 10 µl of reaction buffer. Mix the sample gently.
- Add 2 µl of labelling reagent. Mix the sample gently.
- Dilute 3 µl of cross-linking reagent with 12 µl of water. From this dilution, transfer 10 µl into the labelling mix. Mix thoroughly and spin the sample briefly in a microcentrifuge to collect all solution to the bottom of the tube.
- Incubate the reaction for 30 minutes at 37 °C.
- The probe is ready to be used in a hybridization or can be stored for

up to 2 hours on ice.

Hybridization of Hybond Northern Blots with AlkPhos Direct probes

- Prepare hybridization solution by adding 2.92 g of NaCl (final concentration 5 M) and 4 g blocking reagent (supplied with the kit, final concentration 4%,w/v) to 100 ml of hybridization solution. Mix at room temperature for 1–2 hours until all components have dispersed into a fine suspension. This buffer can be stored at -15 °C to -30 °C in suitable aliquots.
- Pre-heat 10 ml of prepared hybridization buffer to 55 °C.
- Place the blot into the warmed hybridization buffer. Pre-hybridize with continuous mixing for at least 15 minutes at 55 °C. The hybridization buffer should cover all of the blot.
- Remove 1 ml of hybridization buffer and mix all the AlkPhos Direct labelled probe with it. Do not denature the probe before use.
- Mix the probe with the remainder of the hybridization solution. Avoid placing the probe directly onto the blot.
- Hybridize at 55 °C overnight with continuous mixing. Higher hybridization temperatures, up to 75 °C can be used to achieve higher stringency.
- Preheat 300 ml of primary wash buffer to 55 °C.
- Remove the blot from the hybridization buffer.
- Wash the blot twice for 10 minutes at 55 °C in the primary wash buffer. Use gentle agitation.
- Wash the blot twice for 5 minutes at room temperature in the secondary wash buffer. Blot can be left in this buffer for up to 30 minutes.
- Proceed directly to detection of signal.

Detection of hybridization signal with ECF substrate

- Pour the entire contents of the detection buffer bottle into the bottle containing the ECF substrate. Mix for 10 minutes on a rollermixer to dissolve the contents. Store the dissolved substrate in aliquots at -15 °C to -30 °C.
- Drain off any excess wash buffer from the blot and place it sample side up (the unmarked side of the blot) on a clean piece of SaranWrap or plastic.
- Pipette 1.3 ml of the mixed detection reagent onto the blot. Spread the solution evenly onto the blot with the help of a plastic 5 ml pipette. Incubate for 1 minute at room temperature.
- Transfer the blot onto a clean piece of SaranWrap and fold the cling film to cover the blot completely. Alternatively, seal the blot into a plastic bag. In any case, spread the reagent evenly over the blot.
- Incubate the blot at room temperature in the dark, for example in a film cassette. At suitable intervals, scan the blot with a suitable scanner such as the FluorImager or Storm. Place the blot on the scanning bed so that the unmarked side containing the samples faces downwards. Wetting of the surface underneath the blot will improve scanning results. The ECF substrate has a broad excitation spectrum, maximum excitation occurring at 430 nm and an emission maximum at 560 nm. Refer to the instruction manual of your instrument as to how to set up the scan.
- The blot can be scanned several times, at different intervals. The alkaline phosphatase enzyme will continue to convert the ECF substrate into a fluorescent form for up to 24 hours. For abundant mRNAs acceptable results may be achieved in 1 hour, whereas scanning up to 24 hours after addition of the substrate will provide a stronger signal that is suitable for detecting transcripts that are expressed at lower levels.

- ECF signal can be quantitated from the data obtained from the scanning instruments.

Removal of AlkPhos Direct labelled probe

- Wash the blot twice in 50 ml of absolute alcohol (>99% Ethanol) at room temperature for 10 minutes with gentle agitation.
- Wash the blot in 100 ml of 0.5% (w/v) SDS at 60 °C for 1 hour with gentle agitation.
- Rinse the blot in 50 ml of 0.1 M Tris-HCl, pH 8.0 at room temperature for 5 minutes.
- Wrap the blot in SaranWrap and store at -15 °C to -30 °C until further use. Do not allow the membrane to dry.

Interpretation of hybridization results

Expected results with the β-actin cDNA control probe

The amounts of different polyA$^+$ RNAs on the Hybond Northern Blot have been adjusted so that a visible band of 2.0 kb is present in each lane. This transcript is very abundant in most tissues, and consequently a strong hybridization signal should be detectable in a few hours. This signal should be clearly stronger than the general background signal. Pancreas and testes produce lower β-actin signals than other tissues. In heart and skeletal muscle, there are other abundant isoforms of actins, giving rise to bands of 1.6-1.8 kb and 2.0 kb. This is because of cross-hybridization of the β-actin probe with α- and γ-actin mRNAs. Depending on the stringency, other tissues may also show the presence of other actin isoforms. Because of the strong signal produced with this control probe, it is recommended that a hybridization with a probe of interest is performed first, as it may be difficult to remove the β-actin probe and it may mask other less abundant signals.

Hybridization signals

The expression level of gene transcripts can vary significantly between different tissues, on the other hand most genes are expressed in several tissues. In order to detect hybridization signals from tissues expressing the gene of interest at a low level, it is recommended that several film or phosphor screen exposures of differing times are performed. This may necessitate exposures up to several days. If you are using X-ray film for detection, use intensifying screens and perform exposures at -70 °C. Make sure that film developing reagents are fresh and produce clean results. Increased sensitivity of detection can be achieved with the use of radioactive probes, especially with radiolabelled RNA probes. The use of phosphor screens, in conjunction with a scanning instrument such as Typhoon or Storm, offers the benefit of wider dynamic ranges, which decrease the need for multiple exposure times. Do not saturate

the detection capability of either the film or phosphor screen.

Many genes belong to families of closely related genes that share considerable sequence homology. Long DNA probes may not be able to discriminate between these very similar transcripts. This may lead to the appearance of multiple hybridizing transcripts in some tissues, or to the presence of the transcripts in unexpected tissues. The use of oligonucleotide probes allows a more specific detection of related transcripts, if the probe sequences are chosen from regions of low homology between gene family members.

Each time the Hybond Northern Blot is stripped of previous activity with harsh conditions (such as those necessary to remove strongly bound probes), some of the immobilized polyA^+ RNA becomes detached from the blot. This loss may be proportionally greater from some samples than others, resulting in deviation from the original expression pattern observed in the tissues. Hence, the information derived from successive re-hybridizations may not be truly representative of the real situation.

If accurate estimation of the abundance of multiple specific transcripts in different tissues is required, then other more quantitative methods such as ribonuclease protection assay and quantitative reverse transcription polymerase chain reaction are recommended.

Estimation of transcript size

The Hybond Northern Blots show the migration positions of RNAs with sizes varying from 0.24 to 9.5 kb. This information can be used to estimate the size of the transcripts of interest detected on these blots. For the preparation of the Hybond Northern Blots, the separation of mRNAs was performed in 1% formaldehyde gels. These conditions result in the best resolution of transcripts in the range of 1.0–4.0 kb. The size of smaller and larger transcripts will not be estimated optimally. Furthermore, some transcripts may contain secondary

structures that are not fully removed during the run conditions. For accurate analysis of mRNA sizes, at least two different methods of denaturation should be used, as described in (1).

Additional information

Troubleshooting guide

Problems	Possible causes
❶ High background	High background can occur for several reasons: • Only low stringency washes performed. Perform stringency washes with 0.1 × SSC, 0.1% SDS at hybridization temperature. If this fails to remove the high background, then it may be necessary to remove the probe using the probe stripping protocol on page 21. Alternatively, letting the radioactivity on the membrane decay for 2–3 half lives will reduce the background to very low levels. Perform this at -15 °C to -30 °C and do not let the membrane dry. • Unincorporated [α-^{32}P]dCTP is causing background. Purify the probe with ProbeQuant G-50 columns as recommended in this protocol booklet. • Concentrated probe has contacted the membrane directly. Always add probe first to the bulk of the hybridization solution, before letting it come into contact with the membrane. • Probe concentration in the hybridization solution too high. Check that the concentration of DNA to be labelled was correct and that it was diluted correctly.
❷ Hybridization signals absent or very weak	• The specific activity of the probe may have been too low. Use fresh radiolabelled nucleotide to prepare the probe. Check that the correct radiolabelled nucleotide was used for the chosen labelling protocol. Check that the amount of DNA labelled was correct.

Check the incorporation of radioactivity into probe.

- Radioactive probe has not been properly denatured.
  Denature radioactive DNA probes by boiling before use.
- The gene of interest may not be expressed, or is expressed at a very low level in the tissues from which the poly A^+ RNAs were isolated. Perform longer exposure to film at -70 °C with intensifying screens.
  Use the β-actin control probe to verify that the hybridization protocol is working correctly.
- Probe of interest is not detecting mRNA.
  Check that the probe of interest was derived from the coding sequence of the gene of interest.
  Use sequencing to verify that the probe sequence is correct. This is especially important if PCR fragments are being used as probes.
  Test the functioning of the probe with RNA isolated from a tissue that is known to express the gene of interest

❸

Probe not fully homologous to target

If you are using a cross-species probe, reduce the stringency of hybridization by decreasing the hybridization temperature by a few degrees. Perform low stringency washes first, detect signal for a short period of time. If necessary, higher stringency washes can be performed after this.

❹

Inability to strip and reprobe

This may be caused by drying of the membrane. Always minimize the time the membrane is in contact with air. For storage, seal the membrane in a plastic bag.

❺ Signal decreases after two reprobings	This problem is especially common for genes expressed at low levels. Use the probe that is expected to give lowest hybridization signals first. Minimize the time needed to remove radioactive probes from the membrane, by monitoring the stripping process and reducing the level of signal by letting the radioactivity decay.

Reference

1. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

Legal

Product information

Product name	code
Hybond Northern Blot	
Human d 12 poly A^+ RNAs	RPN4800
Human a 8 poly A^+ RNAs	RPN4801
Human b 7 poly A^+ RNAs	RPN4802
Human c 8 poly A^+ RNAs	RPN4803
Mouse a 8 poly A^+ RNAs	RPN4804

Related products

AlkPhos Direct Labelling and Detection System with ECF	RPN3692
Megaprime DNA Labelling System dCTP	
30 reactions	RPN1606
60 reactions	RPN1607
5'end Labelling Kit	RPN1509
Rediprime™ II DNA Labelling System	
30 reactions	RPN1633
60 reactions	RPN1634
Nick Translation Kit dCTP, 20 reactions	N5000
RNA Labelling Kit, 20 reactions	RPN3100
Rapid-hyb Buffer, 1000cm^2 membrane	RPN1635
ProbeQuant G-50 Micro Columns	27-5335-01
Typhoon 8600 Variable Mode Imager	
Typhoon 8600 & ImageQuant Solutions for Windows™NT	63-0027-96
Typhoon 8600 & PC Workstation	63-0027-97
Typhoon 8600 & ImageQuant for Macintosh™	63-0027-95
Storm 860	

Also available

Hyperfilm MP, Hybond membranes & blotting paper

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