Gene Images Random Prime Labelling Module

RPN3540 30 labelling reactions
RPN3541 60 labelling reactions

STORAGE:

On receipt, the random prime module should be stored at -15°C to -30°C.

STABILITY:

The kit components are stable for at least 3 months when stored under the recommended conditions.

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.

Amersham Biosciences

Gene Images random prime labelling and detection system

Gene Images random prime labelling module	
Reagents sufficient for 30 labelling reactions	RPN3540
Reagents sufficient for 60 labelling reactions	RPN3541
Gene Images random prime labelling and CDP-Star detection system	
Regents for 30 labelling reactions and detection on 2500cm^2 membrane (consists of 1x RPN3540 and 1x RPN3510)	RPN3500
Reagents for 60 labelling reactions and detection on 5000cm^2 membrane (consists of 2x RPN3540 and 2x RPN3510)	RPN3501

SYSTEM COMPONENTS

Gene Images random prime labelling module (RPN3540) consists of:

Nucleotide mix: 300µl
5x stock solution of fluorescein-11-dUTP (Fl-dUTP), dATP, dCTP dGTP and dTTP in Tris-HCl, pH7.8, 2-mercaptoethanol and $MgCl_2$

Primers: 150µl
Random nonamers in an aqueous solution

Enzyme solution: 30µl
5 units/µl exonuclease free Klenow, in a buffer solution, pH6.5

Water: 1ml deionised water

Control unlabelled DNA: 50µl
10ng/µl *Hin*dIII digested lambda DNA

Control fluorescein-labelled DNA: 50µl
50pg/µl fluorescein-labelled *Hin*dIII digested lambda DNA in 50ng/µl herring sperm carrier DNA

Liquid block: 50ml
For use in the recommended hybridisation buffer

(RPN3541 consists of 2x RPN3540)

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.

Note that the protocols describe the use of ethidium bromide, formaldehyde, sodium hydroxide and EDTA. Warning: Ethidium bromide is harmful. Formaldehyde is toxic by ingestion and inhalation. Sodium hydroxide is corrosive. EDTA is harmful. Please follow the manufacturers' safety data sheets relating to the safe handling and use of these reagents.

DESCRIPTION

The Gene Images™ random prime labelling module is designed to label nucleic acid probes by random prime labelling. Nonamers of random sequence are used to prime DNA synthesis on a denatured DNA template in a reaction catalysed by the (exonuclease-free) Klenow fragment of *E.coli* DNA polymerase I. Fluorescein-11-dUTP (Fl-dUTP) partially replaces dTTP in the reaction so that a fluorescein-labelled probe is generated. There is net synthesis of probe in this reaction, with up to 350ng probe synthesised from 50ng template.

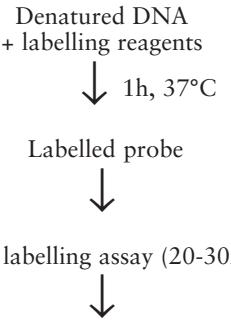
Following labelling, the probe can be denatured and used directly in hybridisation. An advantage of using fluorescein-labelled probes is that it is generally unnecessary to remove unincorporated Fl-dUTP prior to use of the probe in hybridisation. In addition, fluorescein-labelled DNA is stable under standard hybridisation conditions and, as with radiolabelled probes, the stringency of hybridisation can be controlled either with temperature or salt concentration.

Each batch of this module is tested by our quality control group using the Gene Images CDP-Star™ detection module (RPN3510) to ensure that it will detect the following:
A single copy gene in human DNA genomic Southern blots, representing 0.06pg (60fg) target in 0.125µg of DNA.

p53 message in a 1µg loading of human liver messenger RNA in Northern blots.

0.1pg of target on a *Hin*dIII-digest of lambda DNA Southern blots, using the unlabelled control DNA contained in the kit as template for probe labelling.

0.05pg of the control fluorescein-labelled DNA on direct dot blots.



Hybridisation of probe with Southern or Northern blots followed by detection using eg the Gene Images CDP-Star detection module

Please note: The Gene Images CDP-Star detection module (RPN3510) is required for high sensitivity detection of fluorescein-labelled probes.

CRITICAL PARAMETERS

- It is of critical importance to accurately determine the amount of nucleic acid template to be used in non-radioactive labelling reactions.

- Once the blot has been wetted (this is normally at the pre-hybridisation stage) then it must not be allowed to dry out at any stage until detection is completed.

- The fluorescein hapten is sensitive to prolonged exposure to light. For long term storage, solutions and blots containing fluorescein should be placed in the dark.

- Freezers used for storing components should not be of the 'frost-free' type as these may cause damage due to the repeated freeze-thaw cycles of these freezers.

- Successful production of **Northern blots** depends on all solutions and apparatus being RNase-free. To achieve this, where practicable, bake apparatus at 150°C (plastic for 2 hours, glassware for at least 2 hours, paper for 30 minutes). Treat any other equipment with RNase *AWAY*™.

ADDITIONAL EQUIPMENT AND REAGENTS REQUIRED

Equipment

Adjustable pipettes
Sterile pipette tips
Standard laboratory glassware
Shaking and standard water-baths, 37°C (for labelling), 60°C(Southerns), 65°C(Northerns)
Ultraviolet transilluminator (for rapid labelling assay only)
Hybond™-N+ or DE81 paper (for rapid labelling assay)
Polypropylene microcentrifuge tubes
Microcentrifuge
Gloves, preferably powder-free
Plastic boxes/bags for hybridisation

For blotting:
Gel electrophoresis equipment
Heating equipment for agarose, for example microwave oven
Filter paper (for example, Whatman™ 3MM)
Paper towels
UV cross-linker or vacuum oven, 80°C (normal oven is sufficient for nylon membranes)
Blotting membrane (Hybond-N+ is recommended)

Solutions

Note: For successful preparation of Northern blots, all solutions should be prepared using RNase-free water and fresh AnalaR grade reagents. Once the blots have been prepared, these precautions are no longer necessary.

Hybridisation buffer
For rapid hybridisations, use Rapid-hyb buffer (see related products). For overnight hybridisations use the following:
5xSSC
1 in 20 dilution liquid block
0.1%(w/v) SDS
5%(w/v) Dextran sulphate

TE buffer
10mM Tris-HCl
1mM EDTA
pH8.0

EDTA stock
0.2M or 0.5M EDTA pH8.0

SDS stock
10% or 20%(w/v) SDS

Depurination solution (for Southern blotting)
250mM HCl

Denaturation solution (for Southern blotting)
1.5M NaCl
0.5M NaOH

Neutralisation solution (for Southern blotting)
1.5M NaCl
0.5M Tris-HCl
pH adjusted to pH7.5

20xSSC (for Southern/Northern blotting and as stock for 2xSSC for rapid labelling assay)
0.3M Na_3citrate
3M NaCl
Separate (RNase-free) stocks are recommended for Northerns.

5xMOPS (for Northern blotting) (do not store for more than 2 weeks)
0.1M MOPS
40mM sodium acetate
5mM EDTA
Fresh solutions give the most efficient transfer. Storing this buffer in the dark prolongs its life. Do not use once it has turned yellow.

50xTAE (stock for 1xTAE for running gels)
2M Tris-HCl
50mM EDTA
pH adjusted to pH8

10x gel loading buffer
Bromophenol blue 0.05g
Xylene cyanol FF 0.05g
Glycerol 5ml
EDTA 0.186g
Make up to 10ml with TAE buffer
A separate (RNAse-free) stock is recommended for Northern blotting

Reagents

Dextran sulphate (molecular weight 500000, for example, Sigma® Product no. D-6001)

THE GENE IMAGES RANDOM PRIME LABELLING PROTOCOL

It is recommended that the protocols are read thoroughly before using the system.

1. Preparation of labelled probe

This protocol is based on the labelling of 50ng of template DNA. However, the standard reaction can be used to label 25ng-2µg of template DNA. Synthesis of labelled probe is most efficient when using 25-50ng of template, although net synthesis tends to increase with the amount of template (see table 1, page 15).
Solutions required for use with this system are described in the protocols. In addition, there is a full list (including those solutions needed for blotting) preceding this section.

Protocol	Notes
1.1. Dilute the DNA to be labelled to a concentration of 2-25ng/µl in either distilled water or TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0).	1.1. If the DNA solution is too dilute to be used directly, it should be concentrated by ethanol precipitation followed by redissolution in an appropriate volume of water or TE buffer. If an intact plasmid probe is to be used it may be necessary to correct for the proportion of plasmid sequence present during the hybridisation.

1.2. Place the following tubes in an ice bath to thaw: Nucleotide mix Primers Water Leave the enzyme at -15°C to -30°C until required, and return it to the freezer immediately after use.	
1.3. Denature the DNA sample by heating for 5 minutes in a boiling water bath, then chill on ice. It is advisable to denature in a volume of at least 20µl.	1.3. Closed circular double-stranded DNA can be linearised to avoid more rapid re-naturation following this step, although this is not usually necessary.
1.4. To a 1.5ml microcentrifuge tube, placed in an ice bath, add the appropriate volume of each reagent in the following order: Water to a final reaction volume of 50µl — xµl Nucleotide mix — 10µl Primer — 5µl Denatured DNA — 50ng Enzyme solution (Klenow) 5 units/µl — 1µl Total — 50µl	1.4. If desired as a control, 5µl of the unlabelled DNA supplied in the kit can be used in place of the sample DNA. A volume of DNA solution up to 25µl may be used in the case of fragments in low melting point agarose.

Protocol	Notes
1.5. Mix gently by pipetting up and down and cap the tube. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.	1.5. Avoid vigorous mixing which may result in loss of enzyme activity.
1.6. Incubate the reaction mix at 37°C for 1 hour.	1.6. The temperature of incubation and the reaction time can be chosen for convenience as the reaction reaches a plateau and does not decline significantly overnight at room temperature. However, the rate of reaction does depend to some extent on DNA purity so, for samples of lower purity such as miniprep DNA[3,4] or DNA in agarose, a longer incubation period may be required.
1.7. If any of the probe is to be stored, rather than being used in a hybridisation immediately, then the reaction should be terminated by the addition of EDTA to a final concentration of 20mM. Probes can then be stored in suitable aliquots in a freezer at -15°C to -30°C in the dark for at least 6 months. Avoid repeated freezing and thawing.	1.7. Labelled probe can be used directly in a hybridisation (following denaturation) without removal of unincorporated nucleotide. For long term storage, it is advisable to keep probes in the dark as the fluorescein molecule is light-sensitive.

1.8. The yield of probe after labelling 25 or 50ng of template will typically be between 6-8ng/µl. With higher template levels, while the amount of labelled template produced will increase, the overall reaction will be less efficient (see table 1 below). We also recommend that you use the rapid labelling assay as a check to ensure that you have successfully labelled your probe (see next section).	

Table 1. Typical probe yields (when using the Gene Images random prime labelling module) as a function of the amount of starting template. The figures represent the average yield from standard reactions with templates of various lengths (0.45-21Kb). It can be seen that synthesis is most efficient for lower template levels, although the net synthesis tends to increase with the amount of template.

Amount of template per labelling reaction (ng)	25-50	100	200	500	1000	2000
Final total probe concentration (ng/µl)	7	9	13	20	32	54

2. Monitoring incorporation using the rapid labelling assay

Note: The control fluorescein-labelled DNA supplied is too dilute for detection using the rapid labelling assay.

Protocol	Notes
2.1. Prepare 1/5, 1/10, 1/25, 1/50, 1/100, 1/250 and 1/500 dilutions of the 5x nucleotide mix in TE buffer.	
2.2. Dot out 5µl of your labelled probe and 5µl of the 1/5 dilution of nucleotide mix (negative control) on to a strip of Hybond-N+, placed on a non-absorbent backing. Allow the liquid to absorb, (but do **not** allow to dry) and wash the strip with gentle agitation in excess pre-heated 2xSSC at 60°C for 15 minutes.	2.2. A pencil should be used if sample identification is required, since some inks can interfere with the assay. Whatman DE81 paper can also be used as an alternative to Hybond-N+. In this case 2xSSC, 0.1%(w/v) SDS should be used to wash the strip. Following the wash stage, rinse the strip successively with water and then ethanol before handling (DE81 paper is fragile in aqueous solutions).
2.3. Prepare a reference strip by dotting 5µl of each nucleotide mix dilution, except the 1/5, on to a separate strip of Hybond-N+. This reference strip can be re-used and can be	2.3. If using DE81 paper, then after applying the dots, the reference strip can be wetted successively in water and then ethanol to reduce distortion of the filter.

stored, wrapped in SaranWrap™, for several weeks at -15°C to -30°C in the dark.	
2.4. Place both the reference and the washed strips on a piece of Whatman™ 3MM paper lightly moistened with TE buffer and take to the darkroom. Visualise both strips (**sample side down**) on a UV transilluminator. Optimum contrast is obtained using a short wavelength (254nm) transilluminator. The labelled probe should be visible as a fluorescent spot with an intensity between the 1/10 and 1/250 dilutions on the reference strip. Such a result indicates that the probe has successfully incorporated the fluorescein label. The closer that the intensity of the labelled DNA is to the lower dilution (1/10), the more efficient the labelling reaction. The washed negative control should retain little (or no) fluorescence indicating that the fluorescence of the probe is due only to incorporated fluorescein and not unincorporated Fl-dUTP. If significant fluorescein remains in the negative control, wash the filter for a further 15 minutes. This is possible only if the strip has not dried.	2.4. When using DE81 paper, dry Whatman 3MM paper should be used to transport the strips to the darkroom.

Protocol	Notes
2.5. For a hard copy of the results the strips can be photographed. A **Kodak™ Wratten™** No 9 filter (or a similar yellow filter) should be used in conjunction with Polaroid™ 667 black and white film.	

3. Hybridisation and stringency washes for Southern and Northern blots

Please note: Optimum results are obtained by carrying out hybridisations overnight in the hybridisation buffer described below. Alternatively, if the quantity of labelled probe is at a premium, similar sensitivity (with some increase in background) may be obtained by substituting Rapid-hyb™ buffer (RPN1635) in the **overnight** hybridisations and using lower probe concentrations (see note 3.3.). Rapid-hyb buffer was originally developed to allow short hybridisation times when using ^{32}P-labelled probes; if it is used in short hybridisations in the Gene Images system then in many cases satisfactory results will be achieved though there will be some loss in sensitivity.

The following protocol describes the recommended conditions for both Southern and Northern blots (differences are pointed out at the relevant place within the text). The hybridisation and wash conditions are appropriate for a majority of probes. For Southern blots, this allows detection of single copy mammalian genes without significant cross-hybridisation to non-homologous sequences.

Protocol	Notes
3.1. For **overnight** hybridisations prepare the hybridisation buffer as follows: 5xSSC 0.1%(w/v)SDS 5%(w/v) dextran sulphate 20-fold dilution of liquid block (supplied). Combine all the components and make up to the required volume. Gentle heating with continuous stirring will be required to completely dissolve the dextran sulphate. As mentioned above, Rapid-hyb buffer can also be used for overnight hybridisations if desired. However, the probe concentration must be reduced (see note 3.3.) and there may be some increase in background when it is used in this way.	3.1. The hybridisation buffer can be stored in suitable aliquots at -15°C to -30°C. Do not store in a frost-free freezer. Under these conditions the buffer is stable for at least 12 months. The addition of sheared, denatured heterologous DNA (100µg/ml) to the hybridisation buffer can reduce non-specific hybridisation; some reduction in sensitivity may also be observed. Also, it may be convenient to separate any remaining undiluted liquid block at this stage into suitable aliquots for re-freezing.
3.2. Pre-heat the required volume of hybridisation buffer (0.3 ml/cm^2 for hybridisations in boxes and 0.125ml/cm^2 hybridisations in bags or hybridisation ovens) to 60°C for Southern blots or 65°C for Northern blots, then place the blots in the buffer and pre-hybridise for at least 30 minutes at the same temperature with	3.2. Hybridisation can be carried out in boxes, bags or tubes provided there is sufficient buffer to allow adequate access of probe to the blot. **With larger blots (greater than 50cm^2), or if using minimal hybridisation volumes in bags or tubes, the blots should be pre-wetted in 5xSSC.**

Protocol	Notes
constant, gentle agitation.	It is also possible to hybridise several blots in the same solution, again provided that there is adequate volume and circulation of buffer. A maximum of two blots should be hybridised together in a single bag and, when using more than one blot in a tube, it is important that the blots do not overlap one another. High backgrounds can result if there is insufficient hybridisation buffer or if a blot is not totally immersed. Agitation improves access of buffer to the blot, particularly if several blots are to be hybridised in the same solution.
3.3. Remove the required amount of probe to a clean microcentrifuge tube (see note 3.3.). If the volume is less than 20µl, make up to this volume with water or TE buffer. De-nature the probe by boiling for 5 minutes and snap cool on ice.	3.3. The recommended probe concentration is 10ng/ml for low target applications (eg single copy gene detection). [For a 50ng template reaction, this is approximately 1-2µl of reaction product per ml of hybridisation buffer.] If using Rapid-hyb buffer for **overnight** hybridisations, reduce the probe to 2ng/ml. For high target applications lower probe concentrations may be sufficient.

3.4. Centrifuge the denatured probe briefly, then add to the pre-hybridisation buffer, (avoiding placing it directly on the membrane), and mix gently.	3.4. Alternatively, a small aliquot of the hybridisation buffer may be removed and mixed with the probe before returning the mixture to the bulk of the buffer.
3.5. Hybridise overnight, at 60°C for Southern blots or 65°C for Northern blots with gentle agitation.	
3.6. We suggest that all stringency washes are carried out initially at 60°C for Southern blots and 65°C for Northern blots. Prepare the first stringency wash solution of 1xSSC, 0.1%(w/v) SDS and pre-heat to the appropriate temperature. Transfer the blots to this solution (using 2-5ml per cm^2 of membrane) and wash for 15 minutes at this temperature with gentle agitation. Carry out a further wash at the same temperature as the first but using pre-heated solutions of 0.5xSSC, 0.1%(w/v) SDS for Southern blots or 0.1xSSC, 0.1%(w/v) SDS for Northern blots.	3.6. Several blots can be washed in the same solution provided that they can move freely. For Southern blots, the second wash can be varied in the range 1-0.1xSSC to achieve the desired stringency (the SDS concentration should remain constant at 0.1%(w/v)). For Southern or Northern blots, the temperature of these washes may also be increased to achieve the desired stringency. Guidelines on the factors determining stringency are given in reference[6].

We recommend that you proceed immediately with detection of the blot. However, if blots are to be stored at this stage, they should not be allowed to dry out. We would recommend wrapping the moist blot in SaranWrap and storing at 2-8°C in the dark. For detection, we recommend the use of the Gene Images CDP-Star detection module (RPN 3510).

APPLICATIONS

Southern blotting

The following protocol is the method used at Amersham Biosciences for the transfer of DNA on to Hybond-N+.

Protocol	Notes
1. Electrophoresis of DNA is carried out in a neutral agarose gel system[7]. Prepare a 0.8-1% agarose gel containing 1xTAE buffer. Ethidium bromide can be added to a final concentration of 0.2µg/ml.	1. TBE buffer may also be used eg 0.09M Tris-borate, 2mM EDTA pH8.0.
2. DNA samples should be prepared in a solution containing 1x gel loading buffer in 1xTAE. Apply the samples to the gel.	
3. Run the gel in 1xTAE buffer at 4v/cm until the bromophenol blue indicates that the sample has run for a sufficient distance.	3. 1v/cm for electrophoresis overnight.
4. Following electrophoresis, visualise the gel	4. Minimise exposure to UV as this will

under UV transillumination.	damage the DNA. The gel may be photographed during this time.
5. Before capillary blotting, the gel should be processed as follows:	
5.1. Depurination, 10 minutes at room temperature with gentle agitation.	5.1. Depurination need only be considered if target sequences are greater than 10Kb in size. Depurination for too long a time will result in a reduced signal on the resultant blot(s). During depurination, the bromophenol blue dye will turn yellow; the blue colour will return during the denaturation step.
5.2. Denaturation, 25 minutes at room temperature with gentle agitation.	
5.3. Neutralisation, 30 minutes room at temperature with gentle agitation. Ensure that the gel is completely covered in the appropriate solution. Include a rinse in distilled water between each stage.	5.3. When using nitrocellulose membrane, the neutralisation time should be extended to 45 minutes. Replacement with fresh neutralisation solution is suggested at least once during this time.
6. Assemble the capillary blotting apparatus using 10xSSC as the transfer buffer. Allow the DNA to transfer overnight on to Hybond N+.	

Protocol	Notes
7. The following day, disassemble the apparatus. Mark the membrane appropriately and fix the DNA to the membrane by baking (2 hours at 80°C) or by UV cross-linking.	7. If using the Amersham Biosciences UV cross-linker (80-6222-31/80-6222-50), use 700 units of energy. Over-irradiation will lead to damage of the DNA, so when using other UV apparatus, optimisation of time of exposure to UV is important. (For nitrocellulose membranes, UV treatment must **not** be used; in this case, baking for 2h at 80°C **in a vacuum oven** is recommended.)
8. The blot may be stored in a suitable container at room temperature under vacuum until required.	

Northern blotting

Successful production of Northern blots depends on all solutions and apparatus being RNase-free. To achieve this, where practicable, bake apparatus at 150°C, glassware for at least 2 hours, plastic and paper for 30 minutes. Treat any other equipment with RNase *AWAY*. All solutions should be prepared using RNase-free water and fresh AnalaR grade reagents. Wear gloves at all times.

The following protocol is a general method used at Amersham Biosciences for the transfer of RNA on to Hybond-N+.

Protocol	Notes
1. Electrophoresis of RNA is carried out in a denaturing agarose gel system[6]. Prepare a 1.2% agarose gel containing 1xMOPS buffer and 0.66M (2%w/v) formaldehyde (formaldehyde is usually supplied as a 37-40%(w/v) solution).	1.1. Due to the toxicity of formaldehyde, the gel should be poured in a fume cupboard. Addition of ethidium bromide to the gel is not recommended as it may inhibit transfer of RNA to the membrane. 1.2. For visualising the gel under UV after electrophoresis, we use extra tracks which are cut off and stained separately.
2. RNA samples are prepared in a solution containing 1xMOPS buffer, 2.2M (6.7%w/v) formaldehyde, 50%(w/v) formamide.	
3. Denature the RNA samples by heating at 55°C for 15 minutes. Add 10x gel loading buffer to a final concentration of 1x and apply samples to the gel.	
4. Run the gel in 1x MOPS buffer at 4-5v/cm until the bromophenol blue indicates that the sample has run for a sufficient distance.	4. See note 1.2. above on visualisation of the gel after electrophoresis.
5. Wash the gel in an excess of water for 15 minutes with gentle agitation.	

Protocol	Notes
6. Soak the gel in excess 10xSSC for 5 minutes with gentle agitation. Repeat this step with fresh 10xSSC.	
7. Assemble the capillary blotting apparatus using 10xSSC as the transfer buffer. Allow the RNA to transfer overnight on to Hybond-N+.	
8. The following morning, disassemble the transfer apparatus. Mark the position of the wells on the blot with a chinagraph pencil. The RNA should then be fixed to the membrane preferably by UV cross-linking (see note 8.). Alternatively, the RNA can be fixed by laying the blot (**RNA side up**) for 5 minutes on Whatman 3MM which has been soaked in 0.4M NaOH. To remove the NaOH after fixing, briefly rinse the blot in 2xSSC.	8. If using the Amersham Biosciences UV cross-linker (80-6222-31/80-6222-50), use 700 units of energy. Over-irradiation will lead to damage of the RNA so when using other UV apparatus, optimisation of time of exposure to UV is important. (For nitrocellulose membranes, UV treatment must not be used; baking for 2h at 80°C **in a vacuum oven** is recommended.)
9. The blot can be stored in a suitable container at room temperature under vacuum until required.	

Dot/slot blotting

The following is a general protocol for manually preparing dot blots. When using dot/slot blot apparatus, prepare the filters according to the manufacturer's recommendations.

Protocol	Notes
1. Using a pencil, mark the membrane lightly with a $1cm^2$ grid.	1. The minimum recommended distance between 1µl applied samples is 0.5cm on a dry membrane. This distance should be increased to 1cm if a pre-wetted membrane is to be used.
2. Nitrocellulose membranes should be pre-wetted in water followed by 10xSSC before application of the sample. The sample can be applied directly to the wet membrane or the membrane can be allowed to air dry before sample application. No pre-wetting is required for nylon membrane.	
3. Dilute the DNA samples in TE buffer. Denature the sample by heating for 5 minutes in a boiling water bath. Then chill on ice. It is advisable to denature in a volume of at least 20µl. Centrifuge briefly to collect the sample at the bottom of the tube. Keep on ice.	3. Where target levels of DNA are less than 1ng, it is strongly advised that the sample is diluted in TE buffer containing 1ng/µl sonicated carrier DNA (for example herring sperm DNA).

Protocol	Notes
4. Carefully apply 1µl samples of denatured DNA to the membrane, avoid touching the membrane with the pipette tip. Leave the filter to air dry.	
5. Fix the DNA to the membrane using heat or UV cross-linking.	5. If using the Amersham Biosciences UV cross-linker (80-6222-31/80-6222-50), use 700 units of energy. Over-irradiation will lead to damage of the nucleic acid so when using other UV apparatus, optimisation of time of exposure to UV is important. (For nitrocellulose membranes, UV treatment must not be used; baking for 2h at 80°C **in a vacuum oven** is recommended.)
6. The blot can be stored in a suitable container at room temperature under vacuum until required.	

ADDITIONAL INFORMATION

Further system checks

Use of control reagents

Two control DNAs are supplied with the Gene Images random prime labelling module: unlabelled *Hin*dIII-digested lambda DNA and fluorescein-labelled *Hin*dIII-digested lambda DNA. When used in conjunction with the Gene Images CDP-Star detection module, these DNAs can be used to demonstrate the performance of the system or to help trace the cause of any labelling or detection problems that might occur. The following examples illustrate the use of the control DNAs.

Use of the control unlabelled DNA for evaluating labelling and hybridisation problems

The control unlabelled DNA can be used for evaluating labelling problems by subjecting it to the standard labelling reaction and examining in the rapid labelling assay (see section 2 of the main protocol). It can also be used to study blotting and hybridisation problems as follows:

After labelling the test sample and the control (unlabelled) DNA template in the random prime reaction, hybridisation should be carried out against complementary blotted targets. Note: 5µl of the control unlabelled DNA corresponds to 50ng of template.
Separate aliquots (containing 100pg, 10pg and 1pg) of the unlabelled control DNA can be electrophoresed on a 1%(w/v) agarose gel and Southern blotted on to Hybond-N+. Sheared herring sperm DNA (0.5pg per well) may be added as carrier but is not essential. In the 1pg track, the bands are equivalent to 480, 190, 140, 90, 50, 40 and 10fg of DNA respectively.

After probing with the labelled, homologous lambda probe, and detecting using the Gene Images CDP-Star detection module, then the 90fg band should be visible after a 2 hour exposure. A poor result obtained under the same hybridisation conditions with the test DNA would imply that this DNA was of poor quality. Poor results with both the test and control sample would imply that problems were occurring at either the labelling or the hybridisation stage.

If desired, the above experiment can be performed to gain familiarity with the system before using your own DNA. (Also, the fluorescein-labelled control DNA can be used on dot blots to gain familiarity with the detection system alone.)

Use of the fluorescein-labelled control DNA for determining probe yield and detection efficiency of the system.

Protocol	Notes
1. Terminate the labelling reaction by addition of EDTA (as in section 1.7 of the main protocol). Refer to table 1 for the expected yield of DNA then dilute a small (2µl) aliquot of the reaction mixture to a nominal concentration of 50pg/µl in TE buffer.	1. Excess carrier DNA is included in the control Fl-labelled DNA to improve stability during long-term storage. This does not interfere with its detection on Hybond N+.
2. Prepare the following serial dilutions of both the labelled probe and the control Fl-labelled DNA: 5, 1, 0.5, 0.2, 0.1, 0.05pg/µl in TE buffer. The control Fl-labelled DNA is supplied at 50pg/µl.	
3. Apply 1µl aliquots of each dilution to a strip of Hybond-N+ resting on a non-absorbent backing. A control aliquot of TE buffer should also be dotted. Allow to dry for 10-15 minutes.	3. It is not necessary to denature the DNA samples prior to application to a nylon-based membrane. A minimum spacing of 0.5cm between dots is recommended.

Protocol	Notes
4. Fix the DNA to the membrane either by UV or by baking for 1-2 hours in an 80°C oven.	4. These blots should be stored **in the dark** under vacuum.
5. Proceed with detection following the protocol given with the Gene Images CDP-Star detection module. When using this module, the 0.1pg dots of both test and control DNAs should be detectable after a 1-2 hour exposure.	5. If 0.1pg sensitivity is not obtained for the control sample, then a detection problem is likely (for example, inappropriate antibody conjugate dilution or contaminated detection solutions).

The yield of labelled probe can be determined by comparing the intensity of the signals generated to those produced by the control fluorescein-labelled DNA (which is at 50pg/µl). If the labelled test sample is detected less well than the control, the degree of reduction in labelling efficiency can be ascertained. If there is only a few-fold difference, it may still be possible to carry out a successful hybridisation by increasing the volume of probe. If the difference is greater than this, then it may be advisable to repeat the labelling step. Probe yield can often be improved by increasing the reaction time, for example to 2 hours at 37°C or overnight at room temperature.

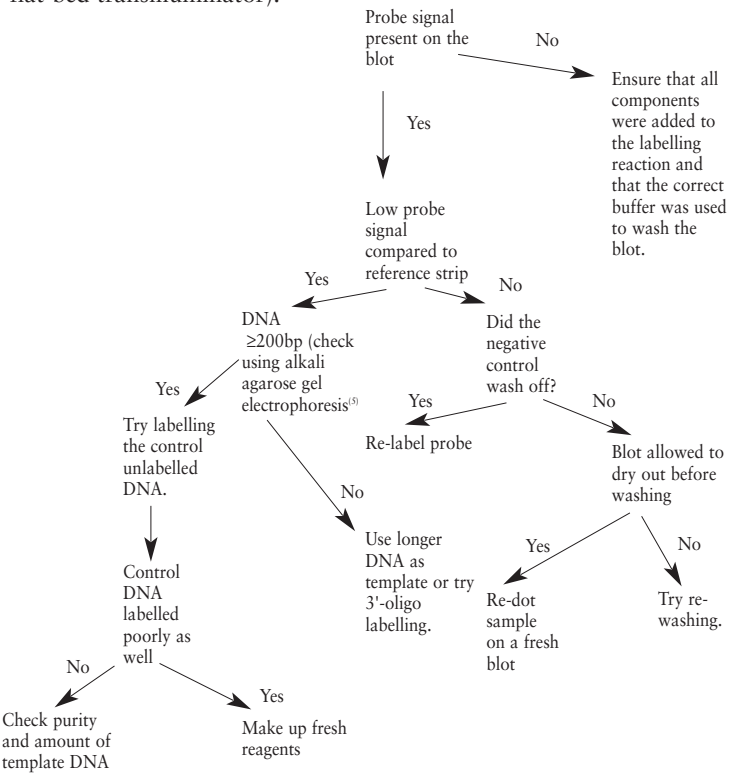
If poor labelling is observed, an additional random prime reaction using the unlabelled control DNA can be included to determine whether the quality of the test DNA is responsible for the reduced efficiency.

Note: the fluorescein-labelled control DNA is not sufficiently concentrated for use as a probe.

Troubleshooting guide

Probe labelling

(Possible problems relating to the results after hybridisation and detection are discussed in the appropriate detection module).
Test quality of probe using the rapid labelling assay. Make sure that the blots are placed on the UV transilluminator with the side containing the dots facing the lamp (eg face down when using a flat-bed transilluminator).



Related products

Gene Images 3'-oligolabelling module RPN5770
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide

Gene Images CDP-Star detection module
for 2500cm^2 membrane RPN3510
for 5000cm^2 membrane RPN3511

Gene Images random prime labelling and CDP-Star detection system
Reagents for 30 labelling reactions and detection on 2500cm^2 membrane (consists of RPN3540 and RPN3510) RPN3500
Reagents for 60 labelling reactions and detection on 5000cm^2 membrane (consists of 2x RPN3540 and 2x RPN3510) RPN3501

Gene Images 3'-oligolabelling and CDP-Star detection system RPN5776
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide; detection reagents for 2500cm^2 membrane (consists of RPN5770 and RPN3510)

Gene Images 3'-oligolabelling and ECF™ signal amplification system RPN5775
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide; signal amplification reagents for 2500cm^2 membrane (consists of RPN5770 and RPN5750)

ECF random prime labelling module RPN5751
30 labelling reactions

ECF signal amplification module RPN5750
Amplification of signal on 2500cm^2 of membrane

ECF random prime labelling and signal amplification system 30 labelling reactions and signal amplification for 2500cm^2 of membrane (consists of RPN5751 and RPN5750)		RPN5752
Hybridisation oven/shaker		RPN2510/ RPN2511
UV cross-linker		80-6222-31/ 80-6222-50
Liquid block (100ml)		RPN3601
Rapid-hyb buffer (125ml)		RPN1635
Rapid-hyb buffer (500ml)		RPN1636

Membranes

Hybond-N+

Positively charged nylon membrane:

Pack of 10 membranes, 20x20cm		RPN2020B
Pack of 10 membranes, 22x22cm		RPN2222B
Pack of 50 discs, 82mm diameter		RPN82B
Pack of 50 discs, 132mm diameter		RPN132B
Roll of membrane, 20cmx3m		RPN203B
Roll of membrane, 30cmx3m		RPN303B

Film

Hyperfilm™-MP

High-sensitivity film for chemiluminescence:

18x24cm	Pack 25 sheets	RPN2103
30x40cm	Pack 25 sheets	RPN2104
5x7 inches	Pack 25 sheets	RPN1674
10x12 inches	Pack 25 sheets	RPN1681

Cassettes

Amersham Biosciences supplies a range of Hypercassette™ products

for autoradiography, the following is a sample from its range:

18x24cm	RPN1642
30x40cm	RPN1644
5x7 inches	RPN1648
10x12 inches	RPN1650

See the current Amersham Biosciences BioDirectory for further details.

References

1. FEINBERG, A.P. and VOGELSTEIN, B., *Anal. Biochem*., **132**, p.6, 1983.
2. FEINBERG, A.P. and VOGELSTEIN, B., *Addendum Anal. Biochem*., **137**, p.266, 1984.
3. HOLMES, D.S. and QUIGLEY, M., *Anal. Biochem*., **144**, pp.193-197, 1981.
4. BIRNBOIM, H.C. and DOLY, J., *Nucl. Acids Res*., **7**, pp.1513-1523, 1979.
5. SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., 'Molecular Cloning: a laboratory manual' Second Edition, Cold Spring Harbor Laboratory, 1989.
6. MEINKOTH, J. and WAHL, G., *Anal. Biochem*, **138**, p.267, 1984.

http://www.amershambiosciences.com

Amersham Biosciences UK Limited

Amersham Place Little Chalfont Buckinghamshire UK HP7 9NA

Amersham Biosciences AB

SE-751 84 Uppsala Sweden

Amersham Biosciences Corp

800 Centennial Avenue PO Box 1327 Piscataway NJ 08855 USA

Amersham Biosciences Europe GmbH

Munzinger Strasse 9 D-79111 Freiburg Germany

RPN3540PL/A

Critical parameters

- It is of critical importance to accurately determine the amount of nucleic acid template to be used in non-radioactive labelling reactions.
- Once the blot has been wetted (this is normally at the pre-hybridisation stage) then it must not be allowed to dry out at any stage until detection is completed.
- The fluorescein hapten is sensitive to prolonged exposure to light. For long term storage solutions and blots containing fluorescein should be placed in the dark.
- Freezers used for storing components should not be of the 'frost-free' type as these may cause damage due to the repeated freeze-thaw cycles of these freezers.
- Successful production of Northern blots depends on all solutions and apparatus being RNase-free. To achieve this, where practicable, bake apparatus at 150°C (plastic for 2 hours, glassware for at least 2 hours, paper for 30 minutes). Treat any other equipment with RNase *AWAY*™.

Gene Images random prime labelling module
Protocol reminder card

RPN3540/RPN3541

Labelling mix	Labelling reaction	Hybridisation buffer	Hybridisation	Stringency washes
Water to 50µl 50ng (or more) **denatured** DNA 10µl Nucleotide mix 5µl Primers 1µl Enzyme (Klenow)	Incubate for 1h at 37°C (or 2-8°C overnight) Terminate reaction with EDTA (20mM final conc.)	5xSSC 0.1%(w/v)SDS 5%(w/v) Dextran sulphate 1/20 volume liquid block	0.125-0.3ml buffer per cm^2 (depending on blot size) Pre-hybridise for 30min (60°C Southerns or 65°C Northerns) Add probe 10ng/ml Hybridise 16h 60°C/65°C	1.1xSSC/0.1%SDS. 2.0.5xSSC/0.1%SDS (Southerns); 0.1xSSC/0.1%SDS (Northerns) 2-5ml/cm^2 (Southerns, 60°C; Northerns, 65°C). Each for 15min with agitation. Proceed with detection using eg the CDP-Star™ detection module RPN 3510

http://www.amershambiosciences.com
Amersham Biosciences UK Limited
Amersham Place Little Chalfont Buckinghamshire UK HP7 9NA