ECF random prime labelling and signal amplification system

RPN 5751
RPN 5752

STORAGE AND STABILITY

Store labelling module (RPN 5751) at -15°C to -30°C.
Store amplification module (RPN 5750) at 2-8°C.
(For convenience, detection bags may be stored at room temperature, if desired). Stable for at least 3 months when stored as above.

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

ECF random prime labelling and signal amplification system

ECF™ random prime labelling and signal amplification system **RPN 5752**
30 labelling reactions and signal amplification for 2500cm^2 of membrane (consists of RPN 5751 labelling module and RPN 5750 signal amplification module)

ECF random prime labelling module **RPN 5751**
30 labelling reactions

ECF signal amplification module **RPN 5750**
Amplification of signal on 2500cm^2 of membrane

COMPONENTS OF THE SYSTEM

ECF random prime labelling module RPN 5751

Nucleotide mix
5x stock solution of fluorescein-11-dUTP (Fl-dUTP), dATP, dCTP, dGTP and dTTP in Tris-HCl, pH7.8, 2-mercaptoethanol and $MgCl_2$, 300µl
Primers
Random nonamers in an aqueous solution, 150µl
Enzyme solution
5 units/µl exonuclease free Klenow, in a buffer solution, pH6.5, 30µl
Water
1ml deionised water
Control unlabelled DNA
10ng/µl *Hin*d III digested lambda DNA, 50µl
Control fluorescein-labelled DNA
50pg/µl fluorescein-labelled *Hin*d III digested lambda DNA in 50ng/µl herring sperm carrier DNA, 50µl
Liquid block
For use in the recommended hybridisation buffer, 50ml

ECF signal amplification module RPN 5750

Anti-fluorescein alkaline phosphatase (AP) conjugate
5000x stock, 150µl
Liquid block
For use in antibody blocking, 2x100ml
Detection bags
For use during application of the detection reagent and subsequent exposure to the FluorImager, 16 bags
ECF substrate
36mg
ECF substrate dilution buffer
60ml, containing diethanolamine at 2.4M in water. See safety data sheet on pages 48-49.

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.

Warning: Signal amplification module RPN 5750 contains diethanolamine. See safety data sheet on pages 48-49.

All chemicals should be considered as potentially hazardous. We therefore 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. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

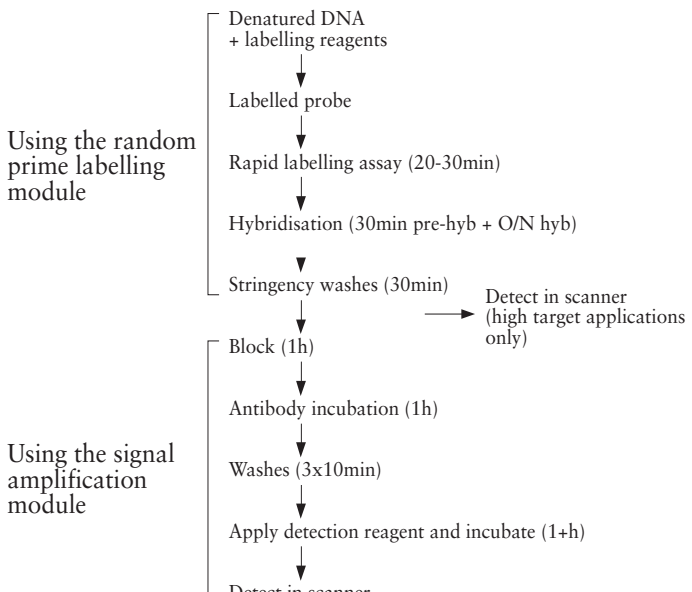
Note that the protocol mentions the use of ethidium bromide and formaldehyde.

Warning: Ethidium bromide may be harmful by ingestion and is a suspected mutagen.

Warning: Formaldehyde is flammable, causes burns and has possible irreversible effects. Please follow the manufacturer's safety data sheets relating to the safe handling and use of these reagents.

DESCRIPTION

The ECF random prime labelling and signal amplification system is a nucleic acid labelling, hybridisation and detection system which is designed as follows:



It allows the production of fluorescein-labelled DNA probes by random prime labelling[1,2] in the presence of fluorescein-11-dUTP. Following hybridisation and stringency washes, where the target levels are high (eg colony or plaque lifts) the hybrids can be detected directly in such instruments as Molecular Dynamics Storm™ and FluorImager™. Where greater sensitivity is needed, then the fluorescent signal can be amplified (after a blocking step) by the use of an anti-fluorescein alkaline phosphatase (AP) conjugate. After washing off the excess conjugate, probe-bound AP is used to catalyse the conversion of the detection reagent to a highly fluorescent product.

This system is tested to ensure that it will detect the following:

1. A single copy gene in human DNA genomic Southern blots, representing 0.25pg (250fg) target in 0.5µg of DNA, 24 hours after addition of detection reagent.
2. 0.1pg of the control fluorescein-labelled DNA on direct dot blots on a normal scan, 1 hour after addition of detection reagent.

CRITICAL PARAMETERS

- If buffer A (see page 10) is not very clean, then spots will appear on the final image. Therefore, it should be made up from fresh ingredients and used within a few days. If problems still occur filtering may help, or if it is desired to keep the buffer for longer than this, it should be autoclaved immediately after making up. For large volumes it is easier to autoclave a 10x solution and dilute it with good quality water immediately before use.
- The random prime reaction works most efficiently with DNA templates of 250bp or greater. Below this a loss of sensitivity will be noticed.
- Accuracy of determination of the amount of nucleic acid to be labelled is critical to the success of non-radioactive labelling systems.
- Once the hybridisation has been started, the entire process should ideally be completed without interruption. If a break in the process is unavoidable, it should be taken after the completion of the stringency washes. Once the blocking stage has begun, other interruptions should be avoided.
- Once the blot has been wetted 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 lead to damage due to repeated freezing and thawing.

ADDITIONAL EQUIPMENT, SOLUTIONS AND REAGENTS REQUIRED

Equipment

Adjustable pipettes, for example Amersham Biosciences pipettes (see related products)
Sterile pipette tips
Standard laboratory glassware
Shaking and standard waterbaths, 37°C and 60°C
Orbital shaker
Polypropylene microcentrifuge tubes
Microcentrifuge
Gloves, preferably powder-free
Plastic boxes/bags for hybridisation
Forceps with non-serrated tips (for handling blots)
Timer

For blotting:
Gel electrophoresis equipment (tanks, power units etc)
Heating equipment for agarose, for example microwave oven
Filter paper (for example, Whatman 3MM)
Paper towels
Vacuum oven 80°C (a normal oven or UV cross-linker is a suitable alternative for nylon membranes)

Solutions

Hybridisation buffer
5xSSC
1 in 20 dilution of 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

Buffer A (1x)
100mM Tris-HCl
300mM NaCl pH7.5
See 'Critical parameters' on page 8 for advice on this buffer.

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
adjust to pH7.5

20xSSC (for Southern blotting and as stock for 2xSSC for rapid labelling assay)
0.3M Na_3 citrate
3M NaCl

5xMOPS (for Northern blotting)
0.1M MOPS
40mM sodium acetate
5mM EDTA

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

10x gel loading buffer
25%(w/v) Ficoll® (Type 400)
0.4%(w/v) bromophenol blue
0.4%(w/v) xylene cyanol FF

Reagents

Bovine serum albumin (BSA) Fraction V (for example, Amersham Biosciences US 70195)
Tween™ 20 (polyoxyethylene sorbitan monolaurate; for example, Amersham Biosciences US 520605).

LABELLING AND DETECTION PROTOCOLS

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

Using the random prime labelling module RPN 5751

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 21). 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. The DNA to be labelled (25ng-2µg) should be in either distilled water or TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) in a volume of at least 10µl and not more than 34µl.	**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 10µl.

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	x µl
Nucleotide mix	10µl
Primers	5µl
Denatured DNA	50ng
Enzyme solution (Klenow) 5 units/µl	1µl
Total	50µl

1.3. Closed circular double-stranded DNA can be linearised to avoid more rapid renaturation following this step, although this is not usually necessary.

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 2µl of 0.5M EDTA. Probes can then be stored in a freezer at -15°C to -30°C in the dark for at least 6 months. Do not use a frost-free freezer.	**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 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.
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 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. Visualise both strips (**sample side down**) either on a UV transilluminator (optimum contrast is obtained using a short wavelength (254nm) transilluminator) or by scanning in the Storm or FluorImager (PMT value 550V). The labelled probe should be visible as a fluorescent spot with an intensity at least as great as the 1/100 dilution 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 (less than the 1/500 dilution) 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.	

3. Hybridisation and stringency washes

The hybridisation and wash conditions given in the following protocol are appropriate for a majority of probes, allowing detection of single copy mammalian genes without significant cross-hybridisation to non-homologous sequences. However, if these conditions are found to be insufficiently stringent for particular probes, then hybridised filters can be washed in 0.2x or 0.1xSSC at 60°C. Alternatively, stringency can be increased by raising the hybridisation or wash temperature to 65°C. Such alterations may lead to some decrease in specific signal, although an overall improvement in signal-to-noise ratio should result.

Protocol	Notes
3.1. The recommended hybridisation buffer consists of the following components: 5xSSC 0.1%(w/v)SDS 20-fold dilution of liquid block (supplied) 5%(w/v) dextran (mw 500000) Combine all the components, make up to the required volume, mix well by stirring until dissolved. Only gentle heating should be required.	**3.1.** For convenience it is recommended that the buffer is made up in a large volume and 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 3 months. Alternatively, use of Amersham Biosciences's Rapid-hyb™ buffer (RPN 1636) will give very good results when used in overnight hybridisations. (When using the rapid-hyb buffer in this manner, the probe concentration should be significantly reduced (to 1-2ng per ml).
3.2. Preheat the required volume of hybridisation buffer (0.3ml/cm^2 for small	**3.2.** Hybridisation can be carried out in boxes, bags or tubes provided there is

blots and 0.125ml/cm^2 or less for large blots) to 60°C, then place the blots in the buffer and pre-hybridise for at least 30 minutes at 60°C with constant, gentle agitation.	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.** 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 3.2.). If the volume is less than 10µl, make up to this volume with water or TE buffer. Denature the probe by boiling for 5 minutes and snap cool on ice.	**3.3.** The recommended probe concentration is 10ng/ml; for a 50ng template reaction, this is approximately 1.5µl of reaction product per ml of hybridisation buffer. (If rapid-hyb buffer is used, the best results will be obtained by retaining overnight hybridisation but reducing the probe concentration to 1-2ng/ml).

Protocol	Notes
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 with gentle agitation.	**3.5.** For high target applications it is possible to use shorter hybridisation times. Some loss of sensitivity will result, but if necessary such loss can be offset by the use of higher probe concentrations.
3.6. Prepare a stringency wash solution of 1x SSC, 0.1%(w/v)SDS and pre-heat it to 60°C. Carefully place the blots in this solution and wash for 15 minutes at 60°C with gentle agitation. Carry out a further wash in pre-heated 0.5xSSC, 0.1%(w/v)SDS at 60°C for 15 minutes. Both solutions should be used at a volume of approximately 2-5ml/cm^2 of membrane for small blots. For a 20x20cm blot, 500ml may be adequate.	**3.6.** For the second wash the SSC concentration can be varied in the range 0.1-1xSSC to achieve the desired stringency (the SDS concentration should remain constant at 0.1%(w/v)). 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]. Several blots can be washed in the same solution provided that they can move freely.

4. Direct detection of fluorescein-labelled probe

For high target applications e.g. colony or plaque lifts, it may be possible to detect the bound probe immediately at this stage by virtue of the intrinsic fluorescence of the fluorescein-dUTP with which the probe has been labelled (see protocol below). If desired, this protocol can be tested prior to progressing to stage 5 without harm to the blot. Otherwise, signal enhancement should be continued by progressing through from stage 5 using the reagents supplied in the signal amplification module RPN 5750. **If blots are to be stored at this stage until ready to proceed, they must not be allowed to dry out.**

Protocol	Notes
4.1. To detect the blot directly at this stage, rinse the blot briefly in 2xSSC and place the blot face down on to the scanner sample holder.	
4.2. Lay a piece of SaranWrap or similar material over the blot to prevent it from drying out.	**4.2.** It is particularly important that the blot does not dry out either if it is found necessary to continue through the amplification stage or if it is intended to reprobe the blot.
4.3. Scan the blot either using the 530nm filter or with no filter in place. (Use the 570nm filter only for the amplified signal).	

Using the ECF signal amplification system RPN 5750

5. Blocking, antibody incubation and washes

The following steps are all performed at room temperature and all the incubations require constant agitation of the blots.

Protocol	Notes
5.1. Following the hybridisation washes, briefly rinse the blots in an excess (2ml/cm^2) of buffer A (1x) at room temperature.	**5.1.** 1x buffer A is a solution of 100mM Tris-HCl; 300mM NaCl pH7.5. See 'Critical parameters' (page 8) for advice on this buffer.
5.2. With gentle agitation, incubate blots for 1 hour at room temperature in approximately 0.75-1.0ml/cm^2 of a 1 in 10 dilution of liquid blocking agent in 1x buffer A.	**5.2.** Diluted block can be stored frozen (in aliquots) for several weeks and is easily thawed when required. However, it is recommended that no aliquot is subjected to repeated thawing and re-freezing.
5.3. Dilute the anti-fluorescein AP conjugate 5000-fold in freshly-prepared 0.5%(w/v) bovine serum albumin in 1x buffer A. Incubate the blots in diluted conjugate (0.3ml/cm^2 of membrane) with gentle agitation at room temperature for 1 hour.	**5.3.** Diluted conjugate should be used immediately. Loss of sensitivity occurs with diluted conjugate that has been stored or been through a freeze-thaw cycle. Several blots can be incubated together but it is important that there is free access of solution to the blot. With larger blots, it is possible to carry out this stage in hybridisation bags or

5.4. Remove unbound conjugate by washing for 3x10 minutes in 0.3%(v/v) Tween 20 in 1x buffer A at room temperature with agitation (2-5ml/cm^2).	tubes to help minimise volume. For a 20x20cm blot, a volume of 50ml can be used. It may be found that the dilution of conjugate can be optimised further. Greater dilutions will give lower signal but may reduce background, whilst more concentrated conjugate will give the opposite effect. Bovine serum albumin fraction V (for example, Amersham Biosciences code US 70195) should be used in the buffer.

6. Signal generation and detection

Please read through this whole section before proceeding.

Wear powder-free gloves or rinse gloved hands with water before use. Avoid any skin contact with detection reagents.

Protocol	Notes
6.1. Pour the entire contents of the bottle containing the detection buffer into the similar bottle which contains the detection reagent. Screw up the top firmly and shake the bottle gently (e.g. on a roller-mixer) for about 10 minutes to fully dissolve the reagent.	**6.1.** Once dissolved, the reagent can be stored at 2-8°C for 2-4 weeks. For longer storage, we recommend that it is divided into aliquots and frozen. Repeated freezing and thawing of any aliquot should be avoided.
6.2. After completion of the antibody incubation stage and subsequent washes, drain off any excess wash buffer from the blots (by touching the corner of the blot against the box used for washing the blots or other convenient clean surface) and place them on a sheet of clear plastic. The bags supplied can be used for this purpose as follows: **6.2.1.** Cut a section from the bag which is large enough to cover your blot(s) with a small border of at least 1cm.	

6.2.2. Cut along the sides of the bag, leaving one side uncut. Open out the bag and after draining off the blots, arrange them (**sample side up**) on one half of the opened bag. Do not allow the blots to dry out.

6.3. Apply the detection reagent to the blots using a pipette. Only enough need be applied (~25µl/cm^2) so that when the bag is closed, the solution can be spread evenly over the blot.

6.4. After applying the reagent, fold the plastic over the top of the blots and immediately spread the reagent evenly over the blot(s). This can be done either by rolling a 5ml pipette over the surface or wiping the surface with a **gloved** hand.

6.5. For best results, the blots can now be transferred to a fresh bag before being placed in the scanner. If it is necessary to keep the blots for many hours after addition of the detection reagent, the bags can be heat-sealed to prevent the blots drying out.

6.5. The signal from the reagent will increase with time. For a high target application, an acceptable image may be achieved on scanning only 1 hour or less after addition of the reagent. Scanning 24 hours after addition of the reagent will provide a much stronger signal suited to low target applications. The optimal time for your particular system

Protocol	Notes
	should be found by re-scanning at various times after addition.
6.6. When you are ready to scan, place the bag containing the blot(s) **face down** on to the scanner sample holder. Ethanol or water placed between the lower surface of the bag and the glass will greatly improve the image obtained.	**6.6.** For full instructions on the use of the FluorImager or Storm scanners, please refer to the manual supplied with the machine. The PMT voltage will need to be adjusted to obtain the best possible image from your blot; we recommend that you use normal scan speed and a 200µm pixel size until the PMT voltage has been optimised. Use of a 570nm filter may improve the signal-to-noise ratio, producing a 2-3 fold increase in sensitivity over unfiltered light; the 530nm filter should not be used except when detecting fluorescein-labelled probe directly. Some adjustment of the PMT voltage may also give some benefits when switching between filters/no filters.
6.7. Blots should not be exposed to bright light for long periods. We suggest that they are stored in the dark e.g. in a drawer or a film cassette.	

APPLICATIONS

Southern blotting

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

Protocol	Notes
1. Electrophoresis of DNA is carried out in a neutral agarose gel system[6]. Prepare a 0.8-1% agarose gel containing 1xTAE buffer. Ethidium bromide can be added to a final concentration of 0.2µg/ml to allow visualisation at step 4).	**1.** TBE buffer may also be used e.g. 0.09M Tris-borate, 2mM EDTA pH8.0.
2. DNA samples should be prepared in a solution containing 1x gel loading buffer in 1x TAE. 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, the gel can be visualised under UV transillumination or under the fluorescent scanner by soaking it	**4.** Minimise exposure to UV as this will damage the DNA. The gel may be photographed during this time.

Protocol	Notes
first in ethidium bromide or SYBR™ Green stain (such soaking is not necessary if ethidium bromide was added to the gel mix at stage 1).	
5. Before capillary blotting, the gel should be processed as follows: **5.1.** Depurination, 10 minutes at room temperature with gentle agitation. **5.2.** Denaturation, 25 minutes at room temperature with gentle agitation. **5.3.** Neutralisation, 30 minutes at room 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.** 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. When using nitrocellulose membrane, the neutralisation time should be extended to 45 minutes; replacement with fresh neutralisation solution at least once during this time is suggested.
6. Assemble the capillary blotting apparatus using 10xSSC as the transfer buffer. Allow the DNA to transfer overnight on to Hybond-N+.	
7. Disassemble the transfer apparatus. Mark the membrane appropriately and fix the	

DNA to the membrane by baking (2 hours at 80°C) or by UV cross-linking. **8.** The blot may be used immediately or wrapped in SaranWrap and stored 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, we bake apparatus at 150°C (plastic for 2 hours, glassware for at least 2 hours, paper for 30 minutes). Any other equipment is treated with RNase *AWAY*™. All solutions are prepared using RNase-free water and fresh AnalaR grade reagents. Gloves are worn at all times.

The following protocol is a general method used at Amersham Biosciences Laboratories 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.** 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. For visualising the gel under UV after electrophoresis, extra

Protocol	Notes
	tracks are used 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 gel loading buffer to a final concentration of 1x and apply samples to the gel.	
4. Run the gel in 1xMOPS buffer at 4-5V/cm until the bromophenol blue indicates that the sample has run for a sufficient distance.	**4.** See note 1 above on visualisation of the gel after electrophoresis.
5. Wash the gel in an excess of water for 15 minutes with gentle agitation.	
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. **9.** The blot can be stored in a suitable container at room temperature under vacuum until required.	**8.** If using the Amersham Biosciences UV cross-linker (RPN 2500/2501), 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.)

Dot/slot blotting

The following is a general protocol for preparing manual 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 (Pre-wetting is not required when using Hybond-N+).
2. 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 10µl. Centrifuge briefly to collect the sample at the bottom of the tube. Keep on ice.	**2.** 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).
3. Carefully apply 1µl samples of denatured DNA to the membrane, avoiding touching the membrane with the pipette tip. Leave the filter to air dry.	

4. Fix the DNA to the membrane using heat (80°C for 2 hours) or UV cross-linking (the time for UV cross-linking needs to be optimised for individual transilluminators or cross-linkers) **5.** The filter may be used immediately or stored under vacuum at room temperature.	**4.** Caution: If using **nitrocellulose** membranes (instead of nylon), UV should **not** be used to fix the DNA; fixing should be done by baking in a **vacuum** oven at 80°C for 2 hours.

ADDITIONAL INFORMATION

Further system checks

Use of control reagents

Two control DNAs are supplied with the ECF random prime module: unlabelled *Hin*d III-digested lambda DNA and fluorescein-labelled *Hin*d III-digested lambda DNA. 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.

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

Protocol	Notes
1.1. 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. **1.2.** Prepare the following serial dilutions of both the labelled probe and the control	**1.1.** 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+.

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.	
1.3. 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.	**1.3.** 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.
1.4. Fix the DNA to the membrane either by UV or by baking for 1-2 hours in an 80°C oven.	**1.4.** Very good results can be achieved by UV cross-linking but beware of too long an exposure to UV light as this can damage the DNA leading to loss of signal.
1.5. Proceed with detection following the protocol given in sections 4 and 5 of the main protocol. The 0.1pg dots of both test and control DNAs should be detectable on scanning 1-2 hours after addition of the detection reagent.	**1.5.** 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. 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.

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

The use of the control unlabelled DNA for evaluating problems during labelling has been mentioned above. It can also be used for evaluating blotting and hybridisation problems as follows.

Protocol

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 10pg track, the bands are equivalent to 4.8, 1.9, 1.4, 0.9, 0.5, 0.4 and 0.1pg of DNA respectively. After probing with the labelled, homologous lambda probe, approximately 1pg should be detectable on scanning 1h after addition of detection reagent. 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 test DNA. Also, the fluorescein-labelled control DNA can be used on dot blots to gain familiarity with the detection system alone.

Re-probing blots

For successful removal of probes, **membranes must not be allowed to dry during or after hybridisation and washing.** With Hybond-N+, it is possible to reprobe the same blot several times. Re-probing is not recommended when using nitrocellulose membranes due to their greater fragility. If you nevertheless wish to try re-probing with nitrocellulose then replace step 2 with a single 1 hour wash with 50%(v/v) methanol: water.

Protocol

1. Following detection, membranes may be stored, wrapped in SaranWrap, in a refrigerator (2-8°C).

2. When ready to commence re-probing, incubate the membrane in absolute alcohol (99+% ethanol) at room temperature with agitation (≥1ml per cm^2), 2x10 minutes. (Note: this step can be omitted if the blot has been used only for direct fluorescence without signal amplification.)

3. Add the membrane to a boiling solution of 0.1%(w/v) SDS using approximately 5ml of SDS solution per cm^2 membrane. Place on a bench-top shaker for 15min. Repeat the operation twice more, **using freshly boiling SDS each time.**

4. Pre-hybridise, probe and detect as in standard protocol.

Troubleshooting guide

Problem	Possible cause	Remedy
1. Low sensitivity (faint bands or no bands)	**1.1.** Insufficient incubation time with detection reagent	**1.1.** Re-scan at a later time.
	1.2. Poor transfer of target DNA from gel to membrane	**1.2.** Stain gel with ethidium bromide to check for any DNA remaining. Include control labelled DNA or labelled DNA MW markers in gel (RPN 2071/2072) and detect directly.

	1.3. Target DNA not effectively fixed	**1.3.** Check UV lamp calibration or temperature of oven.
	1.4. Poor labelling efficiency	**1.4.** Check labelling of probe using rapid labelling assay. Make sure DNA was denatured before labelling. Use control unlabelled DNA to check own DNA. Increase labelling efficiency by a longer reaction, higher enzyme concentration or by purification of DNA template. Check that labelled DNA is not too small (<200 bases) by alkaline agarose gel electrophoresis[5].
	1.5. Low hybridisation efficiency	**1.5.** Check hybridisation by using control unlabelled DNA as target and as template for probe (see page 36). Improve hybridisation efficiency by increasing hybridisation time or probe concentration. Try effect of omitting heterologous DNA. Make sure the probe is denatured before hybridisation.
	1.6. Too high a stringency	**1.6.** If high stringency has been used,

Problem	Possible cause	Remedy
		repeat experiment with 0.5x or 1x SSC wash. Try effect of decreasing hybridisation and wash temperature.
	1.7. Low detection efficiency	**1.7.** The antibody conjugate and detection reagent can be checked using direct dot blots of the control Fl-DNA (see pages 32-33). Try using a more concentrated antibody conjugate solution.
2. High general background	**2.1.** Insufficient blocking of nylon membrane	**2.1.** Ensure blots totally immersed during blocking and that blocking volume is sufficient. Do not let blots stick together. Increase length of blocking stage or concentration of blocking agent during pre-hybridisation and/or antibody blocking stage. If liquid block is old and has been stored unfrozen, it may have lost some of its efficacy. Store liquid block frozen in aliquots. (Liquid block can be ordered separately as RPN 3601).

	2.2. Too high a probe concentration	**2.2.** Decrease probe concentration in this specific application.
	2.3. Insufficient hybridisation buffer	**2.3.** 0.125-0.3ml/cm^2 is recommended. There should be sufficient buffer to allow good coverage of the blots when hybridising in boxes.
	2.4. Insufficient movement of the blot during hybridisation	**2.4.** Increase the rate of movement.
	2.5. Too low a stringency	**2.5.** Increase stringency to 0.2x or 0.1xSSC or increase hybridisation temperature (for example to 65°C). Increase number, length and/or volume of stringency washes.
	2.6. Too high an antibody conjugate concentration	**2.6.** Decrease conjugate concentration.
	2.7. Incomplete removal of free conjugate	**2.7.** Check washes have been carried out as recommended. Ensure wash volume is in excess. Try additional washes.
	2.8. Partial drying of membrane	**2.8.** Ensure membrane remains wet. Partial drying can occur if using low

Problem	Possible cause	Remedy
		volumes of antibody and block solutions in tubes.
3. Patchy or grainy background	**3.1.** Membrane damage	**3.1.** Handle blots carefully with gloved hands and blunt non-serrated forceps. Use clean forceps to handle blot after washing, as contamination with hybridisation buffer prior to detection may elevate background.
	3.2. Probe addition	**3.2.** Avoid adding probe directly on to membrane. If necessary, pre-mix with some of the hybridisation buffer.
	3.3. Membrane dried during process	**3.3.** Do not allow membrane to dry out during or after the hybridisation stage.
	3.4. Solid material in buffers	**3.4.** Check solutions for contaminating particulate matter and ensure that all buffer components are totally dissolved.
	3.5. Contamination from powder in gloves	**3.5.** Wear powder-free gloves or rinse gloved hand with water before use.

4. Spotty background	**4.1.** Buffer A contaminated	**4.1.** Dispose of old buffer. Repeat with freshly made buffer. Once opened, buffer A should be discarded at the end of a working day. If even fresh buffer is too dirty, see critical parameters (page 8) for advice on autoclaving.
	4.2. Dirty glassware or plastic-ware used	**4.2.** Use freshly cleaned, dust-free plasticware or glassware. Rinse with ethanol or boiling water before use to remove any minor bacterial AP contamination.
5. Background in tracks	**5.1.** Insufficient stringency	**5.1.** Increase stringency as in 2.5.) above. Use purified insert to avoid hybridisation by vector sequences. Include heterologous DNA in the hybridisation buffer.
6. Blank patches	**6.1.** Contamination from powder in gloves	**6.1.** Wear powder-free gloves or rinse gloved hands in water before use.

Related products

Gene Images 3'-oligolabelling module RPN 5770
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide

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

Gene Images CDP-Star detection module
for 2500cm^2 membrane RPN 3510
for 5000cm^2 membrane RPN 3511

Gene Images random prime labelling module
30 labelling reactions RPN 3540
60 labelling reactions RPN 3541

Gene Images 3'-oligolabelling and ECF signal amplification system RPN 5775
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide; signal amplification reagents for 2500cm^2 membrane (consists of RPN 5770 and RPN 5750)

Gene Images 3'-oligolabelling and CDP-Star detection system RPN 5776
10 labelling reactions each of $100x10^{-12}$ moles of oligonucleotide; detection reagents for 2500cm^2 membrane (consists of RPN 5770 and RPN 3510)

Gene Images random prime labelling and CDP-Star detection system
Reagents for 30 labelling reactions and detection on 2500cm^2 membrane (consists of RPN 3540 and RPN 3510) RPN 3500
Reagents for 60 labelling reactions and detection

on 5000cm^2 membrane (consists of 2xRPN 3540 and 2xRPN 3510)	RPN 3501
Hybridisation oven/shaker	RPN 2510/ RPN 2511
UV Cross-linker	RPN 2500/ RPN 2501
Liquid Block (100ml)	RPN 3601
Rapid-hyb buffer (125ml)	RPN 1635
Rapid-hyb buffer (500ml)	RPN 1636

Membranes

Hybond-N+

Positively charged nylon membrane:

Pack of 10 membranes, 20x20cm	RPN 2020B
Pack of 10 membranes, 22x22cm	RPN 2222B
Pack of 50 discs, 82mm diameter	RPN 82B
Pack of 50 discs, 132mm diameter	RPN 132B
Roll of membrane, 20cmx3m	RPN 203B
Roll of membrane, 30cmx3m	RPN 303B

Film

Hyperfilm-MP

High-sensitivity film for chemiluminescence:

18x24cm	Pack 25 sheets	RPN 2103
30x40cm	Pack 25 sheets	RPN 2104
5x7 inches	Pack 25 sheets	RPN 1674
10x12 inches	Pack 25 sheets	RPN 1681

Cassettes

Amersham Biosciences supplies a range of Hypercassette™ products for autoradiography, the following is a sample from its range:

18x24cm	RPN 1642
30x40cm	RPN 1644
5x7 inches	RPN 1648
10x12 inches	RPN 1650

See the current Amersham Biosciences catalogue 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.

Safety data sheet and contacts

Australia
Sydney
(02)894-5188

Belgium
Gent
(092)41-52-70

Canada
Oakville
(905)847-1166

Denmark
Birkerød
(45)82-02-22

Asia Pacific
Hong Kong
(852)802-1288

France
Les Ulis
(1)69-1828-00

Germany
Braunschweig
(05307)930-0

Iberica
Madrid
(1)304-42-00

Italy
Milan
(02)5088-220

Japan
Tokyo
(03)5992-2828

Nederland
s'Hertogenbosch
(073)641-85-25

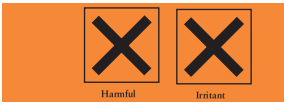
Norway
Gjettum
(67)54-63-18

Sweden
Solna
(08)444-7180

UK Sales
Little Chalfont
(0800)515313

USA
Arlington Heights
IL. (847)593 6300

Safety data sheet SDS204/v08 Date of issue Jul 1998



Amersham Biosciences UK Limited Amersham Place Little Chalfont
Buckinghamshire England HP7 9NA Telephone: (01494) 544000

Product name: Diethanolamine CAS No. 111-42-2

R: 22-36-38	Harmful if swallowed. Irritating to eyes. Irritating to skin.
S: 26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

Composition: Colourless liquid.

Hazards identification: Harmful if swallowed. Irritating to eyes and skin.

First aid measures: In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.

Fire fighting measures: Carbon dioxide, dry chemical powder or polymer foam.

Accidental release: Shut off all sources of ignition. Wear suitable laboratory protective equipment; lab coats, gloves and safety glasses. Absorb on sand or vermiculite and place in a closed container for disposal. Ventilate area and wash spill site after material pick-up is complete.

Handling and storage: Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Do not get in eyes, on skin or on clothing. Do not breath the vapour. Use only in a chemical fume hood. Wash thoroughly after handling. Corrosive.

Personal protection:	See above instructions for handling and storage.
Physical and chemical properties:	Melting point: 27-30°C. Boiling point: 217°C. Vapour pressure: <0.01mm (20°C). Lower explosion limit: 1.6. Flash point: 137°C. Formula weight: 105.14. Density: 1.097. Vapour density: 3.6.
Stability and reactivity:	Incompatible with oxidising agents, copper, copper alloys, zinc or galvanised iron. Avoid contact with acid. Absorbs carbon dioxide from air.
Toxicological information:	LD_{50}: 710mg/kg oral, rat; LD_{50}: 12200mg/kg skin, rabbit.
Ecological information:	Not applicable.
Disposal considerations:	Small quantities may be disposed of by flushing down the sink with copious amounts of water, taking care to avoid inhalation of the vapour. Alternatively use a chemical waste route.
Transport information:	No special considerations applicable.
Regulatory information:	The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.

For further information contact your local office. See page 47.

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 Inc 800 Centennial Avenue
PO Box 1327 Piscataway NJ 08855 USA
Amersham Biosciences Europe GmbH Munzinger Strasse 9
D-79111 Freiburg Germany

RPN5751Pl/AA

ECF random prime labelling and signal amplification system

Protocol reminder card RPN 5751/RPN 5752

Using the ECF random prime labelling module RPN 5751		
Labelling mix Water to 50µl 50ng (25ng-2µg) **denatured** DNA 10µl Nucleotide mix 5µl Primers 1µl Enzyme (Klenow)	**Labelling reaction** Incubate for 1h at 37°C (or 4-6°C overnight) Terminate reaction with EDTA (2µl of 0.5M EDTA)	**Hybridisation** 0.125-0.3ml hybridisation buffer per cm^2 (depending on blot size) Pre-hybridise 60°C/30min Add probe 10ng/ml Hybridise 16h, 60°C See over...

Amersham Biosciences

	Using the ECF signal amplification system RPN 5750	
Stringency washes 1. 1xSSC/0.1%SDS 2. 0.5xSSC/0.1%SDS 2-5ml/cm^2 Each for 15min at 60°C with agitation For high target applications only, scan directly in scanner at this stage	**Antibody development** Rinse in 1x buffer A (100mM Tris-HCl, 300mM NaCl, pH7.5) Incubate in blocking buffer, 0.75-1.0ml per cm^2 (Liquid block diluted 1 in 10 in 1x buffer A) room temp/1h Incubate in Ab conjugate (at 1 in 5000) in 0.5% BSA in 1x buffer A (0.3ml/cm^2) room temp/1h Wash in 0.3% Tween 20 in 1x buffer A, 2-5ml/cm^2 room temp/3x10min	**Detection** Drain blots and place in cut, opened detection bag Apply (dissolved) detection reagent (25µl/cm^2) Fold bag over blots and immediately spread reagent evenly over blots Transfer to clean bags After appropriate time, place in scanner and scan Repeat scan at a later time if stronger signal required

Amersham Biosciences UK Limited Amersham Place
Little Chalfont Buckinghamshire UK HP7 9NA

RPN5751PC/AA