

Amersham ECL™ Direct Nucleic Acid Labeling And Detection Systems

Product Booklet

Codes: RPN3000
RPN3001
RPN3005



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1. Legal

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2. Handling

2.1. 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.

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 material safety data sheet(s) and/or safety statement(s) for specific advice.

You are reminded that certain components in the solutions may cause bleaching on contact with skin.

2.2. Storage

Store hybridization buffer and blocking agent at room temperature. Store remainder of kit at 2–8°C.

2.3. Stability

This system is stable for 3 months when stored under the recommended conditions.

3. Components of the system

	RPN3000	RPN3001	RPN3005
1. DNA labeling reagent Store at 2–8°C	0.5 ml	2 × 0.5 ml	0.5 ml
2. Glutaraldehyde solution Store at 2–8°C	0.5 ml	2 × 0.5 ml	0.5 ml
3. Water Store at 2–8°C	1.0 ml	2 × 1.0 ml	1.0 ml
4. I Hind III control DNA (10 µg/ml) Store at 2–8°C	0.1 ml	2 × 0.1 ml	0.1 ml
5. Gold hybridization buffer Store at room temperature (15–25°C) prior to use. Once this has had blocking agent added it should be stored in aliquots at -15°C to -30°C	500 ml	2 × 500 ml	500 ml
6. Blocking agent Store at room temperature (15–25°C) prior to use.	25 g	2 × 25 g	25 g
7. ECL detection reagent 1 Store at 2–8°C	125 ml	2 × 125 ml	-
8. ECL detection reagent 2 Store at 2–8°C	125 ml	2 × 125 ml	-

All components are stable for 3 months when stored under the above conditions.

4. ECL direct nucleic acid labeling and detection systems

Complete systems

Labeling reagents for 5 µg DNA RPN3000

Hybridization buffer and detection reagent
for 2000 cm² membrane

Labeling reagents for 10 µg DNA RPN3001

Hybridization buffer and detection reagent
for 4000 cm² membrane

Refill packs

ECL direct nucleic acid labeling system RPN3005

Labeling reagents for 5 µg DNA
Hybridization buffer for 2000 cm² membrane

ECL detection reagents

Detection reagents for 2000 cm² membrane RPN3004

Detection reagent for 4000 cm² membrane RPN2105

ECL gold buffer

Hybridization buffer and blocking agent
for 4000 cm² membrane RPN3006

5. Description

The ECL direct nucleic acid labeling and detection system from GE is based on enhanced chemiluminescence (1). The principle technique is outlined in figure 1, page 9.

The system involves directly labeling probe DNA or RNA with the enzyme horseradish peroxidase (2). This is achieved by completely denaturing the probe so that it is in single-stranded form. Peroxidase, which has been complexed with a positively charged polymer, is added and it forms a loose attachment to the nucleic acid by charge attraction. These ionic interactions will be interrupted by the presence of counter ions so it is important that the probe is in a low salt solution. Addition of glutaraldehyde causes the formation of chemical cross-links so that the probe is covalently labeled with enzyme.

Once labeled, the probe is used in hybridization with target DNA or RNA immobilized on a membrane. **From this stage onwards it is important to take care that the enzyme activity is not lost and therefore that the temperature of the hybridization mixture never exceeds 42°C.** A specially optimized hybridization buffer is included in the system which ensures efficient hybridization and protects the peroxidase during this step. A novel rate enhancer is included in the buffer to generate additional sensitivity. The formulation of the buffer includes 6 M urea which is equivalent to 50% formamide in reducing the T_m of hybridization. Therefore when controlling stringency of hybridization the only parameter that may be altered is salt concentration. Recommendations for a suitable initial stringency are given in the protocol.

After hybridization the filters are washed to remove unlabeled probe, again taking care to keep the temperature at or below 42°C. Stringency may be controlled by altering the urea or SSC concentration in the primary wash buffer. If urea is omitted, the temperature of this wash

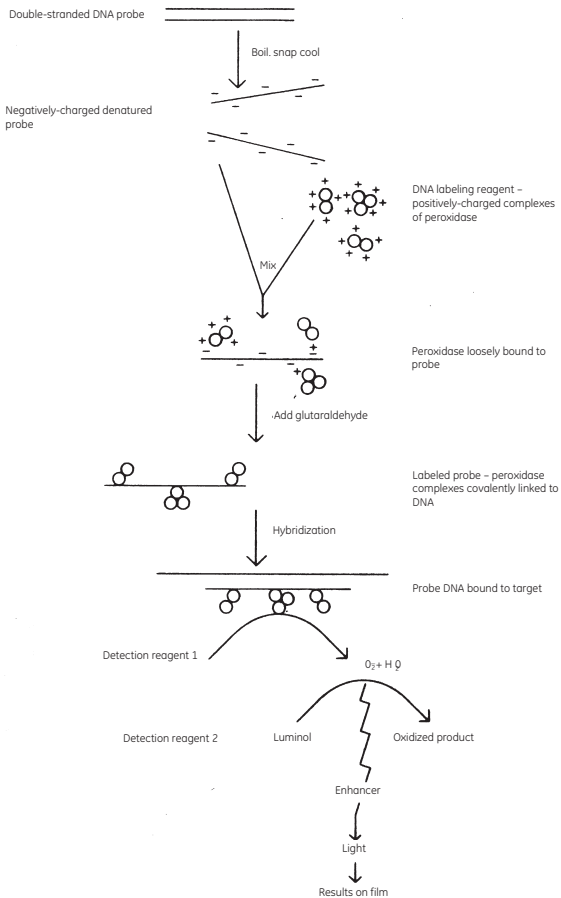


Figure 1. Principles of the ECL direct nucleic acid labeling and detection system.

can be raised to a maximum of 55°C, provided the wash is performed for no longer than 2 × 20 minutes. The washed filters can then be taken directly into the ECL detection step. There are two detection reagents which should be mixed immediately before use.

Detection reagent 1 decays to hydrogen peroxide, the substrate for peroxidase. Reduction of hydrogen peroxide by the enzyme is coupled to the light producing reaction by detection reagent 2. This contains luminol, which on oxidation produces the light. The light output is increased and prolonged by the presence of an enhancer so that it can be detected on a blue-light sensitive film. The ECL direct system has been successfully used in the Southern (3,4), Northern (5) and colony/plaque blotting applications (6).

This booklet contains protocols to all the steps involved in the ECL direct system, from blotting to detection (pages 15–42). It is a simple and rapid technique. It is important, however, to follow the protocol carefully to ensure good results. The protocol summary gives an idea of timescales involved with the system. However, it is recommended that the full protocols are followed until users are familiar with the system. A detachable protocol summary card is provided at the back of this booklet.

6. Critical parameters

- Hybridization temperature must not exceed 42°C to avoid loss of enzyme activity.
- Adequate circulation of the buffer is essential during hybridization as blots should be allowed to move freely.
- Wear powder-free gloves. Powder from the gloves can inhibit the ECL reaction and result in blank patches on film.
- Avoid cross contamination of detection reagents 1 and 2.

7. Additional equipment and solutions required

7.1. Equipment

- Adjustable pipettes for example Pipetman™
- Sterile pipette tips
- Gel electrophoresis equipment (tanks, power units etc)
- Heating equipment for agarose, for example microwave oven
- Large containers for gel boiling
- Standard laboratory glassware
- Filter paper (for example, Whatman™-3MM)
- Waterbaths, 37°C and 42°C shaking
- Ultraviolet transilluminator
- Orbital shaker
- SaranWrap™ (or other suitable cling film)
- Paper towels
- Vacuum oven
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Microcentrifuge
- Plastic boxes/bags for hybridization
- Forceps with rounded non-serrated tips
- Plastic screw-top universal bottles
- X-ray film-cassettes
- Timer
- Film developing facility and reagents

7.2. Solutions

Solutions that are used for DNA electrophoresis, for blotting and for washing the blots after hybridization that are not supplied with the ECL direct nucleic acid labeling and detection system are listed

here. It is recommended that analytical grade reagents and double-distilled water are used throughout.

50× TAE buffer for agarose

Tris (base)	2 M (242 g)
EDTA (disodium salt)	0.05 M (18.6 g)

Adjust to pH8 with glacial acetic acid (~57 ml) and make up to 1 liter.

For use, dilute x50 to give Tris acetate 0.04 M and EDTA 0.001 M.

Reagent for loading DNA samples on to gels

Bromophenol blue	0.05 g
Xylene cyanol	0.05 g
Glycerol	5.00 ml
EDTA	0.186 g

Make up to 10 ml with TAE buffer

Loading buffer for RNA samples

Formamide	0.72 ml
10× MOPS buffer	0.16 ml
Formaldehyde (37%)	0.26 ml
Water	0.18 ml
80% glycerol	0.1 ml
Bromophenol blue (saturated solution)	0.08 ml

Store in a freezer at -15°C to -30°C.

10× MOPS buffer

MOPS [3-(N-morpholino) propanesulphonic acid]	0.2 M
Sodium acetate	0.05 M
EDTA	0.01 M

Depurination solution

HCl	250 mM
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Denaturation solution

NaCl	1.5 M
NaOH	0.5 M

Neutralizing solution

NaCl	1.5 M
Tris HCl	0.5 M

pH adjusted to 7.5

20x SSC

Na ₃ citrate	0.3 M
NaCl	3 M

pH 7.0

Primary wash buffer containing urea

Urea 360 g	6 M
SDS 4 g	0.4%
20x SSC 25 ml	0.5x SSC

Make up to 1 liter. This can be kept for up to 3 months in a refrigerator at 2–8°C. Stringency may be increased by using a lower final SSC concentration, for example 0.1x SSC instead of 0.5x SSC.

Primary wash buffer without urea

SDS 4 g	0.4%
20x SSC 25 ml	0.5x SSC

Make up to 1 liter. This can be kept for up to 3 months in a refrigerator at 2–8°C. Stringency may be increased by using a lower final SSC concentration, for example 0.1x SSC instead of 0.5x SSC.

Secondary wash buffer (2x SSC)

20x SSC	100 ml
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Make up to 1 liter. This can be kept for up to 3 months at 2–8°C.

8. The ECL direct nucleic acid labeling and detection system protocols

8.1. Protocol summary

1. Preparation of nucleic acid blot

2. Hybridization buffer preparation

Add salt, dissolve blocking agent (1.5–2 hours)

3. Pre-hybridization of blots

42°C for 15–60 minutes

4. Probe labeling

Denaturation for 10 minutes

Labeling reaction for 10 minutes

5. Hybridization

42°C for several hours (for example, colony screening) to overnight (for example, single copy gene detection)

6. Blot washing

2 × 20 minutes for stringency washes, 2 × 5 minutes in SSC

7. Detection

Incubation in detection reagents (1 minute)

Exposure to Hyperfilm™ ECL (several minutes–2 hours)

8.2. Protocol A. Labeling, hybridization and detection

Users are recommended to read the complete protocols before starting work. The techniques of capillary or Southern blotting are described in protocol B, page 30, Northern blotting in protocol C, page 37 and colony/plaque blotting in protocol D, page 39. Before commencing labeling, it may be found helpful to prepare the hybridization buffer and begin pre-hybridizing the membrane (see section 8.2.2.).

8.2.1. Preparation of labeled probe

The following protocol is for labeling 100 ng of probe DNA. However, the amount of probe DNA required for a hybridization depends on the size of blot to be probed, the amount of target DNA present and the proportion of probe sequence within the DNA to be labeled. In practice, blot size will determine the hybridization volume to be used (see section 8.2.2.), while the amounts of target and probe sequence will determine the probe concentration. 10 ng probe/ml is appropriate for many applications, although this may be reduced where there is a large amount of available target DNA, for example in many plaque or colony screening experiments (protocol D).

The signal:noise ratio of the final result is sometimes lower when whole clones are used as hybridization probes. Protocols for purifying inserts from vectors can be found on pages 43–45. For labeling probes in low melting point agarose see page 46. The method can be used to label probes greater than 50 bp in length, although for single copy gene detection, probes longer than 300 bp are recommended to give the required sensitivity. The protocol is written for DNA probes, but an identical procedure can be followed to label RNA probes.

Protocol

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| <p>1. Dilute the DNA to be labeled to a concentration of 10 ng/μl using the water supplied.</p> <p>2. Denature 100 ng of the DNA sample (10 μl) by heating for 5 minutes in a boiling water bath.</p> <p>3. Immediately cool the DNA on ice for 5 minutes. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.</p> <p>4. Add an equivalent volume of DNA labeling reagent (10 μl) to the cooled DNA. Mix gently but thoroughly.</p> | <p>1. The concentration of salt in the sample of nucleic acid should not exceed 10 mM. The <i>Hind</i> III control DNA is diluted ready for use (100 ng/10 μl).</p> <p>2. The minimum amount of DNA which should be labeled is 100 ng in 10 μl. If more is required the volume should be increased appropriately while maintaining the concentration at 10 ng/μl. It is essential to use a vigorously boiling water bath rather than a heating block for complete denaturation.</p> <p>3. Maintaining the DNA in a single stranded form is required to ensure a good labeling efficiency. In general denaturation of RNA is also advised to avoid secondary structure.</p> <p>4. The volume of labeling reagent should be equal to the volume of DNA to be labeled. For example 100 ng of DNA in 10 μl will require 10 μl of labeling reagent.</p> |
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Protocol**Notes**

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| <p>5. Add the glutaraldehyde solution, use a volume equivalent to the volume of the labeling reagent (10 μl). Mix thoroughly. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.</p> <p>6. Incubate for 10 minutes at 37°C.</p> <p>7. If not used immediately, the probe can be held on ice for a short period, for example 10–15 minutes.</p> | <p>5. For example, 10 μl DNA solution (10 ng/μl) + 10 μl labeling reagent will require 10 μl glutaraldehyde solution. It is important to maintain the ratio of DNA labeling reagent:glutaraldehyde to ensure a good labeling efficiency. Alteration to this ratio can result in high backgrounds and poor sensitivity.</p> <p>6. When labeling nucleic acids <300 bp, increasing the incubation period to 20 minutes may improve labeling efficiency.</p> <p>7. Labeled probes may be stored in 30% glycerol at -15°C to -30°C (see page 43) for up to six months. Further treatment of the probe before use is not required.</p> |
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8.2.2. Hybridization and stringency washes

The hybridization 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-hybridization to non-homologous sequences.

Protocol	Notes
<p>1. Prepare the hybridization buffer as follows: At room temperature, take the required volume of hybridization buffer. Add solid sodium chloride (analytical grade) to a concentration which is most suitable for effective probe hybridization. Add the blocking agent to a final concentration of 5% (w/v). Immediately mix thoroughly to get the blocking agent into a free suspension. Continue mixing at room temperature for 1 hour on a magnetic stirrer or roller mixer, then heat to 42°C for 0.5–1 hour with occasional mixing.</p>	<p>1. 0.25 ml/cm² is recommended for small blots and 0.125 ml/cm² for large blots. The optimum sodium chloride concentration may be different for different probes. 0.5 M NaCl generally gives acceptable results. If excess buffer is prepared aliquots should be pipetted into sterile plastic containers and stored at -15°C to -30°C. Prepared buffer may be stored for at least three months.</p>
<p>2. If necessary pre-heat the required volume of hybridization buffer to 42°C, place the blots in the buffer</p>	<p>2. Hybridization can be carried out in bags or boxes, provided there is sufficient buffer for the container. Adequate</p>

Protocol

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2. *Continued*

and prehybridize for at least 15 minutes at 42°C with gentle agitation. A 1 hour prehybridization is routinely used at GE.

3. Following pre-hybridization, add the labeled probe to the pre-hybridization buffer. Avoid placing it directly on the membrane and mix gently. Some of the buffer can be withdrawn for mixing with the labeled probe before addition to the buffer. Continue incubation with gentle agitation overnight at 42°C.

2. *Continued*

circulation of the buffer is essential when hybridizing several blots together and it is important that the blots are allowed to move freely. High backgrounds can result if there is insufficient hybridization buffer or movement of the blots. When placing a blot on the surface of the hybridization buffer, allow it to saturate completely. Avoid trapping air between the blot and the buffer, and finally submerge the blot in the buffer. This is particularly important when hybridizing several blots together.

3. It is important not to exceed 42°C during hybridization; higher temperatures are deleterious to the enzyme component of the DNA labeling reagent. It is possible to use shorter hybridization times. Some loss of sensitivity is likely with low target applications, for example

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4. Prepare the primary wash buffer (see page 14) and preheat to 42°C. This is used in excess at a volume of approximately 2–5 ml/cm² of membrane. Carefully transfer the blots to the solution and wash for 20 minutes with gentle agitation, ensuring temperature does not exceed 42°C.
3. *Continued.*
single copy gene analysis but for high target applications, for example colony and plaque blotting (protocol D), shorter hybridizations of 1–4 hours are recommended.
4. Each primary wash should not exceed 20 minutes. If desired, primary wash buffer without urea can be used. This should be pre-warmed to 55°C. 2 × 10 minute washes at 55°C are recommended. The total wash time should not exceed 20 minutes, to protect peroxidase enzymatic activity. Longer washing times at 55°C can result in a significant loss of sensitivity. Stringency may be altered by the SSC concentration in both types of primary wash buffers; 0.5x SSC is low stringency, 0.1x SSC is high stringency (see page 14). Several blots can be washed in the same solution provided that they can move freely. Stocks of the primary wash buffer are stable at 2–8°C for up to 3 months.

Protocol**Notes**

5. Carry out a further wash in fresh primary wash buffer at 42°C for 20 minutes.
6. Place the blot(s) in a clean container and add an excess of secondary wash buffer (See page 14). Wash with gentle agitation for 5 minutes at room temperature. Carry out a further wash in fresh secondary wash buffer at room temperature for 5 minutes.
6. If necessary blots may be left in secondary wash buffer for up to 30 minutes at room temperature before detection. Hybridized blots may be stored overnight wetted with secondary wash buffer wrapped in SaranWrap, in a refrigerator at 2–8°C. Do not allow the blot(s) to dry out.

8.2.3. Signal generation and detection

Please read through this whole section before proceeding.

For optimum sensitivity, it is necessary to work with reasonable speed once the blots have been exposed to the detection solutions, as there is no lag phase with the ECL reaction. (All steps can be carried out in a darkroom if desired; it is necessary to switch off the light only after step 5).

Equipment which is needed includes an X-ray film cassette, a roll of SaranWrap (other 'cling-films' may be suitable), a timer and blue-light sensitive autoradiography film, for example Hyperfilm ECL (RPN2103/2104). If possible wear powder free gloves. Powder from gloves can inhibit the ECL reaction leading to blank patches on film.

The exposure times recommended below are appropriate for Southern blot applications such as the detection of single copy mammalian genes. Shorter exposures can be used for high target applications such as colony or plaque lifts or the detection of amplified target.

Protocol

Notes

1. Mix an equal volume of detection reagent 1 with detection reagent 2 to give sufficient solution to cover the blot. 0.125 ml/cm² is recommended.

1. Mix only the volume of detection reagents necessary to cover the blot. Storage of the mixed solution is not recommended. If not used immediately, prepared detection solution can be held on ice for a short period, for example 30 minutes. Avoid cross-contamination of reagents 1 and 2.

Protocol**Notes**

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| <p>2. Drain the excess secondary wash buffer from the blots and place them in fresh containers or on a sheet of SaranWrap, DNA side uppermost. Add the detection reagent directly to the blot(s) on the side carrying the DNA; do not leave the blots to dry out.</p> <p>3. Incubate for 1 minute at room temperature.</p> <p>4. Drain off excess detection reagents and wrap the blots in SaranWrap. Gently smooth out air pockets.</p> <p>5. Place the blots DNA side up, in the film cassette. Work as quickly as possible to minimize the delay between incubating the blots in substrate and exposing them to the film (next step).</p> <p>6. Switch off the lights and place on a sheet of autoradiography film (for example Hyperfilm ECL) on top of the blots. Close the cassette and expose for 30 seconds.</p> | <p>2. An alternative method is to equilibrate the drained blots in a half quantity of reagent 2 (0.0625 ml/cm²), add to this a similar quantity of reagent 1, and mix by gentle shaking.</p> <p>4. Excess detection reagent can be removed by gently laying the blots, DNA side up, on a folded tissue or Whatman 3 MM paper for a few seconds.</p> <p>5. Ensure there is no free detection reagent in the film cassette; the film must not get wet.</p> <p>6. This should be carried out in a dark room, using red safe lights. Do not move the film whilst it is being exposed. Hyperfilm ECL can be pre-flashed if required (e.g. using</p> |
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6. *Continued.*
a Sensitize pre-flash unit, RPN 2051). This can give a significant improvement in sensitivity at low levels. The DNA side of the filter (wrapped in SaranWrap) must be placed next to the film for maximum sensitivity.
7. Remove the film and develop. If required, expose a second film for an appropriate length of time.
7. For initial experiments with the ECL direct system, an exposure of 30 minutes is recommended with low target applications. A further exposure of 60–120 minutes should give an equivalent image. An alternative approach is to carry out a short 1–2 minute initial exposure and use this to judge the length of subsequent exposure. When undertaking colony and plaque screening it is important to use the minimum film exposure time which enables positive colonies/plaques to be unambiguously detected.

8.2.4. Hybridization and stringency washing in tubes

Nucleic acid hybridization and stringency washing may be conveniently performed in an incubator with an integral rotisserie device or in a hybridization oven. These ovens allow the continuous movement of fluid over the hybridizations which can therefore be performed in minimal volumes so that they are particularly economical on probe usage. The procedure outlined below is intended as a general guideline for the use of hybridization ovens. It may require optimization depending on the application and equipment available.

Protocol

Notes

1. Preheat the hybridization buffer (section 1 page 19) to 42°C.
2. In a suitable container pre-wet the blot in 5x SSC (75 mM Na citrate, 0.75 M NaCl, pH 7.0). Loosely roll the blot and place inside the tube. Add a small amount of 5x SSC to the tube and unroll the blot ensuring no air bubbles are trapped between the membrane and the tube. Do not allow the blot to overlap itself.
2. It is important to exclude any bubbles trapped between the hybridization tube and the membrane as this can lead to black patches on detection. Nylon mesh may be used if there is significant overlap of the blot. Sandwich the blot between two layers of nylon mesh. Roll the sandwich (ensuring no air bubbles are trapped) with the nucleic acid facing inwards. Place the roll in the tube so that it 'unrolls' in the opposite direction from

Protocol

Notes

3. Pour off the 5x SSC and add the appropriate volume of hybridization buffer.
 4. Prehybridize in a rotisserie oven for at least 15 minutes at 42°C.
 5. Prepare the labeled nucleic acid probe (as in section 1).
 6. Add the labeled probe to the prehybridization buffer, but
2. *Continued.*
the movement of the rotisserie. Again ensure that no air bubbles are trapped. Nylon mesh may be reused following washing in a 1% SDS solution and thorough rinsing in distilled water.
 3. A hybridization volume of 0.0625–0.125 ml/cm² is recommended. In practice, the tube size will determine the minimum volume to be used; for example the minimum volume for use with a 4 cm diameter and 30 cm long is 20 ml. Generally more buffer is required than would be used with standard radioactive hybridizations. High backgrounds can result if there is insufficient hybridization buffer.
 6. Some of the buffer can be withdrawn for mixing with the

Protocol**Notes**

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| <p>6. <i>Continued.</i>
avoid placing it directly on to the membrane. Hybridize overnight in a rotisserie oven at 42°C.</p> <p>7. Prewarm the appropriate volume of primary wash buffer to 42°C.</p> <p>8. Discard the hybridization buffer. Add 50–100 ml 5x SSC to the tube and replace in the rotisserie oven for 5 minutes.</p> <p>9. Discard the 5x SSC and replace with primary wash buffer so that the hybridization tube is approximately one-third full. Return the tube to the rotisserie oven and wash the blot for 20 minutes at 42°C.</p> <p>10. Discard the primary wash buffer and replace with an equivalent volume of fresh buffer. Wash for 10 minutes</p> | <p>6. <i>Continued.</i>
labeled probe before addition to the bulk of the buffer.</p> <p>7. If desired, primary wash buffer without urea may be used. This should be preheated to 55°C.</p> <p>8. When washing with primary wash buffer without urea, use this period to increase the temperature of the hybridization oven to 55°C. This should take 5–10 minutes depending on the apparatus.</p> <p>9. When using primary wash buffer without urea, wash for 10 minutes, followed by 2 × 5 minutes at 55°C. The total wash time should not exceed 20 minutes. Stringency may be altered by adjusting the SSC concentration in both types of primary wash buffer (see section 2).</p> |
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10. *Continued.*
at 42°C. Repeat this wash a second time.
 11. Remove the blot from the hybridization tube, place in a suitable container and cover with an excess of secondary wash buffer. Incubate with gentle agitation for a further 5 minutes at room temperature.
 12. Discard the secondary wash buffer and replace with an equivalent volume of fresh secondary wash buffer. Incubate with agitation for a further 5 minutes at room temperature.
 13. Carry out the detection as outlined in section 8.2.3.

8.3. Protocol B. Southern blotting

8.3.1. Gel electrophoresis

Protocol	Notes
<ol style="list-style-type: none">1. Make a 1% agarose solution in 1x TAE buffer (see page 13).	<ol style="list-style-type: none">1. Heating to approximately 90°C is required to dissolve agarose. Low electroendosmosis grade agarose is recommended (for example, Agarose NA, 17-0554-01, -02, -03). Different concentrations of agarose can be used, ranging from 0.8%–2% depending on the size of the fragments to be separated.
<ol style="list-style-type: none">2. Cool agarose solution to 50–60°C. Add ethidium bromide, mix, pour into gel former, and insert gel comb(s) to produce wells of up to 5 mm in width, removing any air bubbles.	<ol style="list-style-type: none">2. Use 1 µl of a 10 mg/ml solution of ethidium bromide per 60 ml of agarose solution. Care should be taken to avoid skin contact with this reagent.
<ol style="list-style-type: none">3. Allow to set and place gel in electrophoresis tank. Fill tank with gel running buffer, sufficient to cover surface of the gel.	<ol style="list-style-type: none">3. Gel running buffer: 1x TAE as for agarose. Gel running buffer must be fresh, do not reuse. The gel running tank must be clean. Failure to ensure cleanliness of the tank and buffer may result in the appearance of a black band at the top of the blot on detection.

Protocol

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| <p>4. Apply DNA samples to wells in gel. The diluted λ DNA should be heated for 5 minutes at 65°C and then snap-cooled on ice before loading. This dissociates the <i>cos</i> ends.</p> <p>5. Apply current to gel and allow to run until bromophenol blue indicates that sample has run for a sufficient distance, for example two thirds of the way down the gel.</p> | <p>4. Loadings of 2–10 μg of restriction enzyme digested genomic DNA are usually sufficient. The control DNA (λ DNA <i>Hind</i> III digest) supplied in the kit (10 $\mu\text{g}/\text{ml}$) should be loaded at 100 pg per well. 1x TAE buffer is a suitable diluent for the DNA and the inclusion of high density mixture (at 20% of the final volume) containing xylene cyanol and bromophenol blue dye (see page 13) aids loading the samples into the gel. Sheared herring sperm DNA (2.5 μg) may be added to the diluted (100 pg) control DNA as a ‘carrier’, but it is not essential.</p> <p>5. A voltage of, for example, 60 volts over 4–5 hours for a 20 cm long gel can be used for genomic DNA. Overnight electrophoresis runs can be achieved by using 1 V/cm of gel.</p> |
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8.3.2. Processing the gel

Protocol

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| <p>1. Slide gel from gel-former into a suitable plastic box or a glass/pyrex dish.</p> <p>2. Cover with depurination solution (see page 13) and begin agitation, for example using an orbital shaker, ensuring that the gel moves freely. Treatment should be stopped immediately the bromophenol blue-dye has turned completely yellow (10–12 minutes).</p> <p>3. Discard depurination solution and rinse gel with distilled water.</p> <p>4. Cover gel with denaturation solution (see page 14) and begin agitation. Agitate for 25 minutes after the bromophenol dye has returned to its blue colour.</p> <p>5. Discard solution and rinse gel with distilled water.</p> | <p>1. With UV illumination, photograph the gel to record the electrophoretic separation of the loaded DNA samples/ controls.</p> <p>2. Approximately 400 ml is recommended for a 20 cm × 20 cm gel. Depurination is not needed if only small fragments (<10 kb) are to be transferred.</p> <p>4. Use a similar volume to that used for depurination.</p> |
|--|--|

Protocol**Notes**

6a. Hybond™ N+: Cover gel with neutralizing solution (see page 14) and begin agitation. Continue agitation for 30 minutes.

6. Use a similar volume to that used for depurination and for denaturation. While the gel is neutralizing begin to prepare for the capillary blotting.

6b. Nitrocellulose membrane:

Cover gel with neutralizing solution (see page 14) and begin agitation. Continue agitation for 30 minutes. Discard solution and replace with fresh neutralizing solution. Continue agitation for a further 15 minutes.

8.3.3. Capillary blotting

Protocol**Notes**

1. Part fill a glass/pyrex dish with 20x SSC, and make a supporting platform (for example using a glass plate or a perspex gel former). Cover it with a wick made from three sheets of filter paper (for example Whatman 3 MM saturated with 20x SSC).
2. Place the gel on the 3 MM paper, taking care to avoid trapping air bubbles.

1. There is a diagram of the capillary blotting stack on page 34. Overlay the filter paper with cling film to enable air bubbles to be smoothed out. A glass rod or a pipette can be used for this and for subsequent similar operations. The cling film should be removed afterwards.

2. *Continued.*

Surround with cling film to prevent the SSC being absorbed directly by the paper towels.

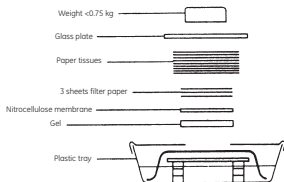
3a. Hybond-N+: Cut a sheet of Hybond-N+ nylon membrane to the size of the gel.

3b. Nitrocellulose membrane: Cut a sheet of nitrocellulose membrane to the size of the gel. Pre-wet the membrane by briefly immersing in distilled water, followed by soaking in 20x SSC for at least 10 minutes.

4. Place the membrane on top of the gel, without trapping air bubbles.
5. Place three sheets of 3 MM paper, cut to size and wetted with 10x SSC on top of the membrane, again avoiding trapping air bubbles.
6. Place a 5–7 cm stack of absorbent paper towels on top of the 3 MM paper.

3. Care should be taken to avoid contaminating the membrane by touching it. Gloves should be worn.

4. The membrane should not come into direct contact with the 3 MM paper wick.



Protocol	Notes
7. Place a glass plate on top of the paper towels and apply a weight (for example approximately 750 g for a 20 cm × 20 cm gel). Leave overnight (or for at least 4 hours).	

8.3.4. Processing the blot

Protocol	Notes
1. Dismantle blotting stack down to membrane, remove membrane and gel together, place membrane side down on a clean piece of 3 MM paper. Peel off gel and discard. Mark the membrane so that the face carrying the DNA is distinguishable.	1. A soft-lead graphite or a chinagraph pencil can be used to mark the membrane.
2a. Hybond-N+ : Place membrane in 6x SSC and rinse for 1 minute with gentle agitation to remove agarose. There are several alternative methods which may be used for DNA fixation, but for ECL detection either baking or UV treatment is recommended. i) Baking (80°C for 2 hours)	2a. Hybond-N+ : If not to be used immediately, fixed blots can be stored dry wrapped in SaranWrap at 2–8°C, or, for up to several weeks at room temperature under vacuum.

Protocol	Notes
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2a. Continued.

as described for Nitrocellulose membrane below. A vacuum is not required with nylon membranes.

ii) UV fixation optimize for each transilluminator/cross linker.

For RNA blots baking is the recommended fixation method as described in i) above. UV fixation can also be used.

2b. Nitrocellulose membrane:

Lay filter, DNA side up, on a clean piece of 3 MM paper and allow surface liquid to drain, then remove to a second piece of 3 MM paper and allow to air dry. Sandwich the blot between fresh 3 MM paper and bake dry (80°C for 2 hours) in a vacuum oven.

2b. Nitrocellulose membrane:

Do not exceed a temperature of 80°C. While blot is baking prepare to label probe and set up hybridization (section 8.2.). If not to be used immediately, baked blots can be stored dry at either 2–8°C or at room temperature under vacuum.

8.3.5. Probe labeling, hybridization and detection

Protocol	Notes
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1. Follow protocol A, sections 8.2.1.–8.2.3. inclusive.

1. Probe concentrations, buffer volumes and detection times

8.4. Protocol C. Northern blotting

Electrophorese RNA in a denaturing system. Formaldehyde/agarose is recommended for use with Hybond membrane

8.4.1. Gel electrophoresis

Protocol	Notes						
<p>1. Prepare gel; for example to prepare 300 ml of gel add:</p> <table><tr><td>Agarose</td><td>3–4.5 g</td></tr><tr><td>10x MOPS buffer</td><td>30 ml</td></tr><tr><td>Water</td><td>219 ml</td></tr></table> <p>Dissolve agarose by heating in a microwave or on a steam bath, mix well and cool to 50°C. In a fume hood, add 16.2 ml of 37% (v/v) formaldehyde, mix well, add 20 µl of a 10 mg/ml solution of ethidium bromide, mix by swirling. Working in a fume hood, pour the gel into the gel former, and allow to set.</p>	Agarose	3–4.5 g	10x MOPS buffer	30 ml	Water	219 ml	<p>1. 250 ml is sufficient for 4000 cm² gel. Thin gels are more desirable than thick gels.</p>
Agarose	3–4.5 g						
10x MOPS buffer	30 ml						
Water	219 ml						
<p>2. Prepare RNA samples: Loadings are normally 10–20 µg of total RNA, or less for an abundant mRNA. 1–2 µg or less is usually sufficient for poly(A)⁺ RNA. The RNA in 5 µl or less of buffer is diluted with 15 µl of RNA loading buffer.</p>							

Protocol**Notes**

2. Continued.

(see page 13). Boil sample, in a vigorously boiling water bath, for 2 minutes. Chill samples on ice and then load on to gel.

- 3. Running the gel:** Running buffer is 1x MOPS buffer. Run gel at 5–7.5 V/cm for 2–3 hours, or until the bromophenol blue dye has migrated three-quarters of the way down the gel. The gel may now be photographed under UV illumination.

8.4.2. Treating the gel

Rinse the gel for 3 × 10 minutes in 10x SSC to remove formaldehyde from the gel. Use a volume sufficient to float the gel in the container.

8.4.3. Blotting the gel

Follow the procedure in Southern blotting protocol, section 8.3.3., 'Capillary blotting', and section 8.3.4., 'Processing the blot', taking care to read the notes for Northern blotting.

8.4.4. Probe labeling, hybridization and detection**Protocol****Notes**

- 1.** Follow protocol A sections 8.2.1.–8.2.3. inclusive.

- 1.** Probe concentrations, buffer volumes and detection times given in protocol A are appropriate for both Southern and Northern blots.

8.5. Protocol D. Colony and plaque blotting

The following protocols describe the application of the ECL direct system to colony and plaque screening. The recommended probe concentrations, hybridization times and film exposure times, whilst generally applicable, are intended as guidelines; some optimization may be necessary for individual systems.

8.5.1. Plating out and incubation

Protocols	Notes
<ol style="list-style-type: none">1. Plate out cells or bacteriophage in the normal way and incubate overnight at the required temperature.	<ol style="list-style-type: none">1. For chloramphenicol amplifiable plasmids it is possible to limit this incubation to 5–6 hours, followed by an overnight amplification on chloramphenicol plates.

8.5.2. Colony/plaque lifts

Protocols	Notes
<ol style="list-style-type: none">1. Remove the agar plates from the incubator. Label a Hybond-N+ disc (RPN 82B) with a chinagraph pencil, or other suitable indelible marker. Place the disc (labeled side down) on the surface of the plate, in contact with the colonies, until the disc is completely wetted.2. Mark the membrane and agar using a sterile needle or other	<ol style="list-style-type: none">1. For plaques (M13/λ) and colonies (which are to be chloramphenicol amplified), leave the discs on the agar plates for 1–5 minutes.2. For plaques (M13/λ) and colonies (which are to be

Protocols

Notes

- Continued.*
means to ensure correct orientation of colonies/ plaques.
- Remove the disc from the agar plate. If no amplification is to be performed, proceed directly to section 8.5.3. step 1.
- For colonies which are to be chloramphenicol amplified, the disc should now be placed colony side up on an L-agar/chloramphenicol plate, and incubated at 37°C overnight.
- Remove the disc from the agar plate.

- Continued.*
chloramphenicol amplified), leave the discs on the agar plates for 1–5 minutes.

8.5.3. Lysis and fixation

Protocols

Notes

- Place the disc, colony/plaque side up, for 5 minutes on to two sheets of Whatman 3 MM paper which have been saturated with 0.5 M NaOH.

- Periods of longer than 1 minute for colonies will increase the intensity of the signal on detection, but may allow the colonies to diffuse slightly. There is no need for this period to be longer than 5 minutes because of the high amounts of target DNA.

8.5.4. Rinsing

Protocols

1. Place the disc into a dish containing 400 ml 5x SSC and agitate on an orbital shaker for 1 minute. However, discs of overnight growth colonies which have not been chloramphenicol amplified, require more vigorous agitation/rinsing to remove unwanted colony material. It is important to remove cellular debris at this stage to avoid non-specific binding of HRP-labeled probes during hybridization. This may be done by holding the disc with forceps and agitating vigorously by hand in the buffer. Discs should be rinsed twice in this manner.
2. Place the disc, DNA side up, on 3 MM paper to dry.
3. The disc may now be used in a hybridization or stored under vacuum at room temperature.

Notes

1. This volume is sufficient for rinsing up to 10 discs, for amplified colonies use fresh 5x SSC every 10 discs. Due to the large amount of bacterial debris that is removed from the discs of overnight growth colonies, 400 ml of 5x SSC is sufficient for only two discs.

8.5.5. Hybridization and detection

A range of conditions have been used;

Plate concentration:	2–10 ng/ml
Length of hybridization:	1–4 hours
Film exposure times:	1 minute–1 hour

The following screening applications have been used:

Colonies: overnight growth (plasmids)
chloramphenicol amplification
(plasmids)
overnight growth (cosmids)

Plaques: M13 and λ

When performing a new screening experiment, it is suggested that a 4 hour hybridization with 10 ng/ml of probe should be used as a starting point. Reducing the hybridization time or probe concentration will reduce sensitivity, but this can be tolerated in most high copy number plasmid or M13 systems, because of the high amount of target DNA. However, there is less target DNA in cosmid or λ systems, so any reduction in hybridization time or of probe concentration may result in the loss of signal strength from the positive hybrids. Any reduction in signal strength can be offset with longer film exposures. For probe labeling, hybridization and detection follow the protocols in section 8.2., 8.2.1.–8.2.3. inclusive.

9. Additional Information

9.1. Quality control

Each batch of the system is checked by our quality control group to ensure that it will detect 0.5 pg of target DNA in a genomic Southern blot.

The performance of the system may be verified using the control DNA provided.

9.2. Reprobing blots

Blots that have been used to generate a signal on film can be reprobbed several times, using different (or the same) DNA probes, labeled in the same way as the original probe. Because the system utilizes an enzyme label, and this becomes inactivated following the chemiluminescent reaction, it is not necessary to 'strip' the blots of old probe before starting second, and subsequent hybridizations. The blot should be kept in the detection reagent for an extended period (for example, overnight) to allow the luminescence to decrease to negligible levels before reprobbed. The reprobbed procedure begins at section 8.2.1. of this protocol. Membranes should be kept moist between reprobbeds, for example wrapped in SaranWrap, and stored at room temperature.

The limit to the number of reprobbeds is likely to be governed by physical damage to the blots. It is therefore recommended that blots are always handled carefully.

The membrane must be blocked by using hybridization buffer containing 5% (w/v) blocking agent during each reprobbed.

9.3. Storage of labeled probes

In our own laboratories a number of DNA probes have been labeled then stored in 50% glycerol at -15°C to -30°C for up to 6 months,

and then successfully used in hybridization on Southern blots. Whilst labeled probes appear to be reasonably stable, the working life of different probe preparations generated by the user may vary.

9.4. Excising inserts from vectors

After insert purification it is important to determine the DNA concentration to ensure efficient labeling (section 8.2.1.). This can be determined spectrophotometrically. Alternatively an aliquot can be electrophoresed in an agarose gel alongside a sample of DNA of known concentration; ethidium bromide staining with UV illumination will allow the DNA concentration to be estimated.

a) Using DEAE-cellulose paper

Vectors containing inserts should be digested with appropriate restriction enzymes and the DNA fragments separated in a 1% agarose gel containing ethidium bromide. Examine the gel over UV light and make horizontal slits above and below the band(s) to be recovered. Insert prepared pieces of DEAE-cellulose paper (detailed overleaf) into each slit to the full depth of the gel and squeeze the gel firmly against the paper to close the incisions. The paper above the band protects against subsequent contamination by DNA fragments of higher molecular weight. Resume electrophoresis until the DNA has entered the DEAE-cellulose paper strips, as verified by examination over UV illumination. Remove the paper, cut off the perimeter which does not contain DNA and wash in cold distilled water for 5–10 minutes. Drain and blot dry with tissues. Place in a 1.5 ml polypropylene microcentrifuge tube, and add 300–700 μ l of extraction buffer (see below). Shred the paper by mixing the tube and contents on a vortex mixer.

Incubate the tube at 37°C for 2 hours, with occasional agitation. If required the tube may be stored at 2–8°C for up to 24 hours prior to this incubation. Transfer the mixture to a capless 0.5 ml polypropylene microcentrifuge tube which has a hole in the bottom,

made by piercing with a fine needle. Place the tube in a 1.5 ml tube and centrifuge for 5 minutes. Centrifuge the filtrate for a further 3 minutes to pellet any remaining fibres. Extract the supernatant, containing the DNA, with 3 volumes of butan-1-ol (saturated with water) to remove ethidium bromide. Precipitate the DNA with 2 volumes of ethanol (from 1 hour to overnight at -15°C to -30°C, centrifuge down the pellet, rinse in 70% ethanol (-15°C to -30°C) and redissolve in distilled water. Up to 80% recovery can be achieved when extracting small fragments of 2 kb and less.

DEAE-cellulose paper preparation

Cut strips of DEAE-cellulose paper (for example Whatman-DE81) to a length equal to the gel thickness and slightly greater than the slot width. Soak for several hours in 2.5 M NaCl. Wash the strips several times in distilled water and store in 1 mM EDTA (pH8.0) at 2–8°C.

Extraction buffer (pH8.0)

1.5 M NaCl

1 mM EDTA

20 mM Tris HCl

b) Using electroelution

As in the above procedure, vectors containing inserts should be digested with appropriate enzymes to release the inserts. The entire digest can be loaded into a preparative well in a 1% agarose gel containing ethidium bromide. The gel should be run until the insert is clearly separated from the vector, as observed using UV illumination. The insert band should be cut out with a scalpel and transferred to a dialysis bag filled with TAE buffer. As much buffer as possible should then be expelled from the dialysis bag, it should be sealed, and then immersed in an electrophoresis tank, parallel to the electrodes, and current should be applied (100 volts, 2 hours).

During this time DNA is electroeluted out of the gel and on to the inner wall of the dialysis bag. It is released from the wall of the bag

by reversing the polarity of the current for 2 minutes. The eluted DNA can then be aspirated from the dialysis bag using a Pasteur pipette, and precipitated by adding 2 volumes of ice cold ethanol and leaving to stand at -15°C to -30°C (1 hour to overnight). After centrifugation the pellet should be rinsed with 70% ethanol (-15°C to -30°C) and redissolved in distilled water.

9.5. Labeling probes in low melting point agarose

Whilst highly purified probes are recommended for use with ECL direct system, it is possible to label probes that have been separated in low melting point agarose, without extracting the nucleic acid from the agarose. Using gels up to 0.7% agarose enables insert bands to be excised and the nucleic acid labeled directly by following the recommended protocol (section 8.2.), but omitting the rapid cooling, section 8.2.1., step 3.

The boiling step both melts the agarose and denatures the DNA. The solution can be left briefly to cool at room temperature, and cold DNA labeling reagent is then added to it. The agarose is now sufficiently dilute not to re-solidify.

The glutaraldehyde cross-linking and 10 minute incubation at 37°C should be carried out as normal. The labeled probe can then be used in hybridizations.

10. Troubleshooting

Problem: No bands visible

Possible cause	Remedy
1. Possible failure in transfer of target DNA from gel to membrane	1.1. Stain gel with ethidium bromide to check for any remaining DNA
	1.2. Check that protocol (section B) has been followed closely
	1.3. Include control λ DNA; for 100 pg loading the fourth band down represents 4.4 kb (9 pg) and the doublet 2.3 and 2.0 kb (4.8 and 4.2 pg respectively).
2. Hybridization and detection not successful	2.1. Check hybridization conditions by including suitable controls, for example control λ DNA provided.
	2.2. Check that probe labeling was successful and that the labeling reagents were used as directed, and in the correct sequence. Check also that the DNA was essentially salt free. Labeling of DNA can be detected by its altered electrophoretic mobility on an agarose gel. Ensure that hybridization temperature did not exceed 42°C.
	2.3. Check that the detection reagents are working; pre-mix small quantities of detection reagent 1 and detection reagent 2 (1 ml of each) and in the dark add 1 μ l of DNA labeling reagent. Visible blue light should be produced.

Problem: Only faint bands visible

Possible cause	Remedy
1. Low level of signal	<ol style="list-style-type: none">1. Pre-flashing the film will increase its sensitivity to the signal and linearize its response. This does however require care as increased backgrounds may result. Pre-flashing involves hypersensitizing the film just before use by pre-exposure to a short flash of light (approximately 1 msec). Conventional electronic photographic flash units are suitable, when attenuated with a diffuser and Wratten 6B filter, to give a flash of the required intensity to increase the 540 nm absorbance of the developed film to 0.15 above that of the unexposed film. Alternatively, an optimized preflash system can be used.2. Check the hybridization temperature did not exceed 42°C.3. Expose the film for an extended period (several hours).4. Poor DNA transfer on to membranes; use control λ DNA as above.

Problem: High background

Possible cause	Remedy
1. Non specific light generation	<ol style="list-style-type: none">1.1. Check that an error has not been made with the quantities of DNA and labeling reagent used in labeling.1.2. The presence of labeled vector sequences in the hybridization may increase 'noise'. Use purified insert as probe whenever possible.1.3. Ensure that the blot is evenly covered with hybridization buffer during the hybridization, and that there are no trapped air bubbles.1.4. Ensure that the labeled probe is not added directly to the membrane. Instead remove an aliquot of buffer from the prehybridization and mix the probe with it, before returning it to the bulk of the buffer.1.5. Ensure that all washing steps have been carried out for the specified times at the specified temperatures.1.6. Expose the film for a minimum period (an initial 1 minute period may be all that is required)1.7. Use high quality membranes: Amersham Protran Premium 0.2 μm Nitrocellulose is the recommended nitrocellulose. Hybond-N+ is the recommended nylon membrane.

Problem: High background *continued.*

Possible cause	Remedy
1. Non specific light generation <i>continued.</i>	1.8. Damage to the membrane can cause non-specific binding of the probe. Handle blots carefully with gloved hands and blunt non-serrated forceps. 1.9. Use clean forceps to handle blot after washing. Contamination with hybridization buffer or primary wash buffer prior to detection will elevate background.
2. Poor blocking of membrane	2.1. Ensure that the blocking reagent is fully dissolved at 5% (w/v). Vigorous stirring may be necessary. 2.2. When reprobing blots, the membrane must be reblocked each time by using hybridization buffer containing blocking agent, from section 6 onwards.

Problem: Blank patches

Possible causes	Remedy
1. Contamination from powder in gloves	1. Wear powder-free gloves.

11. References

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12. Related products

Hyperfilm ECL

Pack of 25 films, 18 cm × 24 cm	RPN2103
Pack of 25 films, 30 cm × 40 cm	RPN2104
Pack of 25 films, 10 inches × 12 inches	RPN1681
Pack of 25 films, 5 inches × 7 inches	RPN1674

Hybond-N+ and Amersham Protran Premium 0.2 μ m Nitrocellulose

Part of an extensive range of membranes available from GE

ECL random prime labeling and detection systems

Based on the incorporation of a hapten (fluorescein) with detection by ECL:

15 labeling reactions; ECL detection reagents for 1000 cm ² membrane	RPN3029
30 labeling reactions; ECL detection reagents for 2000 cm ² membrane	RPN3030
60 labeling reactions; ECL detection reagents for 4000 cm ² membrane	RPN3031

ECL 3'-oligolabeling and detection system

For tailing oligos with fluorescein-dUTP.

Sufficient for 500 pmols oligonucleotides

ECL detection reagents for 4000 cm² membrane RPN2131

ECL 5'-thiol oligolabeling and detection system

For the direct labeling of thiol modified oligonucleotides

with horseradish peroxidase with detection by ECL

Sufficient for 5 × 5 μ g oligonucleotide ECL detection reagents for 4000 cm² membrane RPN2113

C₆-thiol modifier RPN2112

ECL probe-amp reagents

For non-radioactive DNA probe labeling during PCR*

6 reactions	RPN3020
12 reactions	RPN3021

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