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Amersham Gene Images AlkPhos Direct Labelling and Detection System

Product Booklet

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RPN3680PL AC

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1 Introduction

Product codes

RPN3680

RPN3681

RPN3690

RPN3691

RPN3692

About

Direct labelling of DNA probes with alkaline phosphatase for use in conjunction with Chemiluminescent detection with CDP-Star™ Chemifluorescent detection with ECF.

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

All chemicals should be considered as potentially hazardous. For use and handling of the products in a safe way, refer to the Safety Data Sheets.

Note: *The protocols describe the use of Ethidium Bromide, Sodium Hydroxide and EDTA.*

WARNING
Ethidium Bromide is harmful. Sodium Hydroxide is corrosive. EDTA is harmful. Please follow the manufacturers' safety data sheets relating to the safe handling and use of these reagents.

Storage

Store hybridisation buffer and blocking reagent at 15–25°C. Store other components at 2–8°C.

Expiry

Stable for at least 3 months stored as recommended.

2 Components of the system

Contents for RPN3680 - labelling module containing reagents for 25 reactions:

Labelling reagent (containing 0.1%(w/v) Sodium Azide*)	60 µL
Cross-linker solution (containing 4.7%(v/v) Formaldehyde*)	0.5 mL
Reaction buffer	0.5 mL
Water	3 x 1 mL
Control unlabelled DNA (10 µg/ml Hind III digested lambda DNA)	0.1 mL
Hybridization buffer (containing 12% (w/v) urea*)	500 mL
Blocking reagent	2 x 25 g

These reagents can also be ordered as a double pack RPN3681

Note: *The AlkPhos Direct Labelling and Detection system has been developed for use with Hybond™-N+ positively charged nylon membrane from Cytiva. For optimal results its use is strongly recommended.*

3 Critical parameters

- Read the protocol thoroughly before starting
- The concentration of salt in the nucleic acid to be labelled should be as low as possible and must not exceed 50 mM
- The DNA concentration should be accurately determined and adjusted to 10 ng/µL before labelling
- Prepare the hybridization buffer and the stringency wash buffers in advance
- Ensure that the hybridization buffer and stringency wash buffers are warmed to the required temperature prior to use
- Maintaining the DNA in a single stranded form is required to ensure a good labelling efficiency
- Enzyme labelled probes **must not** be denatured prior to addition to the hybridization buffer
- Damage to the membrane can cause non-specific binding of the probe. Handle the blots carefully, with gloved hands and blunt nonserrated forceps
- Wear powder-free gloves or else rinse gloved-hands with water to remove powder before performing the detection procedure

- Bacteria contain alkaline phosphatases which will be detected by this system and will show up as spots on the blots and give rise to spotty backgrounds. Good laboratory practice should keep contamination to a minimum. We recommend that wash buffers should be stored at 2–8°C and that the volume of primary wash buffer required for post hybridization stringency washes should be removed from the bulk before warming.

4 Additional solutions and reagents required

Hybridization buffer

Add NaCl (analytical grade) to the hybridization buffer solution to give a concentration of 0.5 M. Add blocking reagent to a final concentration of 4%(w/v). For best results add the blocking reagent slowly to the buffer solution while stirring. Continue mixing at room temperature for 1–2 hours on a magnetic stirrer or roller mixer. This buffer can be used immediately or stored in suitable aliquots at -15°C to -30°C.

Primary wash buffer (1 litre)

Urea 120 g	2 M
SDS 1 g	0.1% (w/v)
0.5 M Na phosphate pH 7.0 100 mL[1]	50 mM
NaCl 8.7 g	150 mM
1.0M $MgCl_2$ 1 ml	1 mM

Blocking reagent 2 g	0.2% (w/v)

[1] 0.5 M Na Phosphate can be made by using Sodium Dihydrogen Phosphate (monobasic, $NaH_2PO_4.xH_20$) and adjusting the pH to 7.0 with Sodium Hydroxide.

The primary wash buffer can be kept for up to 1 week in a refrigerator at 2–8°C. Avoid reheating.

Secondary wash buffer – 20x stock

Tris base 121 g	1M
NaCl 112 g	2M

Adjust pH to 10.0. Make up to 1 litre with water. This can be kept for up to 4 months in a refrigerator at 2–8°C.

Secondary wash buffer – working dilution

Dilute stock 1:20 and add 2 mL/L of 1 M $MgCl_2$ to give a final concentration of 2 mM Magnesium in the buffer. This buffer should not be stored.

5 Description

The Gene Images AlkPhos Direct labelling and detection system from Cytiva is based on a dioxetane chemiluminescence system. It involves directly labelling probe DNA or RNA with a specially developed thermostable alkaline phosphatase enzyme[1]. This is achieved by completely denaturing the probe so that it is in single-stranded form. The addition of the cross-linker covalently couples the enzyme to the nucleic acid probe.

[1] See licence and patent information in legal section.

Once labelled, the probe is used in hybridization with target DNA or RNA immobilized on a membrane. A specially optimized hybridization buffer containing a novel rate enhancer is included in the system. This ensures efficient hybridization, protects the enzyme against inactivation during this step and generates additional sensitivity. To control the stringency of hybridization the temperature may be altered; recommendations for a suitable initial stringency are given in the protocol.

After hybridization, the blots are washed to remove excess probe. To control the stringency of washing, the temperature of the primary wash buffer may be altered. The washed blots can then be taken directly into the detection step.

There is a choice of methods for the final detection:

- CDP-*Star* chemiluminescent detection reagents utilize the probebound alkaline phosphatase to catalyze the decomposition of a stabilized dioxetane substrate. They have a rapid light output with a short lag phase enabling faster results than some other dioxetane systems. The light reaches its maximum at 4 hours and will last for several days allowing for multiple/long exposures.
- ECF chemifluorescent substrate can be used in conjunction with fluorescence scanning instrumentation e.g. Molecular Dynamics, FluorImager, Storm or Typhoon™. The excitation and emission maxima of the reagent are 430 nm and 560 nm respectively, the large shift allowing high levels of sensitivity to be achieved whilst maintaining a low background level.

- Color detection provides a robust signal detection in systems where high sensitivity is not required. High target blots probed with the AlkPhos Direct system can be detected with NBT/BCIP color detection reagent according to the protocol recommended.

The Gene Images AlkPhos Direct system has been successfully used in Southern, Northern and slot/dot blotting applications.

6 Gene Images AlkPhos Direct Labelling and detection system protocols

Storage and stability

The hybridization buffer and blocking reagent should be stored at ambient temperature (15–25°C). All other components should be stored in a refrigerator (2–8°C). Once the blocking reagent has been added, the hybridization buffer should be used immediately or stored in suitable aliquots at -15°C to -30°C for up to three months.

The kit components are stable for at least 3 months when stored under the recommended conditions. In Cytiva laboratories a number of DNA probes have been labelled and stored in 50% (v/v) Glycerol at -15°C to -30°C for up to 6 months, and then successfully used in hybridization on Southern blots. Whilst labelled probes appear to be relatively stable, the working life of different probe preparations generated by the user may vary.

Preparation of labelled probe

Step	Action
1	Dilute 20 µL cross-linker solution with 80 µL of the water supplied to give the working concentration. ***Note:*** *The working solution can be kept in a refrigerator at 2–8°C for one week.*
2	Dilute DNA (or RNA) to be labelled to a concentration of 10ng/µL using the water supplied. ***Note:*** *The concentration of salt in the sample of nucleic acid should be kept as low as possible and must not exceed 50 mM.*
3	Place 10 µL of the diluted DNA sample in a microcentrifuge tube and denature by heating for 5 minutes in a vigorously boiling water bath. ***Note:*** *The labelling reaction can be scaled up by increasing the volume of all the components of the labelling reaction pro rata; DNA, reaction buffer, labelling reagent and cross-linker working solution.*
4	Immediately cool the DNA on ice for 5 minutes. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
5	Add 10 µL of reaction buffer to the cooled DNA. Mix thoroughly but gently.

Step	Action
	Note: *The reaction should be kept on ice.*
6	Add 2 µL labelling reagent. Mix thoroughly but gently.
7	Add 10 µL of the crosslinker working solution. Mix thoroughly. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
8	Incubate the reaction for 30 minutes at 37°C. ***Note:*** *For color detection this temperature can be increased (see Color detection, on page 23)*
9	The probe can be used immediately or kept on ice for up to 2 hours. For long term storage, labelled probes may be stored in 50% (v/v) Glycerol. ***Note:*** *Further treatment of the probe before use is not required even after long term storage.*

Hybridization

Step	Action
1	Pre-heat the required volume of prepared AlkPhos Direct hybridization buffer to 55°C. The volume of buffer should be equivalent to 0.25 mL/cm^2 of membrane; this may be reduced to 0.125 mL/cm^2 for large blots hybridised in plastic bags or for hybridization in bottles.

Step	Action
	Note: *The AlkPhos Direct hybridization buffer supplied must be prepared for hybridization before use by addition of NaCl and blocking reagent (see* *Chapter 4 Additional solutions and reagents required, on page 6**). It is possible to alter the volume of buffer depending on the size of the container and the number of blots to be hybridised. It is essential that the blots should move freely within the buffer.*
2	Place the blots into the hybridization buffer and prehybridise for at least 15 minutes at 55°C in a shaking water bath (approximately 60 strokes per minute) or hybridization oven.
3	Add the labelled probe to the buffer used for the prehybridization step. Typically use 5–10 ng probe per mL of buffer. ***Note:*** *Avoid placing the probe directly on to the blot. Alternatively, a small aliquot of the buffer may be withdrawn and mixed with the probe before returning the mixture to the bulk of the hybridization buffer. Do NOT denature the probe before use.*
4	Hybridise at 55°C overnight in a shaking water bath or hybridization oven.

Step	Action
	Note: *Stringency can be adjusted by altering the hybridization temperature between 50°C and 75°C – see additional information on hybridization,* *Additional notes, on page 18**.*

Post hybridization stringency washes

Step	Action
1	Preheat the primary wash buffer (see *Chapter 4 Additional solutions and reagents required, on page 6*) to 55°C. This is used in excess at a volume of 2–5 mL/cm^2 of membrane. ***Note:*** *Additional control of stringency can be achieved if desired by altering the temperature of the primary wash.*
2	Carefully transfer the blots to this solution and wash for 10 minutes at 55°C, with gentle agitation. ***Note:*** *Several blots can be washed in the same solution provided that they can move freely. It is important that the required wash temperature is accurately determined. Fluctuations in the temperature will cause changes in the stringency.*
3	Perform a further wash in fresh, primary wash buffer at 55°C for 10 minutes.

Step	Action
4	Place the blots in a clean container and add an excess of secondary wash buffer (see *Chapter 4 Additional solutions and reagents required, on page 6*). Wash, with gentle agitation, for 5 minutes at room temperature.
5	Perform a further wash in fresh, secondary wash buffer at room temperature for 5 minutes. ***Note:*** *Blots may be left in secondary wash buffer for up to 30 minutes at room temperature before detection.*

Signal generation and detection

Chemiluminescent signal generation and detection with CDP-Star

Please read through this whole section before proceeding. Wear powder-free gloves or rinse gloved hands with water before use to remove powder.

Step	Action
1	Drain the excess secondary 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 (sample side up) on a clean, non-absorbent, flat surface. Do not allow the blots to dry out. ***Note:*** *SaranWrap can be used to place the blots upon.*

Step	Action
2	Pipette detection reagent on to the blots (30–40 µL/cm^2) and leave for 2–5 minutes. Drain off excess detection reagent by touching the corner of the blot on to the non-absorbent surface. ***Note:*** *To avoid contamination of the detection reagent, we recommend that a suitable aliquot is aseptically removed from the bulk solution to a separate container before use.*
3	Wrap the blots in SaranWrap. Place the blots DNA side up, in the film cassette. ***Note:*** *Any air pockets created in wrapping the blots should be gently smoothed out. Ensure there is no free detection reagent in the film cassette the film must not get wet.*
4	Switch off the lights and place a sheet of autoradiography film for example Hyperfilm™ ECL™ on top of the blots. Close the cassette and expose for 1 hour at room temperature. The DNA side of the filter (wrapped in SaranWrap) must be placed next to the film for maximum sensitivity. ***Note:*** *This should be carried out in a dark room, using red safe lights. Do not move the film while it is being exposed.*

Step	Action
5	Remove the film and develop. If required, expose a second film for an appropriate length of time. For an initial experiment try a 1 hour exposure. The signal lasts for several days reaching a peak a few hours after addition of the detection reagents. Subsequent exposures can be made with suitably adjusted exposure times to get optimum signal-to-noise ratio. ***Note:*** *Prolonged exposure will increase background and eventually lead to a totally black image.*

Chemifluorescent signal generation and detection with ECF substrate

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.

Step	Action
1	Pour the entire contents of the bottle containing the detection buffer into the bottle which contains the ECF detection reagent. Screw the top on firmly and shake the bottle gently (for example, on a roller-mixer) for about 10 minutes to fully dissolve the ECF substrate. ***Note:*** *Store the dissolved ECF substrate in aliquots, at -15°C to -30°C.*

Step	Action
2	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 (sample side up) on a clean non-absorbent surface. ***Note:*** *SaranWrap can be used to place the blot upon.*
3	Pipette ECF substrate on to the blots (~25 µL/cm^2) and incubate for 1–5 minutes. Do not move the blot during the incubation. Transfer the blots directly on to a fresh sheet of SaranWrap. Fold the plastic over the top of the blots. ***Note:*** *Incubation can be extended for up to 20 minutes to increase the signal obtained, but too long an incubation will result in signal diffusion.*
4	Seal the blot in the SaranWrap by folding back any open edges. This will stop the sample drying out and incubate at room temperature in the dark, for example in a drawer or a film cassette, for the required length of time. The optimal time for your particular system can be found by rescanning at various times. ***Note:*** *For high target levels an acceptable result may be obtained after 1 hour. Scanning up to 24 hours after addition of substrate will provide a much stronger signal suited to lower target applications.*

Step	Action
5	Place the bag containing the blot(s) (sample side down) on to the flat bed, fluorescent scanning instrument. ***Note:*** *Water placed between the lower surface of the bag and the glass will greatly improve the image obtained.*
6	Scan the 'blot' using an appropriate emission filter as available and according to the guidelines for use of the scanning instrument. ***Note:*** *ECF has a broad excitation spectrum (maximum excitation at 430 nm) and an emission maximum at 560 nm.*

7 Additional information

Additional notes

Probe labelling

- The amount of probe DNA required for a hybridization will depend on the size of blot to be probed and the amount of target DNApresent. In practice, blot size will determine the hybridization volume to be used (see *Hybridization, on page 11*), while the amounts of target and probe sequence will determine the probe concentration. 5–10 ng probe/mL is appropriate for many applications, although this may be reduced where there is a large amount of available target DNA. For non-chemiluminescent

methods, the probe concentration can be increased (eg 10–20 ng/mL for ECF). For color detection, probe concentration can be raised as high as 50 ng/mL to maximise sensitivity or speed without incurring unacceptably high backgrounds.

- Use purified insert as probe wherever possible. 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 at the end of this section.
- 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.
- A heating block may not ensure complete denaturation of the DNA to be labelled, the use of a waterbath is recommended. Denaturation of RNA is also advised to remove secondary structure.
- The protocol is written for DNA probes, but an identical procedure can be followed to label RNA probes.

Hybridization and stringency washes

- AlkPhos Direct hybridization buffer has been specially formulated for this system. It is not the same as ECL gold buffer and the two are not interchangeable. It is very important for the correct buffer to be used in the system.

- Poor blocking of membrane may result if the blocking reagent is not fully dissolved. The optimum Sodium Chloride concentration added to the hybridization buffer may vary between 0.25 M and 1.0 M depending on the probe used. 0.5 M NaCl generally gives acceptable results and is recommended as a starting point. 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.
- 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 hybridising several blots together.
- A hybridization temperature of 55°C is recommended for first time users. Stringency can be altered by changing the hybridization (or the wash) temperature. Higher temperatures, up to 75°C, may be used to increase the stringency of hybridization. Temperatures below 50°C are only likely to be appropriate for very short probes. It is very important to ensure that the hybridization temperature is accurately controlled; slight temperature changes between experiments will lead to variations in stringency. Temperatures above 85°C must be avoided.
- Stringency can also be controlled by adjusting the temperature of the primary washes. The normal primary wash temperature is 55°C, but this can be adjusted between 50°C and 75°C.
- Although an overnight hybridization is recommended in most cases, it is possible to use shorter hybridization times (2–4 hours) for high target applications.

- Hybridized blots may be stored overnight, wetted with secondary wash buffer and wrapped in SaranWrap, in a refrigerator at 2–8°C. Do not allow the blot(s) to dry out.
- Hybridization and washes in tubes: Nucleic acid hybridization and stringency washing may be conveniently performed in a hybridization oven with an integral rotisserie device. These devices 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.

Place the blot(s) in a tube. If the blots are dry, they should then be pre-wetted as follows: Add a small amount of hybridization buffer 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. It is important to exclude any bubbles trapped between the hybridization tube and the membrane as this can lead to black patches on detection.

Note: *If there is significant overlap of the blot, nylon mesh may be used as follows: sandwich the blot between two layers of nylon mesh, roll the sandwich with the nucleic acid facing inwards and place the roll in the tube so that it unrolls in the opposite direction from the movement of the rotisserie. Nylon mesh may be reused following washing in a 1%(w/v) SDS solution and thorough rinsing in distilled water.*

To ensure accurate final volume of hybridization buffer and hence probe concentration, pour off the excess hybridization buffer and add the appropriate volume of fresh hybridization buffer. A volume of 0.063–0.125 mL/cm^2 is recommended. In practice, the tube size will determine the minimum volume to be used; for example the minimum volume for use with a tube 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 may result if there is insufficient hybridization buffer.

Quality control and use of control reagents

Each batch of the system is checked by our quality control group to ensure that it will detect 60 fg of a single copy gene in a 120 ng loading of human genomic DNA on a Southern blot when using CDP-Star chemiluminescent detection reagent.

When using ECF chemifluorescent substrate, typical results detect 250 fg of a single copy gene in a 500 ng loading of human genomic DNA on a Southern blot.

The functioning of the labelling system may be verified using the control lambda DNA provided as a probe template at 10 ng/µL.

Probe storage

After labelling, the probe can be used immediately or kept on ice for up to 2 hours. For long term storage, an equal volume of glycerol should be added (i.e. 32 µL for the standard labelling reaction), it should then be mixed, dispensed in aliquots and stored at -15°C to -30°C. The size of individual aliquots should be such that 'freezethaw' cycles are kept to a minimum. The

efficiency of a probe may decrease if subjected to more than 5 'freeze-thaw' cycles. A probe that has been stored in glycerol and stored at -15°C to -30°C can be added directly to the hybridization buffer to the appropriate concentration. Further treatment is not required.

Reprobing blots

Blots which have been used to generate a signal on film can be reprobed several times. Due to the length of the light output in this system, it is first necessary to 'strip' the blots of old probe before starting second, and subsequent hybridizations. Blots can be stripped using the following method. For blots detected with ECF substrate, start at step 1. For blots detected with CDP-Star, step 1 can be omitted.

Incubate blots in absolute alcohol (≥99% Ethanol) at room temperatures with agitation (1 mL/cm^2, 2 X 10 minutes).

Incubate blots in 0.5% (w/v) SDS solution at 60°C for 60 minutes. Rinse blot in 100 mM Tris pH 8.0 for 5 minutes at room temperature. Membranes should be kept moist between reprobings, for example wrapped in SaranWrap, and stored in a refrigerator at 2–8°C. The limit to the number of reprobings is likely to be governed by physical damage to the blots. It is therefore recommended that blots are always handled carefully.

Color detection

For high target applications where high sensitivity is not required, NBT/BCIP color detection can be used. Probe concentrations of 10–50 ng/mL are recommended.

1. Prepare NBT/BCIP substrate as recommended by the supplier.

2. Drain off any excess wash buffer from the blot (by touching the corner of the blot against the box used for washing) and place it (sample side up) on to a non absorbent surface such as SaranWrap.
3. Pipette the detection reagent mix on to the blots (0.125 mL/cm^2 of membrane) and incubate in the dark without agitation. A 24-hour incubation will probably be required for optimum sensitivity, see note below.
4. Once the required color development has been achieved, rinse the blots for 2 x 2 minutes in distilled water and leave on an absorbent surface to dry.
5. It may be desirable to photograph the results as the color will fade over time.

A shorter incubation time can be achieved with this type of substrate if the probe labelling temperature used is increased from 37°C to 50°C for the 30 minute incubation step. It should be noted that probes labelled at 50°C may give higher backgrounds if used with the other detection systems. Increasing the probe concentration during hybridization may also improve the sensitivity of this system.

Excising inserts from vectors

After insert purification it is important to determine the DNA concentration to ensure efficient labelling (see *Chapter 3 Critical parameters, on page 5*). 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.

Using DEAE-cellulose paper

Vectors containing inserts should be digested with appropriate restriction enzymes and the DNA fragments separated in a 1% (w/v) 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 µL 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 this 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%(v/v) 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 height 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 (pH 8.0) at 2–8°C.

Extraction buffer (pH 8.0)

1.5 M NaCl

l mM EDTA

20 mM Tris-HCl

Troubleshooting guide

Problem	Possible cause
High background	• Concentration of cross-linker too high - check whether stock solution used in error. • Labelling reaction left for >30 minutes • Probe concentration too high. • Temperature of waterbath/oven too low. • Hybridization time too long. • Bacterial contamination of CDP-Star detection reagent. • Too much detection reagent left on blot. • Too long an exposure to film.
Spotty background	• Bacterial contamination in wash buffers. • Wash/hybridization containers not clean.
Patchy background	• Insufficient wash buffer used, blots sticking together. • Insufficient hybridization buffer. • Membrane damage. • Blot allowed to dry out. • If hybridised in a tube, airbubbles may have been trapped.

Problem	Possible cause
Low signal	• Initial unlabelled DNA too dilute (<10 ng/µL). • DNA salt concentrations too high. • Cross-linker working solution too old. • <2 µL labelling reagent used. • Reaction time <20 minutes. • Probe concentration too low. • Labelled probe denatured before use - denaturing the enzyme. • Waterbath/oven temperature too high. • Hybridization time too short. • Incorrect hybridization buffer used. • Exposure time too short. • Too high a stringency used.
Low stringency	• Waterbath/oven temperature too low. • Salt concentrations in the hybridization buffer incorrect. • SDS concentrations in the wash buffer incorrect.

8 Related products

AlkPhos Direct Hybridization Buffer	RPN3688
Gene Images	
Gene Images Random Prime	RPN3540
Gene Images CDP-Star Detection	RPN3510
ECL	
ECL 3'-Oligolabelling	RPN2130
ECL Random Prime	RPN3040/1
ECL Detection Reagents	RPN2105
	RPN3004
ECL Direct Labelling and Detection System	RPN3000/1
Other	
Chemiluminescence Detection Film	See catalogue for details

Hyperfilm ECL

Hybond M N_+

A range of hybridization and detection accessories including UV cross linker, hybridization oven, film cassettes, dark room torch and film processor are all available (see Cytiva catalogue for details).

For further details on these products and for information on products for in situ hybridization, immunocytochemistry, CyDye™ fluorescence products, ECL detection and Western blotting see the current Cytiva catalogue or contact your local Cytiva office.

9 Product protocol card

Probe labelling

- Denature DNA (10 ng/µL, 10 µL) for 5 minutes (boiling waterbath).
- Cool on ice (5 minutes). Centrifuge briefly.
- Add reaction buffer (10 µL) and mix.
- Add labelling reagent (2 µL) and mix.
- Add cross-linker at working dilution (10 µL) mix and centrifuge briefly.
- Incubate at 37°C for 30 minutes.
- Place labelled probes on ice.

Hybridisation

- Prepare the AlkPhos Direct hybridization buffer (add NaCl/ block).
- Prehybridize blots (15 minutes at 55°C), 0.125–0.25 mL of buffer/cm^2 of membrane.
- Add probe (5–10 ng/ml).
- Hybridize overnight at 55°C with gentle agitation.

Note: *Stringency can be changed by altering the hybridization. temperature between 50°C and 75°C.*

Stringency washes

- Pre-warm primary wash buffer to 55°C.
- Place blots in 1–2 mL/cm^2 primary wash buffer.
- Wash for 2 x 10 minutes with agitation at 55°C.
- Prepare secondary wash buffer.

- Wash blots in 1–2 mL/cm^2 at room temperature for 2 x 5 minutes with agitation.

Detection CDP-Star detection reagent

- Drain blots. Apply CDP-Star detection reagent (use 30–40 µL/cm^2).
- Leave for 2–5 minutes at room temperature.
- Wrap the blots in SaranWrap.
- Expose to film.

Detection ECF substrate

- Prepare the ECF substrate.
- Drain blots. Apply ECF substrate (use ~25 µL/cm^2).
- Leave for 1 minute at room temperature.
- Seal the blots in a detection bag.
- Scan the blot on fluorescent scanning instrument.

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For local office contact information, visit cytiva.com/contact

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