Gene Images CDP-*Star* Detection Module

For detection of fluorescein-labelled probes in Northern, Southern and dot blots

RPN3510
RPN3511

STORAGE:

Store at 2–8 °C

STABILITY:

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

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Amersham Biosciences

SYSTEM COMPONENTS

Gene Images™ CDP-Star™ detection module (RPN3510) consists of:

Anti-fluorescein alkaline phosphatase conjugate: 150 µl, 5000× stock

Liquid block: 2 × 100 ml, for use in antibody blocking

CDP-Star* detection reagent: 100 ml, ready for use

Detection bags: 8 bags for use during application of the detection reagent and subsequent exposure to film.

RPN3511 consists of 2 × RPN3510.

*See licensing information on back cover.

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.

DESCRIPTION

The Gene Images CDP-Star detection module is an improvement over the original Fluorescein Gene Images dioxetane module. The new substrate (CDP-Star) has a more rapid light output which minimizes the effect of the lag phase previously associated with decomposition of stabilized dioxetanes. Consequently, exposures to film made in the first few hours after addition of the substrate to a membrane will produce a much stronger signal than previously. Following hybridization, this detection module allows detection of hybrids (after a blocking step) by incubation with an anti-fluorescein alkaline phosphatase (AP) conjugate. After washing off the excess conjugate, probe-bound AP is used to catalyze light production by enzymic decomposition of a stabilized dioxetane substrate. The principle of the system is outlined in figure 1.

Each batch of this module is tested by our quality control group using the Gene Images random prime labelling module (RPN3540) to ensure that it will detect the following:
A single copy gene in human DNA genomic Southern blots, representing 0.06 pg (60 fg) target in 0.125 μg of DNA.

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

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

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

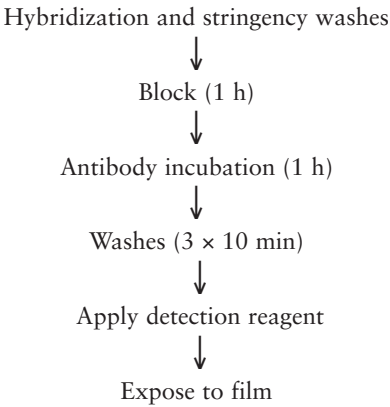

Figure 1. Outline of stages and approximate timescales in the Gene Images CDP-Star detection system

CRITICAL PARAMETERS

- Buffer A (see 'solutions required' section) should be autoclaved to avoid spots (produced from bacterial contamination) on the final image on film.
- Once the hybridization has been started, the entire process should ideally be completed without interruption. However, if a break in the process is unavoidable, then the user should progress to the end of the stringency washes before stopping. Once the blocking stage has begun, other interruptions should be avoided.
- Once the blot has been wetted (this is normally at the pre-hybridization stage) then it must not be allowed to dry out at any stage until detection is completed.
- Fluorescein is sensitive to prolonged exposure to light. For long term storage, solutions and blots containing fluorescein should be placed in the dark.
- Freezers used for storing components should not be of the 'frost-free' type as these may cause damage due to the repeated freeze/thaw cycles that these freezers undergo.

ADDITIONAL EQUIPMENT AND REAGENTS REQUIRED

Equipment

Adjustable pipettes, for example Amersham Biosciences pipettes
Sterile pipette tips
Standard laboratory glassware
Orbital shaker
Gloves, preferably powder-free
Plastic boxes/bags
Autoradiography film such as Hyperfilm™-MP
Autoradiography cassettes such as Hypercassette™
Film developing facility and reagents

Reagents

Buffer A
100 mM Tris-HCl
300 mM NaCl pH 9.5
Autoclave in 500 ml aliquots in 1 litre bottles for 15 min at 105 kPa (15 psi). Once opened after autoclaving, do not use for more than one day.

Bovine serum albumin (BSA) fraction V
Source:
Amersham Biosciences US70195

Tween™ 20 (Polyoxyethylene sorbitan monolaurate
Source:
Amersham Biosciences US20605

1. Blocking, antibody incubation and washes

The following steps are all performed at room temperature and all the incubations require constant agitation of the blots. All containers should be rinsed with ethanol before use to remove any bacterial alkaline phosphatase contamination.

Protocol	Notes
1.1. Following the stringency washes, incubate blots with gentle agitation for 1 hour at room temperature in approximately 0.75–1.0 ml/cm^2 of a 1 in 10 dilution of liquid blocking agent in buffer A.	1.1. Buffer A is a solution of 100 mMTris-HCl; 300 mM NaCl (pH 9.5). It should be autoclaved or at least made up with fresh reagents and filtered. Once opened, unused solution should be discarded at the end of the working day to avoid contamination with exogenous alkaline phosphatase. Diluted block can be stored frozen (in aliquots) for several months and is easily thawed when required. However, it is recommended that no aliquot is subjected to repeated thawing and

	refreezing. Also, it may be convenient to separate any remaining undiluted liquid block into appropriate aliquots for refreezing.
1.2. Dilute the anti-fluorescein-AP conjugate 5000-fold in freshly-prepared 0.5%(w/v) bovine serum albumin in buffer A. Incubate the blots in diluted conjugate (0.3 ml/cm^2 of membrane) with gentle agitation at room temperature for 1 hour.	1.2. Bovine serum albumin fraction V (for example, Sigma Product no. A-2153) should be used in the buffer. 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 bags or tubes to help minimise volume. For a 20 × 20 cm blot, a volume of 50 ml can be used.
1.3. Remove unbound conjugate by washing for 3 × 10 minutes in 0.3%(v/v) Tween 20 in buffer A at room temperature with agitation. An excess volume is again used (2–5 ml/cm^2).	1.3. If not proceeding immediately to stage 5, the blot(s) should be rinsed in buffer A to remove the Tween 20 and then stored in SaranWrap™ at 2–8 °C. The blots must not be allowed to dry out.

2. Signal generation and detection

Please read through this whole section before proceeding.

Wear powder-free gloves or rinse gloved hands with water before use to remove powder.

Protocol	Notes
2.1. 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 (sample side up) on a sheet of SaranWrap on a flat surface.	2.1. Instead of using SaranWrap, a section can be cut from one of the bags supplied.
2.2. Pipette detection reagent on to the blots (30–40 µl per cm^2) and leave for 2–5 min.	2.2. 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.

2.3. Drain off excess detection reagent by touching the corner of the blot(s) on to the SaranWrap.	
2.4. The blots can either be wrapped in a fresh piece of SaranWrap or for best results, they can be transferred to one of the bags supplied as follows:	2.4. For long exposures it may be useful to heat-seal the bag to prevent drying out of the blot.
2.4.1. Cut a section from the bag which is large enough to cover the blot(s) with a small border of at least 1 cm.	
2.4.2. Cut along the sides of the bag, leaving one side uncut. Open out the bag and after draining the blots, arrange them on one half of the open bag. Fold the bag over the blot(s). Ensure the outside of the bag is dry before exposing to film. Place the bag containing the blots (sample side up) in a film cassette and take to a dark-room.	

Protocol	Notes
2.5. Switch off the lights and place a sheet of Hyperfilm-MP on top of the blots. Close the cassette and expose initially for 1 hour or more for low target/high sensitivity applications. Shorter exposures should be used for higher target applications. Remove film and develop.	2.5. The exposure times quoted are only a guide eg if the image is too dark (it may even appear completely black), then a shorter exposure may still give an excellent result. For very high target applications, an exposure time as short as 30 sec may be sufficient. The light output increases rapidly during the first few hours. After this time, the light output will be more stable, making it easier to judge the optimum exposure time. The light output will begin to decline 2–3 days after addition of the detection reagent. Adjust exposure times to compensate.

3. Reprobing blots

It is necessary to remove the previous probe from the membrane before reprobing. 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. Reprobing is not generally recommended with nitrocellulose membranes because of their greater fragility. Stripping of the probe is generally less successful with Northerns due to the strength of the bonding of the probe to the RNA target.

Protocol

3.1. Prior to reprobing, membranes may be stored, wrapped in SaranWrap, in a refrigerator (2–8 °C).

3.2. When ready to commence reprobing, rinse membrane in 5× SSC for 1–2 minutes.

3.3. Add the membrane to a boiling solution of 0.1%(w/v)SDS using approximately 5 ml of SDS solution per cm^2 membrane. Place on a bench-top shaker for 10 min. Repeat the operation twice more, **using freshly boiling SDS each time.**

3.4. Pre-hybridize, probe and detect as in standard protocol.

ADDITIONAL INFORMATION

Troubleshooting guide

Problem	Possible cause	Remedy
1. Low sensitivity	1.1. Wrong choice of probe	1.1. Check homology of probe to target.
	1.2. Poor transfer of target from gel to membrane	1.2. Stain gel after transfer with ethidium bromide to check for any nucleic acid remaining.
	1.3. Northern blots: degradation of target by RNase	1.3. Ensure equipment is treated to remove RNase (see labelling module booklet).
	1.4. Target not effectively fixed	1.4. Check UV lamp calibration or temperature of oven.
	1.5. Poor labelling efficiency	1.5. For all probe types: check labelling of probe using rapid labelling assay. For random prime labelled probes: make sure dsDNA 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. For oligonucleotide probes: check the purity of the oligonucleotide. Use control reagents to test labelling reaction.
	1.6. Low hybridization efficiency	1.6. For Southerns, check hybridization by using control unlabelled DNA as target and as template for random prime labelling. Improve hybridization efficiency by increasing hybridization time or probe concentration. Make sure the probe is denatured before hybridization.
	1.7. Too high a stringency	1.7. Repeat experiment using lower stringency wash. Try effect of decreasing hybridization and wash temperature.

Problem	Possible cause	Remedy
	1.8. Antibody too dilute	1.8. Check the antibody dilution step.
	1.9. Antibody-AP conjugate inactivated	1.9. Check or replace developer and/or fixer. Ensure film is next to the sample side of the membrane. Pre-flashing the film will increase its sensitivity and linearise response at low light levels.
	1.10. Film exposure problem	1.10. Check or replace developer and/or fixer. Ensure film is next to the sample side of the membrane. Pre-flashing the film will increase its sensitivity and linearise response at low light levels.
	1.11. Too short an exposure time	1.11. Expose the film for an extended period.
2. High general background	2.1. Over-exposure of film.	2.1. If signal is strong, use a shorter exposure period (on a significantly over-exposed film, the blot may even appear completely black).
	2.2. Insufficient blocking of nylon membrane	2.2. 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 hybridization 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 RPN3601).
	2.3. Too high a probe concentration	2.3. Decrease probe concentration in this specific application.
	2.4. Insufficient hybridization buffer	2.4. Make sure that there is sufficient buffer to cover the blot during shaking.
	2.5. Insufficient movement of the blot during hybridization	2.5. Increase the rate of movement.
	2.6. Too low a stringency	2.6. Test the antibody and signal generation by use of control labelled DNA provided in the labelling module.

Problem	Possible cause	Remedy
	2.7. Too high an antibody conjugate concentration	2.7. Decrease conjugate concentration.
	2.8. Incomplete removal of free conjugate	2.8. Check washes have been carried out as recommended. Ensure wash volume is in excess. Try additional washes.
	2.9. Excess detection reagent	2.9. Ensure excess detection reagent has been removed and that there is a layer of plastic between blot and film.
	2.10. Partial drying of membrane	2.10. Ensure membrane remains wet. Partial drying can occur if using low volumes of antibody and block solutions in tubes. Seal detection bags for long exposures.
3. Patchy or grainy background	3.1. Membrane damage	3.1. Handle blots carefully with gloved hands and blunt non-serrated forceps.
	3.2. Probe addition	3.2. Avoid adding probe directly on to membrane. If necessary, pre-mix with some of the hybridization buffer.

	3.3. Membrane dried during process	3.3. **Do not allow membrane to dry out** at any stage.
	3.4. Contamination from powder in gloves	3.4. 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 fresh, autoclaved buffer. Remember that once opened, buffer A should be discarded at the end of a working day.
	4.2. Dirty glassware or plasticware used	4.2. Use freshly cleaned, dust-free plasticware or glassware. Rinse with ethanol before use to remove any minor bacterial AP contamination.
5. Background in tracks	5.1. Insufficient stringency	5.1. Increase stringency as in 2.6. above. Use purified insert to avoid hybridisation by vector sequences. Include heterologous nucleic acid in the hybridization 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 RPN5770
10 labelling reactions each of 100×10^{-12} moles of oligonucleotide

Gene Images random prime labelling module
30 labelling reactions RPN3540
60 labelling reactions RPN3541

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

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

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

ECF random prime labelling module RPN5751
30 labelling reactions

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

ECF random prime labelling and signal amplification system 30 labelling reactions and signal amplification for 2500 cm^2 of membrane (consists of RPN5751 and RPN5750)		RPN5752
Hybridization oven/shaker		RPN2510/ RPN2511
Liquid block (100 ml)		RPN3601
Rapid-hyb buffer (125 ml)		RPN1635
Rapid-hyb buffer (500 ml)		RPN1636
Membranes **Hybond-N+** Positively charged nylon membrane:		
Pack of 10 membranes, 20 × 20 cm		RPN2020B
Pack of 10 membranes, 22 × 22 cm		RPN2222B
Pack of 50 discs, 82 mm diameter		RPN82B
Pack of 50 discs, 132 mm diameter		RPN132B
Roll of membrane, 20 cm × 3 m		RPN203B
Roll of membrane, 30 cm × 3 m		RPN303B
Film **Hyperfilm-MP** High-sensitivity film for chemiluminescence:		
18 × 24 cm	Pack 25 sheets	RPN6K
18 × 43 cm	Pack 25 sheets	RPN36K
24 × 30 cm	Pack 25 sheets	RPN2115K
30 × 40 cm	Pack 25 sheets	RPN7K
35 × 43 cm	Pack 25 sheets	RPN8K
5 × 7 inches	Pack 25 sheets	RPN1676K
8 × 10 inches	Pack 25 sheets	RPN1677K

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

18 × 24 cm	RPN11642
30 × 40 cm	RPN11644
5 × 7 inches	RPN11648
10 × 12 inches	RPN11650

See the current Amersham Biosciences catalogue for further details.

References

1. Sambrook, J., Fritsch, E.F. and Maniatis, T., 'Molecular Cloning: a laboratory manual' Second Edition, Cold Spring Harbor Laboratory, (1989).

2. Meinkoth, J. and Wahl, G., *Anal. Biochem.* **138**, 267 (1984).

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RPN3510PL/AB

Critical parameters

- Buffer A (see solutions required section) should be autoclaved to avoid spots (produced from bacterial contamination) on the final image on film.
- Once the hybridization has been started, the entire process should ideally be completed without interruption. However, if a break in the process is unavoidable, then the user should progress to the end of the stringency washes before stopping. Once the blocking stage has begun, other interruptions should be avoided.
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Gene Images CDP-Star detection module

Protocol reminder card **RPN3510/RPN3511**

Antibody development	Detection
Incubate in blocking buffer (liquid block diluted 1 in 10 in buffer A) room temp/1 h. Incubate in Ab conjugate (at 1 in 5000) in 0.5% BSA in buffer A (0.3 ml/cm^2) room temp/1 h. Wash in 0.3% Tween™ 20 in buffer A, 2-5 ml/cm^2 room temp/3 × 10 min.	Place blots on SaranWrap™ or cut bag. Pipette detection reagent on to blot at 30–40 µl/cm^2 and leave for 2–5 min. Place blot in cut bag or clean SaranWrap. Expose to film.

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