

NUCLEIC ACID SQUASH AND DOT BLOT HYBRIDIZATION METHODS

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SQUASH BLOT HYBRIDIZATION:

1. Using cork borer No. 4, cut 2 disks from each plant to be tested, use completely folded new young leaves.
2. Using a flamed glass rod, squash completely the disk onto a nylon membrane. Try to use small membranes approximately 10X10 cm. Use two disks for each plant. Briefly pass the rod after dipping in alcohol through flame to sterilize, but do not overheat the rod, this would damage the tissues.
3. Air dry the membranes on a filter paper then keep in a dry place, until the time of denaturing.

DOT BLOT HYBRIDIZATION METHOD:

1. Prepare DNA sample in liquid form:
Plant tissues to be tested are: tomato yellow leaf curl virus (TYLCV) positive control (+), unknown sample (S), and tissue from tomato not infected with TYLCV (H).
 - a) Grind two 1cm diameter leaf discs in 100µl Dellaporta buffer using microfuge tube and plastic pestle.
 - b) Grind two 1cm diameter leaf discs in 100µl TE buffer using microfuge tube and plastic pestle.
2. Spin tubes at 10K for 10 seconds in microcentrifuge.
3. Transfer 75µl of supernatant of each tube to new tube, mark with the same sample number and treatment code.
4. From Dellaporta buffer/heat extraction method (the supernatant) make dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32. Keep the rest of supernatant as 1/1 dilution.
5. Heat the tubes for 10 min in boiling water (95-100C) then keep on ice.
6. Spot 5µl of each sample on neutral nylon membrane. Air dry for 30 min.
Diagram of membrane loading is as follows:

TE buffer/heat	+	+	S	S	H	H
Dell buffer/heat	+	+	S	S	H	H
Dilutions	1/1	1/2	1/4	1/8	1/16	1/32

MEMBRANE LYSING:

1. In a large glass tray, saturate 3 layers of Whatman 3 MM paper with 0.5N NaOH. (20 ml 5N NaOH + 180 ml H²O) Remove any excess.
2. Lay membranes DNA side up in saturated paper; create no bubbles or folds. Leave for 5 minutes. (Saturate paper again before lysing another set of membranes).
3. Transfer membranes to tray with 1M Tris pH 7.4., leave for 5 minutes agitating occasionally.

4. Transfer membranes to a tray with 2X SSC. (20 ml 20X SSC + 180 ml H²O) (20X SSC: Dissolve 175.3g of NaCl +88.2g Sodium Citrate in 800 ml H²O. Adjust pH to 7.0 with 10N NaOH. Adjust volume to 1 liter with H²O. Autoclave)
5. Leave for 5 minutes agitating occasionally.
4. Transfer membranes to tray with 95% ethanol. Leave for 5 minutes agitating occasionally. Ethanol will get cloudy, replace if lysing many membranes.
5. Air-dry membranes.

DNA Fixation on Membrane

There are two methods:

1. UV crosslink DNA by selecting optimal crosslink on machine or manually by exposing to UV light with a transillumination device for 3 min.
2. Bind the DNA to the nylon membrane by backing for 2 hours at 80 C.

AlkPhos Direct Labeling and Detection System

DESCRIPTION

The Gene Images™ AlkPhos Direct™ labeling and detection system from Amersham Pharmacia Biotech is based on a dioxetane chemiluminescence system. It involves directly labeling probe DNA or RNA with a specially developed thermostable alkaline phosphatase enzyme. 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.

Once labeled, 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 unlabelled probe, again taking care to control the stringency by adjusting the temperature of the primary wash buffer. The washed blots can then be taken directly into the detection step.

Final detection is as follows:

- The CDP-Star™ chemiluminescent detection reagent utilizes the probe-bound alkaline phosphatase to catalyze the decomposition of a stabilized dioxetane substrate. It has a rapid light output with a very 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.

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

CRITICAL PARAMETERS

- Read the protocol thoroughly before starting
- The concentration of salt in the nucleic acid to be labeled should be as low as possible and should not exceed 50mM
- The DNA concentration should be accurately determined and adjusted to 10ng/μl before labeling
- 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 labeling efficiency
- Enzyme labeled probes **should 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 non-serrated forceps
- Wear powder-free gloves or else rinse gloved-hands with water to remove powder before performing the detection procedure
- Bacteria contain alkaline phosphates, 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.

Solutions

Hyb Buffer : 1L mix NaCl with hyb until into solution, slowly add blocker to dissolve

Hyb Buffer	500ml bottle	(add NaCl to hyb buffer to give final concentration of 0.5M)
NaCl	14.61g	(add blocking reagent to give a final concentration of 4%)
Blocking reagent	20g	mix at RT for 1-2 hours on magnetic stirrer until clear

Store in aliquots at -20 C

Primary Wash: 500mL

Urea	60g	
SDS	0.5g	
0.5M NaH ₂ PO ₄		50mL *
NaCl	4.35g	
1.0M MgCl ₂	0.5mL	
Blocking reagent		1g

Store 1 week in 4 C

* monobasic sodium phosphate, adjusted to pH7.0 with NaOH

Secondary Wash: 500 ml 20X Stock

Tris base 60.5g

NaCl 56g

Adjust pH to 10.0

Store 4 months at 4 C

Secondary Wash: 500mL Working Solution

Dilute 1:20

20X Stock 25mL

dH₂O 475mL

1M MgCl₂ 1mL

Additional Notes:

Use 22 ml hyb solution per 10 cm² in large 10 cm diameter X 20 cm length tube

Prehyb blots for 30 minutes to 1 hour to completely saturate blot

For general probes, hyb at 55 C. For specific probes, hyb at 65 C

Use 30-40 ml of primary wash in hyb bottle at hyb temperature, wash 2X 20 minutes

Use 100 ml of secondary wash per 10 cm² in container at RT, wash 2X 10 minutes

Use 2 ml detection reagent per 10 cm²

Preparation of Probe

Protocol	Notes
<p>1) Dilute 20µl cross-linker solution with 80µl of the water supplied to give the working concentration.</p> <p>2) Dilute DNA (or RNA) to be labelled to a concentration of 10ng/µl using the water supplied.</p> <p>3) Place 10µl of the diluted DNA sample in a microcentrifuge tube and denature by heating for 5 minutes in a vigorously boiling water bath.</p> <p>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.</p> <p>5) Add 10µl of reaction buffer to the cooled DNA. Mix thoroughly but gently.</p>	<p>1) The working solution can be kept in a refrigerator at 2-8°C for one week.</p> <p>2) The concentration of salt in the sample of nucleic acid should be kept as low as possible and should not exceed 50mM.</p> <p>3) 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.</p> <p>5) The reaction should be kept on ice.</p>
<p>6) Add 2µl labeling reagent. Mix thoroughly but gently.</p> <p>7) Add 10µl of the cross-linker working solution. Mix thoroughly. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.</p> <p>8) Incubate the reaction for 30 minutes at 37°C.</p> <p>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 at -15°C to -30°C for up to six months.</p>	<p>8) For color detection this temperature can be increased (see protocol on page 26)</p> <p>9) Further treatment of the probe before use is not required even after long term storage.</p>

Hybridization

Protocol	Notes
<p>1) Pre-heat the required volume of prepared AlkPhos Direct hybridisation buffer to 55°C. The volume of buffer should be equivalent to 0.25ml/cm² of membrane; this may be reduced to 0.125ml/cm² for large blots hybridised in plastic bags or for hybridisation in bottles.</p> <p>2) Place the blots into the hybridisation buffer and prehybridise for at least 15 minutes at 55°C in a shaking water bath (approximately 60 strokes per minute) or hybridisation oven.</p> <p>3) Add the labelled probe to the buffer used for the pre-hybridisation step. Typically use 5-10ng probe per ml of buffer.</p> <p>4) Hybridise at 55°C overnight in a shaking water bath or hybridisation oven.</p>	<p>1) The AlkPhos Direct hybridisation buffer supplied must be prepared for hybridisation before use by addition of NaCl and blocking reagent (see page 9). 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.</p> <p>3) 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 hybridisation buffer. Do NOT denature the probe before use.</p> <p>4) Stringency can be adjusted by altering the hybridisation temperature between 50°C and 75°C - see additional information on hybridisation, pp.20-22.</p>

Post hybridization stringency washes

Protocol	Notes
1) Preheat the primary wash buffer (see page 9) to 55°C. This is used in excess at a volume of 2-5ml/cm ² of membrane.	1) 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.	2) 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	
4) Place the blots in a clean container and add an excess of secondary wash buffer (see page 9). 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.	5) 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.

Protocol	Notes
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.	1) Either SaranWrap™ or a section cut from a Gene Images detection bag (RPN 3609) can be used to place the blots upon.
2) Pipette detection reagent on to the blots (30-40µl/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.	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.
3) Wrap the blots in SaranWrap or place in a detection bag. Place the blots DNA side up, in the film cassette.	3) 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. Detection bags can be heat-sealed to prevent drying out during long exposures.

Protocol	Notes
<p>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.</p>	<p>4) This should be carried out in a dark room, using red safe lights. Do not move the film while it is being exposed,</p>
<p>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.</p>	<p>5) Prolonged exposure will increase background and eventually lead to a totally black image.</p>

References:

Potter, J.L., Nakhla, M.K., Mejía, M., and Maxwell, D.P. 2003. PCR and hybridization methods for specific detection of bean-infecting begomoviruses in the Americas and Caribbean. Plant Dis. 87:1205-1212.