SOUTHERN BLOTTING AND HYBRIDISATION PROTOCOL
(For Hybond NTM membranes)

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SOUTHERN BLOTTING

1. SOLUTIONS
20 x SSPE 500 ml-1
3.6M NaCl 105.15 g
0.2M NaH2PO4.2H2O 15.6 g
0.02M EDTA, pH 8.0 20 ml 0.5M EDTA, pH 8.0

20 x SSC 2.5 l-1
3M NaCl 438.25 g
0.3M Na3 citrate 220.55 g

Depurination 2 l-1
0.25M HCl 43 ml conc HCl

Denaturation 2 l-1
1.5M NaCl 175.3 g
0.5M NaOH 40 g

Neutralisation 2 l-1
1.5M NaCl 175.3 g
1.0M Tris-Cl pH 8 242.3 g

2. BLOTTING PROCEDURE
(i) Wash gel in depurination solution 2 times for 15 min shaking at r.t.
(ii) Rinse in dH2O
(iii) Wash in denaturation solution for 30 min shaking at r.t.
(iv) Rinse in dH2O.
(v) Wash in neutralisation solution 2 times for 15 min shaking at r.t.
(vi) Rinse briefly in 20 x SSC.
(vii) Fill a tray with 20 x SSC, make a platform with a glass sheet and
cover with a wick of 3 sheets of 3MM paper saturated in 20 x SSC.
(viii) Place the gel, bottom up, onto the wick removing any air bubbles,
surround the gel with Parafilm.
(ix) Cut a sheet of Hybond N membrane to the exact size of the gel, wet
the membrane in 20 x SSC, and place on top of the gel. Avoid trapping
air bubbles.
(x) Place 3 wetted sheets of 3MM paper, cut to size, on top of the
membrane.
(xi) Place absorbent paper towels on top (5 cm deep) cover with a glass
plate and place a weight on top.
(xii) Transfer o/n.
(xiii) Dismantle the apparatus and rinse the membrane with 2 x SSPE.
Blot excess liquid from the membrane.
(xiv) Stratalink membrane at energy 2400.
(xv) Allow membrane to air dry between two sheets of 3MM paper then
bake under vacuum for 2 h at 80oC.

FILTER HYBRIDISATION
1. SOLUTIONS
FILTER HYBR’N SOLUTION 500 ml-1
20 x SSPE 6.25 ml
100 x Denhardt's solution 1.25 ml
10% SDS 1.25 ml
dH2O 16.25 ml

5 x OLB
Mix solutions A, B, and C in the ratio 2:5:3
* Store at -20oC.

SOLUTION A: dNTP mix
2M Tris-HCl, pH 8.0 625 ul
5M MgCl2 25 ul
2-b-Mercaptoethanol 18 ul
0.1M dATP 5 ul
0.1M dTTP 5 ul
0.1M dGTP 5 ul
* DNTPs dissolved in 3mM Tris 0.2mM EDTA, pH 7.0
* Store at -20oC.

SOLUTION B
2M HEPES, pH 6.6
* Store at 4oC.

SOLUTION C
Hexadeoxyribonucleotides (Pharmacia P-L cat No. 27-2166-xx) evenly suspended in 3mM Tris-HCl 0.2M EDTA, pH 7.0 at 90 O.D. units ml-1.
* Store at -20oC.

2. PREHYBRIDISATION
(i) Denature 0.5 ml of a 1 mgml-1 solution of Salmon Sperm DNA by heating to 100oC for 5 min. Chill on ice and add to hybridisation solution and prewarm at 65oC.
(ii) Prehybridise membrane in 20 ml of hybridisation solution in a hybridisation tube in a rotisserie hybridisation oven for 3 h at 65oC.

3. PREPARATION OF PROBE
(i) Take 100 ng of probe DNA and dilute to 20 ul in TE. Denature probe by boiling at 100oC for 5 min, chill on ice for 5 min.
(ii) Label probe by random priming following Feinberg and Vogelstein standard protocol with 50 uCi of 32P dCTP.
(iii) Denature 100 ng of probe DNA dissolved in 1 x TE to a volume of 32.5 ul in a pierced Eppendorf tube by boiling for 5 min in a boiling water bath, then immediately cool on ice.
(iv) Add 10 ul of 5 x OLB, 2 ul 10 mgml-1 BSA, 1 ul 1 unitul-1 Klenow, and vortex.
(v) Spin down mix in microfuge add 5 ul of 32P DCTP. Mix briefly and incubate at 25oC for 3 h
(vi) Remove unincorporated nucleotide by passing the labelled probe, diluted to 100 ul with 35 ul of Orange/Dextran Blue dye, over a Sepharose CL-6B spin column.

4. HYBRIDISATION
(i) Denature probe by boiling at 100oC for 5 min, chill on ice for 5min.
(ii) Add probe to 5 ml hybridisation solution, pre-warmed to 65oC.
(iii) Pour off prehybridisation solution and add probe.
(iv) Hybridise o/n at 65oC.
(v) Pour off probe.

5. WASHES
   (i) Wash membrane 2 times with 100 ml of a pre-warmed solution of 1 x SSPE, 0.1% SDS at 65°C for 20 min.
   (ii) Wash membrane 2 times with 100 ml of a pre-warmed solution of 0.1 x SSPE, 0.1% SDS at 65°C for 20 min. Or, alternatively, 2 times in plenty of 5mM Na₂PO₄, 1mM EDTA, 0.2% SDS pH 7.0 for 30 min at r.t. * The latter is more convenient for processing large numbers of filters.
   (iii) Rinse membrane in 2 x SSPE. Place membrane on 3MM paper wetted with 2 x SSPE and wrap in 'Saran wrap' or seal membrane in a plastic bag.

6. AUTORADIOGRAPHY
   (i) Place membrane in an empty cassette, place an Trimax (3M) intensifying screen over the membrane, overlay the screen with a Kodak X-OMAT AR film, and place a second screen over this.
   (ii) Expose film at -70°C.
   * A signal should be seen within 3-5 h with a good probe or o/n.

7. STRIPPING BLOTS FOR REPROBING
   (i) Shake the membrane in a solution of 0.1% SDS at 65°C for 15-20 min.
   (ii) In the case of a strong signal, shake the membrane in 0.4N NaOH for 30min at 45°C.
   (iii) Transfer the membrane to 0.2M Tris-Cl pH 7.5, 0.1 x SSC, 0.1% SDS for 30min at 45°C.
   (iv) Store the membrane sealed in a plastic bag in 2 x SSPE at 4°C.