

Southern Blot

12-1-05

v.4

NDJ

This protocol has been optimized for probing with pcp2, but works for all that has been tried.

Probe Preparation:

The purpose of this step is to prepare a DNA fragment that can be used as a template for generating the radioactive labeled probe.

1. Digest to remove desired probe from plasmid. Digest PCEVII with Xba I and Not I with React 2 buffer under standard conditions (Approx 1 to 2 mg). Digest to remove
2. Run out on 1% agarose gel
3. Cut band out of probe (For pcp2 probe is at 600).
4. Perform gel extraction. See DNA gel extraction protocol.
5. Spec DNA to determine concentration.
6. Make sure there is P32 CTP isotope. You will need it soon (tomorrow).

Digestion of Genomic DNA that will be probed:

This step is to separate the DNA into predictably sized pieces in order that it can run out on a gel. The DNA in this gel will eventually be transferred to a membrane where it can be probed using DNA hybridization. A plasmid control can be used 6pg≈1genome equivalent for a 10 ng southern.

| | | |
|------------------|-------|------|
| DNA 10ug and H2O | | 35ul |
| React 3 | 4.1ul | |
| EcoR1 | | 2ul |

May use other enzymes to get genomic DNA. You want it to transgene once so you will see finger print of insertion site. Akt transgenic mice use EcoRV and React 2.

*Can make master mix to assist in pipetting. If testing for homozygosity, digest 30ul of un-OD tail DNA, then OD it. Then add equal DNA amounts. This OD after digestion allow for more consistent DNA OD readings.

Vortex and spin down. Incubate at 37°C for two hours.
Spike with 1ul EcoRI and incubate at 37°C O.N. Vortex and spin down.

Run out on 1% Agarose gel. The gel should be 300ml. See gel protocol. 100v for 2hours.

Take picture of gel with ruler in order to determine size. Ruler lining up to gel

Preparation of DNA for Transfer to Membrane:

1. Rock gel gently in 0.1 M of HCl for 30 minutes at R. T. This incubation partially deurinates the DNA. Prepare 0.1 M HCl by adding 50ml of 1M HCl to 450ml of H2O. Incubate in casserole pan. The bands of loading dye in gel should turn green and yellow. Pour down drain when finished.

2. Replace acid solution with basic salt solution #2 for 15 minutes and rock at R. T. This denatures DNA and makes strands available for hybridization. Pour solution down drain when finished.
3. Replace basic solution with neutralization solution #3. Rock at R.T. for 30 minutes. The step is done because the a high pH will not allow the binding of the DNA to the nitrocellulose membrane. The dye bands should return to normal colors.

Solutions:

These solutions are prepared in bulk and kept near entrance of the lab.

Solution #2

| | |
|------|------|
| NaOH | 0.5M |
| NaCl | 1.5M |

Solution #3

| | |
|----------|------|
| NaCl | 1.5M |
| Tris pH7 | 1 M |

| | | |
|-----------|---------|-----------|
| Prepare: | | |
| Tris HCl | 127g/l | |
| Tris Base | 23.6g/l | |
| NaCl | | 174.12g/l |

18X SSC + 1m NH₄Ac

| | |
|--------------------|----------|
| NaCl | 157.8g/l |
| NaCit | 79.4g/l |
| NH ₄ Ac | 77.1g/l |

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|----|
| 23 |
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Transfer to Membrane

1. Cut
 - 2 13.5 x 7 inch Whatman filter paper
 - 4 9.5 x 7 inch Whatman filter paper
 - 1 9 x 6 inch Nitrocellulose membrane (N+ hybond, Amersham Pharmacia Biotech)
2. Pour 18X SSC into glass baking dish to a depth of 1.5 inch.
3. Wet both sheets of 13.5 X 7 papers and 2 9.5 x 7 inch filter paper
4. Place a plate of glass over the dish. See picture at end. Then place the two 13.5 x 7 papers over the glass with ends in SSC. Put two wet 9.5 x 7 inch filter paper on top. Then place the gel upside down so it will appear on the blot with the same orientation as the gel picture. Overlay plastic wrap over gel and all of dish. Cut out center part over gel. Put the labeled membrane on top. The nitrocellulose membrane should be pre-wet for a second in water and two minutes in 18X SSC. Place one wet 9.5 x 7 inch piece of filter paper and one dry, in that order, on top of membrane. Then put four inches of paper towels. Put a tray on top and place two half filled 500ml bottles evenly on top.
5. Let go overnight.

Prepare for Hybridization (following day)

1. Dissassemble tower. Put in UV Autolinker and push run
2. Turn on hyb oven to 65°C. Heat block to 37 and 100°C.
3. Assay for radioactively and take necessary radioactive precautions

- Put 15ml hyb buffer in hyb oven in hyb tube.
- Put membrane in bottle. Make sure it is laying flat against the tube.

24a

Radiolabelled Probe Production

- Start water boiling and get bucket of H₂O ice.
- Get screw top tube from radioactive room
- Add TE 10mM Tris 1 mM EDTA to screw cap tube so amount of TE and the DNA added in step 6 equal 50ul.
- Add probe 20ng probe. (0.5ul PCP2 40ng/ul; TRE probe 0.85ul 23ng/ul; 0.5ul of TTA probe; 0.5ul Akt probe).
- Boil in container for 5 minutes
- Bury in ice for 5 minutes
- Remove rediprime tube from deli cooler
- Spin down probe with rediprime as balance
- Add probe to rediprime tube
- Take to radioactive room.
- Take special radioactive precautions (If you do not know what they are, you need to trained).
- Add 5 ul 32P + 50uCi
- Mix well until purple
- Log amounts used
- Incubate 37°C for 30 minutes
- Add 5ul 0.2M EDTA to stop reaction (this step may be omitted)
- 100°C for 5 minutes. Make sure it boils so it the DNA strands melt apart
- Put probe in ice for 5 munte

Hybridization

- Add probe to buffer at bottom of top (not to blot)
- Make sure no bubbles of membrane on tube.
- Hyb for 2 hours
- Turn on water shaking bath to 65°C.
- Assay for radiation.
- Make membrane wash 1 and 2.

Wash # 1

| | |
|------------------|-------|
| 10% SDS | 5ml |
| 25XSSC | 40ml |
| H ₂ O | 455ml |

| | |
|------------------|-------|
| 25% SDS | 2ml |
| 25XSSC | 40ml |
| H ₂ O | 458ml |

24b

Wash #2

| | |
|------------------|-------|
| 10% SDS | 5ml |
| 25XSSC | 4ml |
| H ₂ O | 491ml |

| | |
|------------------|-------|
| 25% SDS | 2ml |
| 25XSSC | 4ml |
| H ₂ O | 494ml |

Wash Membrane

- Pour off waste in liquid radioactive waste.
- Incubate in wash #1 at RT for 20 minutes. Shaking in glass dish.r.
- Add wash #1 to tube and but back in hyb oven. (Cleaning).
- Put membrane in new glass dish with Wash #2.
- Incubate for 20 minutes at 65°C for 20 minutes. Wash longer in desired. Check background by using Geiger counter on edges (background) versus areas that

should be labeled. There should be a noticeable difference. Repeat step five is excessive

Northern: Geiger on 10x, The reading should be ≈ 2.5 . Exposure time for this level of activity is approximately 45 minutes.

6. Put waste down drain.
7. Wrap radiation screen in plastic wrap.
8. Place blot on plastic wrap and wrap again with plastic wrap. Fix plastic wrap to screen with tape. Place another screen facing the plot.
9. Expose blot to standard Kodak film (20 min to ON depending on probe)
10. Clean radioactive room thoroughly and assay for radiation.
11. Use two screens and expose at -80°C .

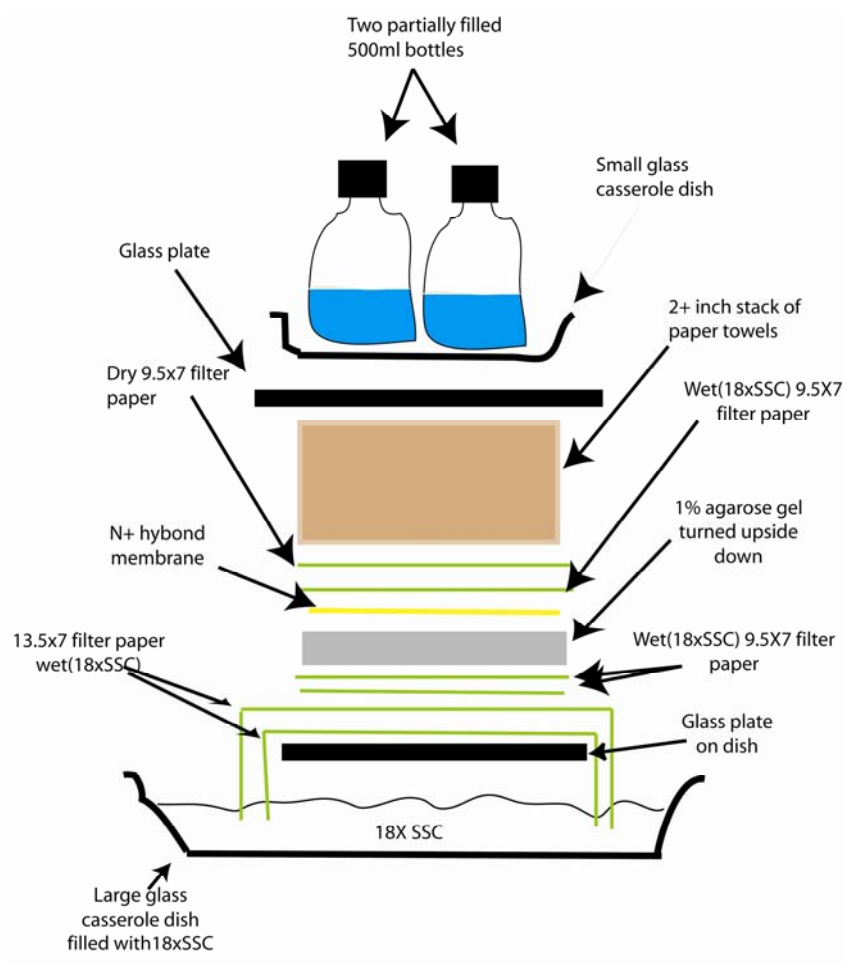


Figure 1. Construction of transfer tower.