Andes Lab Southern Protocol 6/03

Gel

1. Wash the gel rig, comb, caster, etc. with ddH20.
2. Prepare 1% agarose gel with EtBr in 1XTAE; total volume ~80 mL.
3. Let gel sit at least 15 m after it is poured.
4. Place gel with tray in gel rig. Fill gel rig with 1XTAE so that it barely covers the gel.
5. Equilibrate the gel by letting it sit for 5 m in the buffer.
6. Load DNA sample (10ug max, 500 ng – 1 ug ideal) with blue juice. Use HindiIII restricted lambda DNA as standard.
7. Run gel ~60 – 70 V.
8. Periodically check with UV lamp.
9. Stop running when blue juice front is a centimeter from the end of gel.
10. Take photograph of gel with ruler; 0 is reference at the well.

Blotting

11. Depurinate the DNA by soaking in 0.25 M HCl 10-15 m.
    (Acid depurination for fragments > 4 Kb.)
12. Denature DNA by placing in bath of 0.5 N NaOH, 1 M NaCl 30m RT on rotator.
13. Neutralize gel by bathing in 1.5 M Tris-HCl, pH 7.4, 3M NaCl 30 m RT on rotator.
14. Rinse gel with ddH20.
15. Soak gel 10XSSC 15m RT on rotator.
16. Cut one sheet of nylon membrane and two sheets of Whatmann 3MM paper to the size of the gel.
17. Cut two lengths of whatmann paper wider than the gel and long enough to fit from end to end in transfer buffer tray.
16. Wet the nylon membrane in water.
17. Soak the nylon membrane and whatmann paper in 20X SSC for 1-2min
18. Set up the capillary transfer
    -fill large pyrex dish with 20X SSC.
    -place a support in the middle.
    -place the two long whatmann wicks over the support and remove air bubbles.
    -place gel upside down on whatmann wick.
    -place plastic wrap around the edges of gel to prevent short circuits.
    -place presoaked nylon membrane over gel and remove air bubbles.
    -place the two presoaked sheets of whatmann over nylon membrane and remove air bubbles.
    -place a stack of 5-10cm of dry/cut to gel size paper towels.
    -place a piece of glass on top of paper towels.
    -place 1kg weight on top of glass.
    -let proceed for 12-18hr, replacing the wet paper towels at least once.
19. Disassemble the capillary transfer set up.
20. Carefully peel away the gel from the membrane.
21. Mark the lanes with a blunt ended pencil.

22. Check transfer on UV box and mark in pencil the ladder positions.
23. Soak membrane in 3X SSC for 10min.
24. Autocrosslink with stratalinker in Proctor Lab.
25. Place in seal a meal bag if not using right away and store at 4C.

Probe preparation

26. Order Prime-a-Gene Labeling System from Promega (Cat # U1100).
27. Order from CORD Perkin Elmer Life Science alpha-32P-dCTP, 3000 CI/mmol, 10mCI/ml (Cat # BLU-513H).
28. PCR the gene of interest and clean up with Qiagen PCR purification kit.
29. Spec to determine concentration (usually 10-50ng/ul).
30. Place 25ng of clean PCR rxn in 0.5ml tube and bring up to 5ul with nuclease free H2O.
31. Denature for 2 min at 95C in PCR Machine.
32. Rapidly chill in ice water bath for at least 2 min.
33. Spin down to bring everything to bottom.
34. Make dNTP mixture
    -from the kit, mix an equal amount of dATP, dGTP, dTTP.
35. Assemble reaction in the order shown in a 0.5ml tube
    -25ul Nuclease free H2O.
    -10ul 5X Labeling buffer.
    -2ul of dNTP mixture (dATP, dGTP, dTTP).
    -5ul of denatured template.
    -2ul Nuclease free BSA.
    -5ul alpha-32P-dCTP.
    -1ul Klenow Polymerase.
36. Mix gently and centrifuge briefly to bring down contents.
37. Incubate reaction at room temp for 60min.
38. Terminate reaction by heating to 95C for 2min and rapidly chilling in ice water bath.
39. Add 2.083ul of 0.5M EDTA.
40. Prepare G-50 spin column by spinning in 1.5ml tube at 2Xg for 1 min.
41. Blot the extra drop of water off of the end of the column after spinning and place in a new 1.5ml tube.
42. Load the 50ul probe sample above directly to the middle of the slanted G50 column.
43. Spin at 2Xg for 1 min.
44. Take one microliter of sample and check with Geiger counter.

Hybridization

45. Heat Ultrahyb to ~65C to dissolve precipitant.
46. Carefully place blot in hybridization bottle with DNA side facing inward.
47. Add 10ml Ultrahyb/100cm^2 blot area to a clean hybridization bottle.
48. Close tightly (make sure that no part of the blot overhangs the edge of the bottle and gets into the threaded areas ….this will end up in wicking of Ultrahyb out of bottle and you will have a mess).

49. Pre-Hybridize at 42C in Hybridization Chamber for at 30-60min.
50. Add Radioactive probe directly to Ultrahyb buffer in the Hybridization Bottle (no need to change buffer).
51. Close tightly again.
52. Place back in hybridization chamber at 42C for 14-24 hrs.
53. Next day, wash 2x5min with Low Stringency Wash Buffer.
54. Wash 2x15min with High Stringency Wash Buffer.
55. Carefully! take the blot out of bottle after last wash and wrap in Saran.
56. Place blot in Saran into Phosphorimaging cassette (make sure the screen has been erased).
57. Let the blot expose in cassette at least 3-4 hours (longer if small signal expected).

Solutions

10% SDS

10gm SDS
Bring up to 100ml with ddH2O water
Autoclave if desired

20X SSC

210.4gm NaCl
105.8gm NaCitrate
ddH2O water to about 1.1 Liters
Adjust pH to 7.0
Bring up to 1.2L with ddH2O
Autoclave if desired

0.25M HCl

18mL 3M HCl
198mL ddH20

0.5M NaOH, 1 M NaCl

10gm NaOH Pellets
29.22gm NaCl
500ml ddH2O water

1.5M Tris-HCl, pH 7.4, 3 M NaCl

90.83gm Tris
250 mL ddH20
87.66 gm NaCl
pH to 7.4
Bring up to 500 mL with ddH2O

Low Stringency Wash Solution (2XSSC, 0.1%SDS)

5ml 10%SDS
50ml 20XSSC
Bring up to 500ml with ddH20 water

High Stringency Wash Solution (0.1SSC, 0.1%SDS)

5ml 10%SDS
2.5ml 20XSSC
Bring up to 500ml with ddH20 water