

Amino-allyl dUTP Labeling for cDNA Microarray Probes

Notes:

- Can be used for as little as 3 µg of total RNA. The Following is for up to 40 µg of total RNA but adjust as necessary.
- Adaptation of protocol from Joe DeRisi original protocol.
- Hybridization buffer recipe from HGMP Hinxtton
- Per microarray chip you should have two target samples. One labeled with Cy3 and one with Cy5 dyes.
- The dyes are not photo-stable and care needs to be taken to reduce exposure to light.
- **Microcon YM-30 column steps are approximate, and need to be optimized for your particular centrifuge. If you centrifuge too long and the pellet is dry, reload waste and re-centrifuge.**

Reagents to mix and aliquot:

- 50x dNTP mix:
 - 10 µl dATP (100 mM stock)
 - 10 µl dCTP (100 mM stock)
 - 10 µl dGTP (100 mM stock)
 - 4 µl dTTP (100 mM stock)
 - 6 µl amino-allyl dUTP (100 mM)
 - Mix together in tube and keep on ice to add to the Master mix below.
- Cy3 and Cy5 dyes
 - Pre-aliquoted form Amersham RPN5661
- Master Mix (for 50 samples)
 - 300 µl 5X First strand buffer (comes with Transcriptor RT Enzyme, Roche)
 - 300 µl RNase-free water
 - 30 µl 50x dNTP mix made above.
 - Mix and make aliquots of 63 µl and store at -20°C until used
- Formamide Hybridization Buffer:
 - 20 ml deionised formamide
 - 5 ml 50x Denhardt's solution
 - 12.5 ml 20x SSC
 - 0.022g Sodium Pyrophosphate
 - 2.5 ml 1 M Tris-HCL pH7.4
 - 500 µl 10% SDS
 - Fill to 50 ml with DEPC treated H₂O and filter through 0.22 µm filter. Store at -20°C in aliquots.

Experiment:

1. RNA is isolated and purified using the protocol RNA Isolation.Doc.
2. Turn on a heat block (or water bath) to 70°C, one to 42°C, one to 65°C and one to 95°C (only needed in the final steps).
3. Adjust the volume of RNA to 14.5 µl with DEPC treated water and add to a 0.5 ml RNase/DNase free PCR tube.

4. Add 1 μ l of Olio dTV (5 μ g/ μ l, custom 17-mer Sigma-Genosys) primer to each RNA sample.
5. Place the tubes at 70°C for 10 minutes.
6. Place the tubes immediately on ice for 5 minutes.
7. Add 12.6 μ l of the master mix to each tube (made above) and place at 42°C to pre warm for 2 minutes.
8. Add 1 μ l Transcriptor Reverse Transcriptase (20U/ μ l stock, Roche) to each tube and incubate at 42°C for 1 hour.
9. Add another 1 μ l Transcriptor Reverse Transcriptase (20U/ μ l stock, Roche) to each tube and incubate again at 42°C for 1 hour.
10. Remove the tubes from heat block, and add 10 μ l 0.5M EDTA and 10 μ l of 1N NaOH. Place at 65°C for 15 minutes.
11. Remove from heat and place on ice for 5 minutes.
12. Add 25 μ l of 1M Tris-HCl (pH 7.5), mix.
13. Prepare three microcon-YM30 filters, by placing one column into a waste collecting tube. Two will be used next and the other one will be used in step 40.
14. Add 450 μ l of DNase free water to each sample and add each sample to a microcon-YM30 concentrator. Add sample without touching the membrane.
15. Centrifuge at RT at 13,000 rpm for about 8 minutes (or until ~50 μ l of water left in reservoir), empty waste, and then add another 400 μ l of water.
16. Centrifuge again at RT at 13,000 rpm for 8 minutes.
17. Empty waste and add 400 μ l water for a third time.
18. Centrifuge both tubes at RT at 13,000 rpm for 10 minutes (until volume left reached ~ 10 μ l).
19. Invert the columns into new 1.5 ml collection tubes (provided by Millipore).
20. Centrifuge at RT at 3,000 rpm for 4 minutes.
21. Place the tubes in the speed vac to dry 10 minutes on high heat.
22. Re-suspend the pellet in 4.5 μ l DNase-free water.
23. Re-suspend one aliquot of Cy3 and one aliquot of Cy5 dye in 4.5 μ l of 0.1M Bicarbonate buffer (pH in range of 8.5-9.0).
24. Mix one sample with Cy3 (usually control/reference RNA) and the other with Cy5
25. Place in the dark for 1 hour at 23°C (in a heat block).
26. Add 4.5 μ l of 4M hydroxylamine to each sample to stop reaction and incubate in the dark for 15 minutes at 23°C.
27. Prepare the Sigma GenElute PCR kit column by adding 500 μ l of column preparation buffer and centrifuge at 13,000 rpm for 1 minute.
28. Add 35 μ l of 3M sodium acetate (pH 5.2) to each reaction, and mix both samples in a new 1.5 ml Eppendorf tube.
29. Add 500 μ l of Binding buffer from a Sigma GenElute PCR kit. Apply the sample to a column.
30. Centrifuge for 1 minute at 13,000 rpm.
31. Re-apply the flow through on to the column and centrifuge at 13,000 rpm for 1 minute.
32. Discard flow through, and add 750 μ l of wash buffer to the column.
33. Centrifuge for 1 minute at 13,000 rpm. Discard flow through.
34. Repeat step 32 and 33 two more times.
35. Centrifuge column for 1 minute at 13,000 rpm.
36. Place column into a new 1.5 ml Eppendorf tube and add 100 μ l elution buffer.
37. Incubate at RT for 1 minute in dark.

38. Centrifuge for 1 minute at 13,000 rpm.
39. Add another 100 μ l of elution buffer to column and centrifuge for 1 minute at 13,000 rpm. Collect in same tube.
40. Add 300 μ l DNase-free water to sample and apply to a microcon-YM30 column.
41. Centrifuge at 13,000 rpm until sample is approximately 10 μ l (about 10 minutes).
42. Invert the column into a new 1.5 ml collection tube.
43. Centrifuge at RT at 3,000 rpm for 4 minutes.
44. Dry in the speed vac completely (about 10 minutes).
45. Add 31.5 μ l Formamide based hybridization buffer, 2.2 μ l yeast tRNA (4mg/ml), 2.2 μ l Poly dA (8 mg/ml, Sigma Custom) and 1.1 μ l 10% SDS to sample.
46. Place at 95°C for 2 minutes.
47. Centrifuge for 3 minutes at 13,000 rpm.
48. Keep Dark until use.