Reverse transcription and indirect labelling of total RNA for cDNA and oligo arrays

Overview

The samples to be compared are each labelled with a different fluorescent dye and then subjected to paired competitive hybridisations. Indirect labelling involves two steps, firstly, incorporation of amino-allyl dUTP by reverse transcription, and then attachment of the fluorescent dyes.

The following is an adaptation of a protocol from the Ajioka group within the Department of Pathology, University of Cambridge (Ajioka). This protocol can be used to label as little as 3 µg of total RNA. However, the following is for up to 30 µg of total RNA. For each microarray slide you should have two target samples; one labeled with Cy3, the other with Cy5.

Microcon YM-30 column steps are approximate, and need to be optimized for your particular centrifuge. If you centrifuge for too long and the pellet is dry, reload waste and re-centrifuge.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Amino-allyl dUTP (Sigma; Cat. No. A0410)
- Oligo (dT)23 anchored (Sigma; Cat. No. 04387)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC-treated MilliQ water
- Cy3 and Cy5 monofunctional dyes, pre-aliquoted (Amersham; Cat. No. RPN5661)
- RNAsin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- EDTA (BDH; Cat. No. 100935V)
- Sodium hydroxide, (BDH; Cat. No. 102525P)
- Tris-HCl solid (BDH; Cat. No. 443864E)
- Sodium bicarbonate (Sigma; Cat. No. S-7277)
- Microcon YM-30 concentrators (Millipore; Cat. No. 42410)
- MinElute PCR Purification Kit (Qiagen; Cat. No. 28004)
- Hydroxylamine (Sigma; Cat. No. H-2391)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac

Removal of RNase

All materials should be autoclaved and only handled using gloves to avoid RNase contamination. Glassware should be baked at 180 °C overnight. MilliQ water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If possible, keep a set of pipettes purely for RNA work.

Procedure

Reagents to mix and aliquot:

1. Make a 50 x 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)

Then mix together and add to the master mix as below

2. Master Mix for 1 sample (total 14.6 µl)
   - ♦ 6 µl First strand buffer (comes with Superscript III)
   - ♦ 0.6 µl 50x dNTP mix made above
   - ♦ 3 µl 0.1 M DTT (provided with Superscript III)
   - ♦ 0.25 µl RNAsin
   - ♦ 2.75 µl RNase-free water
   - ♦ 2 µl Superscript III

You can make a pre-mix without Superscript III and make aliquots before storing at -20 °C. Add Superscript III before use.

Reverse transcription for amino-allyl dUTP incorporation:

3. RNA is isolated and purified using our standard protocol
4. Adjust the volume of RNA to 14.5 µl with DEPC treated water and add to a 1.5 ml RNase/DNase free microfuge tube
5. Add 1 µl of anchored Oligo dT primer to each RNA sample
6. Place the tubes at 65 °C for 10 minutes
7. Place the tubes immediately on ice for 5 minutes
8. Add 14.6 µl of the master mix to each tube (made above) and incubate at 42 °C for 2 hours
9. Remove the tubes from hot block, and add 10 µl 0.5 M EDTA and 10 µl of 1 M soduim hydroxide. Place at 65 °C for 15 minutes
10. Remove from heat and place at room temperature for 2 minutes
11. Add 25 µl of 1 M Tris-HCl (pH 7.5), mix
12. Add 450 µl of DEPC water to each sample and add each sample to a microcon-YM30 concentrator. Add sample without touching the membrane.
13. Centrifuge at room temperature at 13,000 rpm for about 7 minutes (or until 50 µl of water left in reservoir), empty waste, and then add another 400 µl of water.
14. Centrifuge again at room temperature at 13,000 rpm for 7 minutes
15. Empty waste and add 400 µl water for a third time
16. Centrifuge tube at room temperature at 13,000 rpm for 8 minutes (until volume left reached 10 µl).
17. Invert the column into new 1.5 ml collection tube.
18. Centrifuge at RT at 3,000 rpm for 4 minutes.
19. Place the tubes in the speed vac to dry 10 minutes on high heat. Can be stored at -20 °C.

Dye attachment:

20. Resuspend the pellet in 4.5 ul DEPC water
21. Resuspend one aliquot of Cy3 or Cy5 dye in 4.5 µl of 0.1 M Sodium Bicarbonate (pH in range of 8.5-9.0).
22. Mix resuspended dye with resuspended pellet.
23. Place in the dark for 1 hour at 23 °C (in a hot block).

Dye quenching and removal of unincorporated dye:

24. Add 4.5 µl of 4 M hydroxylamine to each sample to stop reaction and incubate in the dark for 15 minutes at 23 °C.

Clean up samples using Qiagen MinElute PCR Purification Kit:

25. Add 35 µl of 3 M sodium acetate (pH 5.2) to each reaction, and mix both samples (Cy3 and Cy5) in a new 1.5 ml microfuge tube
26. Add 5 volumes (500 µl) of buffer PB and mix.
27. Place a MinElute column in a provided 2 ml collection tube.
28. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 minute at 13,000 rpm.
29. Discard flow through, and add 750 µl of Buffer PE to the column.
30. Centrifuge for 1 minute at 13,000 rpm.
31. Repeat steps 29 and 30.
32. Discard flow through and place MinElute column back in same tube. Centrifuge column for 1 minute at 13,000 rpm.
33. Place column into a new 1.5 ml microfuge tube and add 10 µl elution buffer (DEPC water).
34. Incubate at room temperature for 1 minute in dark.
35. Centrifuge for 1 minute at 13,000 rpm.
36. Add another 10 µl of DEPC water to column and centrifuge for 1 minute at 13,000 rpm. Collect in same tube.
37. Dry in the speed vac to a volume of 2 to 5 µl (about 10 minutes).
38. Add 2 µl sonicated salmon sperm DNA (from 10 mg / ml stock).

The two samples (i.e. sample and control) have been combined together for hybridisation to a microarray and the blocking agent, sonicated salmon sperm DNA, has been added. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (17-02-2006).