Cy Labelling, Hybridization, and Slide Washing

Pombe Post-Genomics

1. Set temperature blocks to 70°C and 42°C (or waterbath). Place all required reagents and samples on ice (experimental and reference RNA aliquots, bacterial RNA 'cocktail', LabellingMix*, oligo(dT) primer, and DEPC water, not Superscript enzyme or Cy dyes).
2. Add 1 µl of bacterial RNA 'cocktail' (in water) and 2.5 µl of anchored oligo-dT_{17} primer (2 µg/µl) to each 12.9 µl RNA sample (or 1.25 µl each of random nonamer (dN_9) and oligo(dT) primer). Prepare a separate tube with the corresponding reference pool RNA for each timepoint. (For polyA RNA use less water and additional random hexamers).
3. Incubate the reaction mixtures at 70°C for 10 min. Place Cy dyes on ice for step 8.
4. Snap-chill the tubes on ice. Spin down reaction mixtures for 15 sec and place the tubes on ice.
5. Make sure the LabellingMix* is completely thawed, then thoroughly vortex, and add 9.6 µl of this mix to each tube with RNA (change tips between tubes).
6. With Gilson P2 pipette add 2 µl of dCTP Cy3 or Cy5-labelled nucleotide to each tube. Remember which dye you use for experiment vs reference and stick to it for the timecourse. Change tips between tubes. Keep exposure to light of Cy dyes to minimum.
7. With Gilson P2 pipette add 2 µl of Superscript II reverse transcriptase (Invitrogen) taken freshly from freezer. Make sure that there is no drop outside of tip after taking enzyme. Change tips between tubes. When enzyme gets low, spin down briefly before use. Put enzyme back to freezer immediately after use.
8. The total volume of reaction mixture should now be 30 µl. Vortex reactions and spin down.
9. Incubate the reactions at 42°C for 1.5 hrs. Cover with lid for light protection.
10. Add 1.5 µl of 1 M NaOH and incubate at 70°C for 15 min to hydrolyze the RNA (in meantime prepare columns as described in 12.).
11. Add 1.5 µl of 1 M HCl and mix to neutralize. Set temperature block to 100°C for step 23.
12. Prepare AutoSeq G-50 columns (Amersham), 1 column for each labeling reaction:
    - Resuspend the resin in the column by vortexing gently.
    - Loosen the cap $1/4^{th}$ turn and snap off the bottom closure.
    - Place the column in a 1.5 ml screw-cap microcentrifuge tube for support. Alternatively, cut the cap from a flip-cap tube and use this tube for support. Note: If using a flip-cap tube, 10-20 µl of fluid will remain in the tip of the column after spinning. Blot this fluid from the column using a clean paper towel before applying sample into the column.
    - Spin the column for 1 min at 2000 x g. Start the timer and the centrifuge simultaneously. Use the column immediately after preparation to avoid drying of the matrix.

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* LabellingMix: Prepare for 20 labelling reactions, store at -20°C:

- 120 µl 5x first strand buffer (Invitrogen)
- 60 µl 0.1M DTT (Invitrogen)
- 12 µl dNTP mix (25 mM dATP, dTTP, dGTP, 10 mM dCTP)

13. Purify the labeled cDNA as follows:
    - Place the column in a new 1.5 ml tube and slowly apply the sample to the centre of the angled surface of the compacted resin bed, being careful not to disturb the resin. Do not allow any of the liquid to flow around the sides of the bed.
    - Spin the column for 1 min at 2000 x g. Start the timer and microcentrifuge simultaneously. The purified sample is collected at the bottom of the tube. Discard the column.
14. Pool purified experimental cDNAs with corresponding reference cDNAs (for timecourse experiments first pool and mix all reference samples, then add an equal aliquot of reference to each experimental sample).
15. Add 1/10th volume of 3M NaAc pH 5.2 (6 µl) and 3 volumes of RT 100% EtOH (180 µl), mix, and precipitate at RT or -70°C for 30 min (RT minimizes nucleotide precipitation).
16. Centrifuge at RT for 15 min at 14,000 rpm. Pellet should appear purple. Discard SN, add 100 µl of 70% EtOH (4°C), mix gently by tipping with finger, and spin for 5 min (same tube orientation!). Aspirate most SN, spin 5 sec, and remove rest of liquid with pipette.
17. Air dry for 5 min at RT. Make a master mix for all your reactions (calculate some extra, e.g. for ½ reaction): ~45 µl/reaction of hybridization buffer (5 x SSC, 6 x Denhardt's 60 mM TrisHCl pH 7.6, 0.12% sarkosyl, 48% formamide; filter sterilized) and 3 µl/reaction of polyA DNA (2 µg/µl, Sigma P0887). Add to each ss cDNA pellet and resuspend pellet with pipette.
18. Place hybridization mixtures in 100°C temperature block for 5 min. Let the tubes cool down at RT for 10 min. Spin the tubes for 15 sec to remove evaporated liquid from lids. Gently vortex.
19. Immediately before use, clean microarrays and coverslips with dust gun. Add hybridization mixture onto middle of inverted clean 60x25 mm LifterSlip. Slowly lower a labeled microarray with the DNA side onto the LifterSlip to prevent bubbles and misplacement.
20. Prepare Boekel humid chamber with 8 round Whatman GF/D 25 mm filters, and add 300 µl of 15 x SSC to each filter. Put microarrays into chamber and incubate at 49°C for ~16 hrs in Grant Boekel hybridization oven (or alternative hybridization chamber).
21. Remove microarrays from chamber and immediately place into a staining jar filled with wash solution 1 (2 x SSC, filter sterilized, HPLC water). Let coverslip fall off by itself (~15 sec).
22. Put microarrays in slide rack. Wash in a staining jar with ~350 ml of solution 1 at RT for 5 min with gentle shaking.
23. Transfer microarrays in slide rack to wash solution 2 (0.05 x SSC, 0.1% SDS, filter sterilized, HPLC water). Wash at RT for 15 min with gentle shaking as before.
24. Repeat step 23.
25. Transfer microarrays in rack to wash solution 3 (0.05 x SSC, filter sterilized, HPLC water). Wash at RT for 5 min with gentle shaking as before.
26. Exchange wash solution 3 once to get rid of all SDS.
27. Quickly transfer microarrays in slide rack to a centrifuge and spin at 1000 rpm for 1 min to dry the slides.
28. Store microarrays in light protected slide box at RT. Scan as soon as possible.