

mRNA expression microarrays

(By Yakun Wan, 8-22-2007)

Part I Total RNA preparation

1. Logarithmic phase cells in 15 ml liquid medium were harvested at an OD_{600} between 0.8 and 1.0 by centrifugation at 3,500g for 5 minutes.
2. Cell pellets were resuspended in 1 ml cold H₂O, washed with cold H₂O, pelleted, frozen in liquid N₂, and stored at -80 °C.
3. Cell pellets were dissolved in 700 ul of TES buffer and put into 2 ml tubes. 700 ul of acid phenol was added and the samples were vortexed.

<u>TES buffer:</u>	<u>Stock</u>	<u>Final</u>
(50 ml)	2.5ml of 1M Tris-HCl 1ml of 500mM EDTA 2.5ml of 20% SDS	50mM Tris-HCl (pH 7.5) 10mM EDTA 1% SDS

4. Samples were incubated at 65°C for 1 hour with vortexing every 10min.
5. Tubes were incubated on ice for 5 min and centrifuged at 15,000g for 5 min at 4 °C.
6. 550ul of the upper solution was transferred to a new snap-cap tube containing 500ul fresh water saturated phenol, mixed and centrifuged as above, and this phenol extraction was repeated once more.
7. For each example, the aqueous layer (about 375ul) was transferred into a clean 2 ml snap cap tube containing 500ul chloroform and vortexed.
8. Samples were centrifuged at 15,000 rpm for 5 min.
9. 200 ul of the upper solution was put into new 2 ml snap cap tubes containing 20 ul (1/10th sample volume) of 3M sodium acetate (pH 5.2), tubes were mixed and 500ul (2.5 times sample volume) of 100% ethanol was added.
10. The samples were again mixed and then incubated for 1 hr at -80 °C to facilitate RNA precipitation.

11. Precipitated RNA was pelleted by centrifugation at 15,000 g for 20 min at 4 °C, mixed in 1 ml ice cold 70% ethanol, and centrifuged at 15,000 g for 2 min at 4 °C.
12. The pellets were allowed to air dry for 15 min after which they were resuspended in 100 ul of TE, vortexed, and spun down.
13. RNA purification by Qiagen Mini-prep kit.
 - (1) Add 350ul of RLT to the above tubes and mix.
 - (2) Add 250ul 100% ETOH, mix, and transfer into columns.
 - (3) Spin at 10,000 rpm for 1 min. Discard the flow through.
 - (4) Prepare DNase solution: 10ul DNase stock+70ul RDD/sample.
 - (5) Wash the columns with 350ul RW1, spin at 10,000 for 1 min, and discard the flow through.
 - (6) Add 80ul of DNase solution and incubate for 15 min at RT.
 - (7) Add 350ul of RW1 and spin at 10,000 for 1 min. Discard the flow through.
 - (8) Add 500ul of RPE and spin at 10,000 rpm for 1 min. Discard the flow through.
 - (9) Add 500ul of RPE and spin at 10, 000 rpm for 2 min. Discard the flow through.
 - (10) Place the columns in a new tube and centrifuge for an additional 1 min.
 - (11) Place the columns in a new tube and add 300 ul of DEPC treated H2O. Spin at 10,000 rpm for 1 min.
 - (12) Add 30ul of DEPC treated H2O and spin at 10,000 rpm for 1 min.
 - (13) Quality and yield of total RNA are determined by spectrophotometry.
 - (14) Dilute the samples to 0.5ug/ul.

Part II

cDNA labeling

The protocol is designed to convert 5-20 ug of total RNA into first strand cDNA. The kit is **SuperScript™ indirect cDNA labeling system (from Invitrogen)** Catalog: L1014-01, L1014-02, L1014-03.

1. Mix and briefly centrifuge components before use.
2. Prepare reactions as follows, using 0.2ml RNase free PCR tubes.

Component	sample
Total RNA	16 ul (total 8 ug)
Anchored Oligo (dT)20 primer (2.5ug/ul)	2 ul

- Mix and spin down.
- Incubate tubes at 70 °C for 5 min, and then place on ice for at least 1 minute.
- Add the following to each tube on ice:

Component	sample
5* First-strand buffer	6 ul
0.1M DTT	1.5ul
dNTP mix (including amino-modified nucleotides)	1.5ul
RNaseOUT™ (40U/ul)	1 ul
SuperScript™ III RT (400U/ul)	2 ul
Final volume	30ul

- Mix gently and collect the contents of each tube by brief centrifugation. Incubate tube at 46 °C for 3 hours. Note: A 3 hour incubation results in 20-30% higher cDNA yield than a 2 hour incubation.
- Incubate at 70 °C for 5 min. Centrifuge the tubes briefly in a microcentrifuge to collect contents.
- Cool tube at 37 °C, and then add 0.2 ul RNaseH (10 units/ul), mix well by pipetting. Vortex, then centrifuge the tube briefly to collect the contents.
- Incubate at 37 °C for 15 min. Centrifuge the tubes briefly to collect the contents.
- Vortex QuickClean resin vigorously for 1 min.
- Add 0.5 ul 0.5M EDTA (pH 8.0) and 3 ul of QuickClean resin to tube, vortex for 1 min. Centrifuge the tubes briefly to collect the contents.
- Insert a 0.22 um spin filter into its accompanying collection tube, and pipet the sample into the spin filter. You need only ensure complete transfer of the liquid portion. Centrifuge at maximum speed for 1 min.
- Remove and discard the spin Filter. Add 2.2 ul 3M sodium acetate, and vortex.
- Add 55ul of ice-cold 100% ethanol to the sample, then vortex.
- Place tube in a -20 °C freezer for 1 hr to precipitate the cDNA.

(Note: for the maximum yield, precipitate cDNA overnight at -20 °C).

16. Centrifuge the tube at maximum speed for 20 min at room temperature.
17. Carefully pipet off the supernatant, and wash the pellet once with 200ul of -70 °C ethanol.

(Note: if desired, you may stop at this point and store cDNA, covered with ethanol, overnight at -20 °C. For storage, do not remove ethanol and freeze the dry pellet. After overnight storage, centrifuge sample to ensure complete recovery of cDNA.)

18. Dry pellet and resuspended pellet in 5 ul of H₂O.
19. Add 3 ul of 300mM NaHCO₃ sodium bicarbonate.
20. Add 8 ul of DMSO to each vial of dye (4ul per labeling reaction).
21. Add dye to cDNA/sodium bicarbonate mixture. Mix well and place the tube at the room temperature in the dark or wrapped in aluminum foil. Incubate at room temperature for 45 min.

Note: the remaining DMSO/dye solution can be stored tightly capped at -20 °C for at least 1-2 months without noticeable degradation.

22. Vortex FluorTrap Matrix vigorously for 1 min.
23. Add 100 ul FluorTrap Matrix to the completed coupling reaction. Vortex for 1 min.
24. Insert a 0.22 um spin filter into its accompanying collection tubes, and pipet the sample into the spin filter. Centrifuge at 10,000 rpm for 1 min.
25. Mix the hybridization samples. For example, to label 4742 strain from YPD medium with Dye 555, and label 4742 strain from SCIM with oleate with Dye 647. After purification of labeled samples, mix equal volumes (100ul + 100 ul). Speed vacuum to dry until the volume is 15 ul.
26. The labeled and mixed samples are now ready for microarray analysis.