Preparation of Fluorescent DNA Probe from Total mRNA for Oligo Arrays - Direct Label of Total RNA

Kate Rubins/Brown Lab – 02/10/2006 (update 05/18/2006)

RT Reaction

1. To anneal primer, mix 20-50μg of Total RNA with 4μg of oligo dT and random hexamer (30μg recommended):

	Cy3	Cy5
Total RNA	20-50μg	20-50μg
Oligo dT (2μg/μl)	2μl	2μl
Random Hexamer (4μg/μl)	1μl	1μl
ddH_2O (DEPC)	to 15.4μl	to 15.4μl
Total volume:	**15.4μl**	**15.4μl**

2. Heat to 65°C for 10 min and cool on ice.

3. Add 14.6μl reaction mixture (Master Mix or separate) to each RNA/hexamer mixes:

Reaction mixture	μl
5X first-strand buffer	6.0
0.1M DTT	3.0
Unlabeled dNTPs (low dTTP)	0.6
Cy3 or Cy5 (1 mM, Amersham)	3.0
Superscript II (200 U/μl, Gibco BRL)	2.0
Total volume:	**14.6μl**

Making unlabeled dNTPs (low dTTP) stock (Update 05/18/2006)

Unlabeled dNTPs	Vol.	Final conc.
dATP (100 mM)	25 uL	25 mM
dCTP (100 mM)	25 uL	25 mM
dGTP (100 mM)	25 uL	25 mM
dTTP (100 mM)	15 uL	15 mM
DdH2O	10 uL	
Total volume:	**100 uL**	

4. Incubate at 42°C for 1 hour.

5. Add 1μl SSII (RT booster) to each sample and thoroughly mix reaction with a pipette.

6. Incubate for an additional 1 hour.

7. Degrade RNA by addition 15μl of 0.1N NaOH and incubate at 65-70°C for 10 minutes.

8. Neutralize by addition of 15μl of 0.1N HCl.

Cleanup MinElute

1. Keep Cy5 and Cy3 separate for MinElute cleanup (QIAGEN Cat. #28004) to measure CyDye incorporation, if desired. If using thin coverslips with small probe volume, you may need to purify Cy3 and Cy5 together or Speedvac to reduce volume after elution.
2. Add 600μl of Buffer PB (Binding Buffer) to each sample.
3. Assemble the MinElute column on the provided 2ml collection tubes.
4. Add the entire 720μl to a MinElute column.
5. Centrifuge for 1 minute @ 10,000 RCF. Discard flow-through and reuse 2ml tube.
6. Add 750μl of WASH Buffer PE to the column.
7. Centrifuge at 10,000 RCF for 1min. Discard flow-through and reuse 2ml tube.
8. Centrifuge again at maximum speed for 1minute to remove residual EtOH.
9. Place column in a fresh 1.5ml tube. Add 10μl of H_20 to elute.
10. Allow elution buffer to stand for at least 2 minutes before spinning.
11. Centrifuge at maximum speed for 1 minute. Add 10μl of H_20 to elute.
12. Allow elution buffer to stand for at least 2 minutes before spinning.
13. Centrifuge at maximum speed for 1 minute.

Probe Preparation

1. Measure how many uL eluted for each sample (still keep Cy 5 and Cy 3 separate) – should be around 18uL for each column. Bring up to volume with water or TE, if needed.
2. Spec 1uL each sample on the Nanodrop to determine dye incorporation. Combine Cy 5 and Cy 3 sample
3. Concentrate Cot-1 DNA from 1ug/uL to 10ug/uL in a speedvac. Check that the Yeast tRNA and PolyA RNA are at 10ug/uL.
4. Make Cot, Poly A and yeast tRNA mix:

	uL
10 ug/uL Cot1 human DNA (Gibco-BRL)	2
10 ug/uL polyA RNA (Sigma, #P9403)	2
10 ug/uL tRNA (Gibco-BRL, #15401-011)	2

5. Add Cot, Poly A and yeast tRNA mix, 20X SSC, 10% SDS to the combined Cy5 and Cy3 probe.

Cover Slip Size (mm)	**Total Hyb Volume (μl)**	**Probe Vol (μl)**	**Cot, PolyA, tRNA Mix**	**20x SSC (μl)**	**10% SDS (μl)**	**1M (ph 7.0) HEPES**
22x60 regular thin coverslips	35	28	6	5.95	1.05	0.84
22x60 Erie M-series thick Lifter Slips	55	36	6	9.35	1.65	1.32

6. Denature probe by heating for 2 minutes at 100°C
7. Spin at 14,000 RPM for 5 to 10 minutes.

Notes:

- **Thin coverslips are not recommended, due to coverslip bending issues.**
- **Avoid introducing bubbles.**
- **Do not vortex after adding SDS.**
- **HEPES is recommended for all probes.** (Update 05/18/2006)