RNA PROBE LABELING (*E. coli*)

NOTES:

- Read this entire protocol before starting.
- Start with minimum of 20ug total RNA for each labeling reaction.
- Better quality data can be achieved if replicate hybridizations are performed using dye swapping (i.e. if 1st hybridization is control (Cy3) vs. experimental (Cy5), switch dyes for 2nd hybridization)
- Cy dyes are light sensitive (especially Cy5) and should ALWAYS be handled in dim light.
- All procedures should be performed using RNA handling precautionary measures.
- All solutions that can be filtered should be.

RNA PREPARATION

1- **A.** If RNA is in ethanol, spin down 20ug of RNA for each labeling reaction, 30 min. at 4°C (proceed to step2).
B. If RNA is stored in H2O it must be at a concentration of ≥ 1.5ug/ul. (proceed to step 5)
2- Carefully pipette off supernatant and wash pellet with 200ul 70% ETOH. (prepared with DEPC H_2O)
3- Spin 5 min. and remove supernatant without disturbing pellet
4- Air dry pellet 15-20 min at room temperature (**Caution**- if pellet is over dried it is hard to resuspend!)
5- Bring each RNA sample to a final volume of 14ul with DEPC H_2O
6- To each RNA sample (14ul) add 2ul of 5mg/ml random hexamer (Pharmacia) + 1ul of control RNA (yeast RNA mix). If no controls are being used add milliQ H_2O in place of controls.
7- Heat to 70 °C 5 min → ice 2 min → pulse spin
(It has been suggested that heating to 98°C (rather than 70 °C) for 5 minutes can lead to stronger hybridization signals. This is likely due to RNA fragmentation during the heating step. If this method is to be used it should be tested prior to using as a standard protocol)

LABELING

1- Prepare labeling mix (2.5X for 2 reactions). Using BRL SuperScript II reverse transcriptase.

1X labeling mix		2.5X labeling mix
8ul 5X first strand buffer	(Gibco BRL)	20ul 5X first strand buffer
4ul 0.1M DTT	(Gibco BRL)	10ul 0.1M DTT
4ul 10X dNTPs (low dTTP)**		10ul 10X dNTPs(low dTTP)
1ul RNAsin (Promega)		2.5ul RNAsin
1.5 ul DEPC H_2O		3.75 ul DEPC H_2O
18.5 ul total volume		46.25 ul total – use 18.5ul/rxn

2- To the RNA/hexamer mix add 18.5ul of labeling mix and incubate 10min at RT
3- Add 1.5ul SSII reverse transcriptase (Gibco BRL) followed by 2 ul of appropriate CyDye dUTP (1mM stock), mix well by tapping and pulse spin
4- Incubate 1hr at 42 °C in the dark
5- Add an additional 1ul SSII reverse transcriptase, tap, pulse spin and continue incubation 1hr
6- Degrade RNA by addition of 2ul 1N NaOH, vortex, pulse spin and incubate 15 min at 65 °C
7- Neutralize by addition of 2ul 1N HCl + 4ul 1M Tris pH 7.4, vortex and pulse spin

CLEANUP LABELED PROBE

1- Add 450ul miliQ H_2O to each of the probe samples. Mix thoroughly by pipetting up and down. Transfer samples to separate Microcon-30 microfilters. (Amicon)
2- Spin at 12,000 RPM in variable speed microfuge for ~12 minutes or until 20-40ul remains in the filter. (Spin times should be empirically determined.)
3- Combine probes by carefully inverting one microfilter (Cy5) into a fresh tube (supplied with Microcon kit) and spin for 1 minute at max speed.
4- Transfer recovered probe to the filter containing the partner probe (Cy3).
5- Add 400ul miliQ H_2O to the mixed probes and gently mix by pipetting up and down. Be careful not to touch the filter at the bottom of the filtration unit.
6- Spin ~12 minutes at 12,000 RPM to concentrate combined probe to 20-40ul
7- Repeat step 5 once but this time concentrates probe to <8ul. (This must be determined after spinning the probe out of the filtration unit.)
8- Invert column into a fresh tube and spin 1 minute at maximum speed to recover probe. Carefully measure recovered probe volume.

9- At this point probes can be hybridized immediately (see hybridization protocol) or stored in dark at 4°C

Reagents and Suppliers for labeling and hybridization

***Note- The GEC sells NEN Cy3 and Cy5 - dUTP at a discounted price (please inquire)**

Cy3-dUTP:	1 mM	Amersham	Cat # PA53022
Cy5-dUTP:	1 mM	Amersham	Cat # PA55022
SuperScript II:	200 U/µl	GIBCO-BRL	Cat # 18064-014
RNAsin	20-40 U/µl	Promega	Cat # N2515
Yeast tRNA	4 µg/µl*****	Sigma	Cat # R8759
100 mM dNTP set	10X**	Pharmacia	Cat # 27-2035-01
$pd(N)_6$ (Hexamer)	5mg/ml*	Amersham	Cat#27-2166-01
. Microcon YM-30 columns		Amicon	Cat # 42410
Hybridization Chambers		Telechem	Cat # AHC-1
Perfecthyb Plus buffer		Sigma	Cat # H7033
Plastic Cover Slips (Hybri-Slip)		Sigma	Cat # Z36,590-4

Other reagents: 20X SSC, TE pH7.4, 10% SDS, 500 mM EDTA, 1M NaOH,
1M Tris-HCl pH7.5, sterile dH2O and DEPC H2O. Filter all solutions.

***** comes lyophilized, must be resuspended at specified concentration
******for 10X stock: 5 mM each of dA, dG, dC and 2 mM of dT in DEPC H2O

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