Institute for Systems Biology Protocol
Direct Incorporation of Cy3/Cy5 During Reverse Transcription
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Materials Needed:

Isolated and quantitated total RNA samples
Microarray slides (70-mer plus-strand oligomers)
RNase-free water
oligo dT primer (16- to 18-mer) at 1 μg/ul
Coverslips, 22 x 40mm size from Corning
100 mM DTT (dithiothreitol)
low dTTP dNTP mix (10 mM each dATP, DCTP, dGTP, **1 mM dTTP**)
Cy3-dUTP and Cy5-dUTP (1 mM each [separately])
3 M Ammonium Acetate, pH 5.2
100% Ethanol, 70% Ethanol

Superscript II Reverse Transcriptase, 5X first strand buffer
RNase A (4 mg/ml)
RNase H (2 unit/μl)

Reverse Transcription and Cy-dye Incorporation

1. In duplicate, aliquot 50 μg total RNA (one for each treatment)
    a. already checked with denaturing agarose gel
    b. quantitated with UV spectrophotometer
    c. precipitated (eg. 1/10 volume 3M NaOAc pH 5.2, 2 volumes EtOH)
2. To each tube, add 2.5 μg oligo dT
3. Adjust volume to 11 μl with DEPC-treated H_2O
4. Heat to 75°C for 10 min
5. Cool slowly to room temperature and spin down (Note: keep at RT from this point on)
6. Add the following in order:
    a. 4 μl Superscript first strand 5X buffer
    b. 2 μl DTT (100 mM)
    c. 1 μl dNTPs (10 mM each dATP, dCTP, cGTP and **1 mM dTTP**)
    d. 1 μl Cy-dye labeled dUTP (1 mM) (One gets Cy-3 dUTP and one gets Cy-5 dUTP)
    e. 1 μl Superscript Reverse Transcriptase II (200 units/ ul; make sure this is Exonuclease-free)
7. Mix gently and incubate at room temperature for 10 min
8. Incubate at 42°C for 2-3 hours (no more than ~5 hours; do not do this step overnight)
9. Heat sample to 95°C for 2 min
10. Place samples on ice, spin down (can store at –20°C at this point if necessary)

Degrade RNA

1. Make sure contents of tubes are spun down
2. Add 0.5 µl of RNase A (4 mg/ml) at room temperature (Promega)
3. Add 0.5 µl of RNase H (2 U/µl) (Fermentas) (Note: Not clear if really necessary to use both enzymes; the RNase H is fairly expensive)
4. Incubate at 37°C for 15-30 min

(Alternative RNA Degradation)

1. Add 3.5 µl 0.5 M NaOH/50 mM EDTA, then
2. Heat at 65 C, 10 minutes
3. Add 5 µl 1 M Tris 7.5

Purification (Using Qiagen PCR CleanUp Kit)

1. Add 25 µl high-quality H_2O to samples (add only 17.5 ul if alternative RNA degradation was done)
2. Add 2.7 µl 3 M Sodium Acetate, pH 5.2
3. Add 250 µl QIAquick buffer PB
4. Apply each sample to a QIAquick column (the DNA should stick to the column here)
5. Centrifuge for 30 sec at full speed
6. Take the column flow-through and replace back onto the top of the column and spin a 2nd time
7. Place the flow-through back in the original tube and save in case of problems with the purification.
8. Wash with 400 µl QIAquick buffer PE, spin 30 sec at full speed and discard flow-through (your DNA remains on the column)
9. Repeat step 8, discarding flow-through
10. Spin the column briefly once more to get rid of remainder of wash solution
11. Place column in a clean, well-labeled 1.5 ml elution tube
12. Apply 30 µl buffer EB to center of column without touching the membrane
13. Wait one min, then centrifuge 1 min at full speed (gradually increase from 0 to full speed to avoid shearing off the eppendorf tube lids). Your DNA is in the flow-through this time!
14. Again apply 30 µl buffer EB to center of column without touching the membrane. DNA should be in a volume of 60 µl.

Determining the incorporation of labeled nucleotides

1. Use a 384-well spec plate (if available) so that small volumes can be used
2. Use undiluted labeled sample and buffer as the blank (~40 µl of each). Read absorbances at 260 nm, 280 nm, 550 nm for Cy3 and 650 for Cy5.
3. Calculations:

a. Extinction coefficients are 150,000 for Cy3 and 250,000 for Cy5
b. Purity: Corrected A260/A280 (want this to be ~1.8)
c. Pmol dye/ µl of sample = A(dye)/(extinction coefficient *10^6)
d. dsDNA = 50 µg/ml
e. ssDNA = 33 µg/ml
f. ssRNA = 40 µg/ml

Example:
Total dsDNA (µg) = 50 µg/ml * 1 ml/ 1000 µl * A260 * Volume (µl) * DF

4. If labeling efficiencies are fairly close for each dye and the nucleic acid yield is also similar, then about 30 pmol of dye of the sample per hybridization is a good starting point to product good intensity on most arrays. It is better to control the amounts of starting RNA/cells and have a good internal control and normalization scheme to deal with incorporation discrepancies.

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Cost Analysis

Reagent	**Supplier**	**Startup Cost**	**Number of Two Label Expts**	**Cost per Two Label Expt**
oligo dT	Operon	$75 for 100 ug	20	$3.75
Superscript II	Invitrogen Life Technologies	$220 for 10,000 units	25	$8.80
Cy3–dUTP	Amersham	25 nmoles for $295	25	$11.80
Cy5-dUTP	Biosciences	25 nmoles for $304	25	$12.16
Rnase A	Promega	$212 - 1 ml, 4 mg/ml	1000	$0.22
Rnase H	Promega	$146 for 50 units	25	$5.84
QIAquick	Qiagen	50 columns for $76	25	$3.04
Totals		**$1328 for 25 Experiments**		**$45.61 per Expt**