

Institute for Systems Biology Protocol

Direct Incorporation of Cy3/Cy5 During Reverse Transcription

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Introduction

This procedure begins RNA isolated from two types of cells that are to be compared. The RNA should have been checked for quality by measurement of UV absorbance. The A_{260} / A_{280} ratio is determined by measurement of the samples in 10 mM Tris pH 7.5 and should be between 1.8 and 2.2. Lower values may indicate protein contamination and higher values may results from organics carried over from the isolation. The RNA should also be checked by denaturing agarose electrophoresis. For eukaryotic RNA, 28S and 18S rRNA bands should appear in a 2:1 ratio over a faint background smear of mRNA. Degraded RNA will appear as low molecular weight smear. The first step in preparation of labeled cDNA for use in microarray hybridization is reverse transcription in the presence of labeled nucleotides. This process uses reverse transcriptase to produce complementary (-) strands from the transcripts in the sample. Oligo dT primes the reactions from poly(A) tails at the 3' ends of transcripts. A nucleotide mix that is low in dTTP is used in combination with labeled UTP. Cy3 is used to label one of the two samples and Cy5 is used for the other. After reverse transcription, the RNA is removed by a combination of RNaseH, which degrades RNA that is hybridized to DNA, and RNase A, which degrades single stranded RNA. Alternatively, base hydrolysis can be used to degrade the RNA. The resulting cDNA is then purified using the QiaQuick kit. Samples are loaded onto silica gel membranes in high salt, washed, and eluted in low salt. The efficiency of the labeling procedure can be measured by spectrophotometry, as described below.

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 $\mu\text{g}/\mu\text{l}$
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

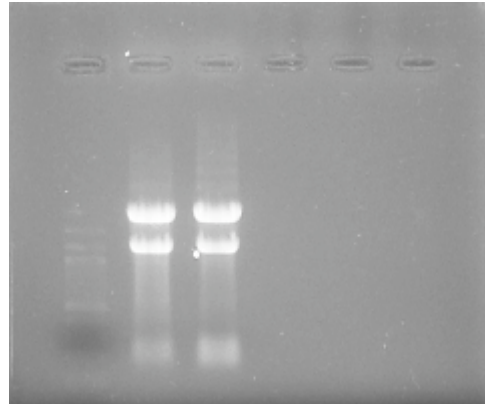
Superscript II Reverse Transcriptase, 5X first strand buffer
RNase A (4 mg/ml)
RNase H (2 unit/ μl)

Precautions

1. RNA handling procedures should be used until after the reverse transcription protocol. Wipe working area with RNase away or lab disinfectant, use gloves and RNase free reagents.
2. Since they are photolabile, the Cy3 and Cy5 cyanine dyes should be kept from light. Keep both the dyes and the dye-incorporated cDNA samples in the dark as much as possible. Suggestions include lowering the lab lighting and using tin foil wraps and tents.

RNA Purification and Measurement

1. Isolate RNA from yeast cultures or other cells.
2. Check quality by running 1 ug on a 1% denaturing agarose gel (non-denaturing gels may be substituted). Ethidium bromide may be added to gel and buffer at 0.5 ug/ml for staining. As seen at right, two bright rRNA bands should be visible on a background smear of mRNA with a minimum of small molecular weight degradation products.
3. Measure RNA with UV spectrophotometer at 260 and 280 nm. The ratio of A_{260} to A_{280} should be between 1.8 and 2.2.
4. Precipitate 50 ug aliquots of RNA for use in labeling procedure using 1/10 volume 3M Na[OAc] and 2 volumes ethanol. Centrifuge at high speed for 10 minutes and air dry pellets (or use speed vac).



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 µl (2.5 µg) oligo dT
3. Add 8.5 µl DEPC-treated H₂O
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 μ l Superscript Reverse Transcriptase II (200 units/ μ l; make sure this is Exonuclease-free)
7. Mix gently by flicking with finger.
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 1 μ l of RNase cocktail (RNase A at 4 mg/ml and RNase H at 1 unit/ μ l).
(Note: it is not clear whether or not both enzymes are really needed; the RNase H is fairly expensive)
3. Incubate at 37°C for 15-30 min

You can save some time at this point by beginning to incubate the microarrays slides at 55 C, as described in Hybridization Protocol.

(Alternative RNA Degradation)

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

Purification (Using Qiagen PCR CleanUp Kit)

1. Add 25 μ l high-quality H₂O to samples (add only 17.5 μ l if alternative RNA degradation was done)
2. Add 2.7 μ l 3 M Sodium Acetate, pH 5.2
3. Add 250 μ l 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 μ l 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 tube lids). Your DNA is in the flow-through this time.
14. Reapply 30 µl buffer EB to the column, wait 1 minute, and spin. Eluted DNA should be in a total volume of 30 µl.

Determining the incorporation of labeled nucleotides (optional)

Note: If a UV-visible spectrophotometer equipped with a small volume (50 µl) cuvette or a Nanodrop spectrophotometer are unavailable, assume good cDNA synthesis and dye incorporation for both samples. Simply mix the two together and proceed with the hybridization.

1. Use a UV-visible spectrophotometer to determine yield of cDNA and incorporation of Cy dyes. The samples must be measured directly or with minimal dilution. If small volume cuvettes (eg. 50 µl) are used, make sure to recover the samples for use in hybridization. Alternatively, a Nanodrop instrument can be used to measure only 2 µl of each sample.
2. Use undiluted labeled sample (or two-fold dilution in EB) and buffer EB as the blank.. 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 to 2.2)
 - c. total ssDNA (µg) = 33 µg/ml * 1 ml/ 1000 µl * A₂₆₀ * Volume (µl)
 - d. Pmol dye/ µl of sample = [A(dye)/(extinction coefficient)] * 50 x 10⁶
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. An alternative is to control the amounts of starting RNA/cells and have a good internal control and normalization scheme to deal with incorporation discrepancies.

Cost Analysis (based on 2005 prices) – Direct Cy Dye Incorporation

Reagent	Supplier	Startup Cost	Number of Two Label Expts	Cost per Two Label Expt
oligo dT	Operon	\$75 for 100 µg	20	\$3.75
Superscript II	Invitrogen	\$209 for 10,000 units	25	\$8.36
Cy3–dUTP	Amersham	25 nmoles for \$308	25	\$12.32
Cy5–dUTP	Biosciences*	25 nmoles for \$318	25	\$12.72
RNase A	Promega	\$200 - 1 ml, 4 mg/ml	1000	\$0.20

RNase H	Promega	\$154 for 50 units	25	\$6.16
QIAquick	Qiagen	50 columns for \$81	25	\$3.24
Totals		\$1345 for 25 Experiments		\$46.75 per Expt

*Now part of GE Healthcare

Note: Several of these companies use Fisher, Thomas or VWR as distributors. You may be able to get some of these cheaper if your institution has a blanket quote with one of the distributors.

For comparison, a 5 reactions 3DNA kit from Genisphere is available for \$295.