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

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Introduction

This procedure begins with 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. Before proceeding with the microarray procedures, checking the RNA samples with RT-PCR for one or several genes is also a good idea (See Bradford *et al.*, (2005) An inexpensive gel electrophoresis-based polymerase chain reaction method for quantifying mRNA levels 4: 157-168 for an RT method). During the direct incorporation of Cy-dyes procedures, 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 quantified total RNA samples
Microarray slides (70-mer plus-strand oligomers)
RNase-free water (DEPC treated)
oligo dT primer (16- to 18-mer) at 1 μg/μl
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 Sodium 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) (optional)
Aluminum foil

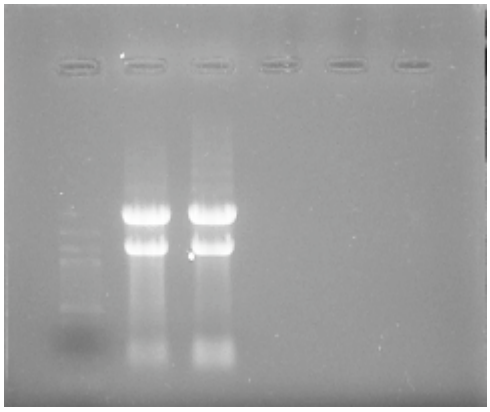
Precautions

1. ***The most important variable involved in the production of quality microarray data is the quality and quantity of starting RNA material.*** It cannot be overemphasized that effort should be devoted to the preparation and quality control of the RNA samples before proceeding with a microarray experiment. 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 aluminum foil wraps and tents.

RNA Purification and Measurement

1. Isolate RNA from yeast cultures or other cells. RNA can be prepared from traditional methods involving hot phenol or Trizol (see GCAT website), and there is a variety of commercially available kits for RNA purification (Ambion, Qiagen, BIO101, Epicentre).
2. Check quality by running 1 μg on a 1% agarose gel (denaturing loading buffers may be used). Ethidium bromide may be added to gel and buffer at 0.5 μg/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 μg 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. quantified 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 H_2O
4. Heat to 75°C for 10 min
5. Cool slowly to room temperature and spin down (Note: keep at room temp 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/ μl; 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

Stopping Point: Can store at –20°C or -80°C at this point if necessary.

Degrade RNA

1. Make sure contents of tubes are spun down
2. Add 1 μl of RNase cocktail (RNase A at 4 mg/ml and RNase H at 1 unit/μl). (Note: the RNase H is fairly expensive and may be omitted without significant effect)
3. 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

Stopping Point: Can store at –20°C or -80°C at this point if necessary.

Purification (Using Qiagen PCR CleanUp Kit)

1. Add 25 μl high-quality H_2O to samples (add only 17.5 μl 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. Wash with 400 µl QIAquick buffer PE, spin 30 sec at full speed and discard flow-through (your DNA remains on the column)
8. Repeat step 8, discarding flow-through
9. Spin the column briefly once more to get rid of remainder of wash solution
10. Place column in a clean, well-labeled 1.5 ml elution tube
11. Apply 30 µl buffer EB to center of column without touching the membrane
12. Wait 1 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.
13. Reapply the ~30 µl cDNA eluate from the previous step to the column, wait 1 minute, and spin. Eluted cDNA should be in a total volume of ~30 µl.

Determining the incorporation of labeled nucleotides (optional)

Cy dyes are designed to emit fluorescence but can also be detected by absorption of visible light. The following are characteristics of Cy3 and Cy5.

Cy3 Pink in Color Green Fluorescence, 532 nM
Cy5 Blue in Color Red Fluorescence, 635 nM

Note: If a UV-visible spectrophotometer equipped with a small volume (50 µl) cuvette or a Nanodrop spectrophotometer are unavailable, assume reasonably 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 10^6
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 2007 prices) **– Direct Cy Dye Incorporation**

Reagent	**Supplier**	**Startup Cost**	**Number of Two Label Expts**	**Cost per Two Label Expt**
oligo dT16	Operon	$75 for 100 μg	20	$3.75
	Catalog number SP230			
Superscript II	Invitrogen	$209 for 10,000 units	25	$8.36
	Catalog number 18064-014			
dATP	Invitrogen	$60 for 100 mM, 250 μl	100	$0.60
	Catalog number 10216-018			
dCTP	Invitrogen	$60 for 100 mM, 250 μl	100	$0.60
	Catalog number 10217-016			
dGTP	Invitrogen	$60 for 100 mM, 250 μl	100	$0.60
	Catalog number 10218-014			
dTTP	Invitrogen	$60 for **10 mM**, 250 μl	100	$0.60
	Catalog number			
DEPC-water	Invitrogen	$40 for 4 x 1.25 mL	62.5	$0.64
	Catalog number 10813-012			
Cy3–dUTP	GEHealthcare	$307 for 25 nmoles	25	$12.28
	Catalog number PA53022			
Cy5-dUTP	GEHealthcare	$317 for 25 nmoles	25	$12.68
	Catalog number PA55022			
QIAquick	Qiagen	$88 for 50 columns	25	$3.52
	Catalog number 28104			
RNase A/T1	Ambion	$61 for 1 ml	320	$0.19
	Catalog number 2286			
RNase H	Ambion	$66 for 200 units	25	$2.64
	Catalog number 2293			

*Yeast tRNA	Ambion	$58 for 0.5 ml@10 mg/ml	200	$0.29

**DIG Easy Hyb

Totals **~$1165 for 25 Experiments** **~$46.75 per Expt**

*for hybridizing with the microarray – part of hybridization mix

**Also part of the hybridization mix. Package comes with dry granules to reconstitute for 6 x 100 ml of hybridization solution – a ton since we only use ~40 µl / array! Check on GCAT-L to see if someone is willing to send you some. Lasts forever dry. If you want to order some for yourself: Roche, Catalog No. 11796895001, for $193.

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

2nd note: We have not included some of the more common reagents in this list, including things like sodium acetate, ethanol, or DTT, nor have we included the cost of RNA preparation (depends on which method you choose to use), or consumables (pipet tips, 50 ml conical tubes, 1.5 ml microtubes), etc.

For comparison, a 5 reaction 3DNA kit from Genisphere is available for $305 (or $61/reaction)