

Protocol for Reverse Transcription and Amino-allyl Coupling of RNA

The following is a slight modification* of a protocol developed by Joe DeRisi (UCSF) and Rosetta Inpharmatics (Kirkland, WA). *Original document can be obtained at www.microarrays.org.

A. RT Reaction

1. To anneal primer, mix 1-2 μ g mRNA with 5 μ g of anchored oligo-dT [(dT) 20 -VN] (Operon, HPLC purified) in a total volume of 18 μ L. One reaction for sample mRNA and one for reference mRNA.

oligo dT	5 μ g of 2.5 μ g/ μ L	2 μ L
mRNA/water	1-2 μ g	16 μ L

□

- Heat to 70C for 10 minutes. Cool on ice for 5 minutes.
- Add 11.6 μ L of nucleotide mix to each of Cy3 and Cy5 reactions.

Nucleotide Mix for one reaction

5X RT buffer		6.0 μ L
50X dNTP stock solution		0.6
DTT	0.1M	3.0
Superscript II RT (Gibco)	200U/ μ L	1.5
RNasin (Gibco, optional)	40U/ μ L	0.5

50X dNTP stock solution using a 4:1 ratio aminoallyl-dUTP to dTTP***:

10 μ L each 100 mM dATP, dGTP, dCTP (Pharmacia)
8 μ L 100 mM aminoallyl-dUTP** (Sigma, #A0410)
2 μ L 100 mM dTTP

**Dissolve 10 mg aminoallyl-dUTP in 170 μ L water. Add approx. 6.8 μ L 1N NaOH. Final pH is roughly 7.0 using pH paper.

***Altering the ratio of aminoallyl-dUTP to dTTP will affect the incorporation of Cy dye. □

□

1X dNTP final concentration during labeling

500 μ M each dATP, dCTP, dGTP
400 μ M aminoallyl-dUTP
100 μ M dTTP

4. Incubate reaction for 1 hour at 42C. Add additional 1 μ L reverse transcriptase and continue incubation at 42C for an additional 1 hour.

B. Hydrolysis

- Degrade RNA by addition of 15 μ L of 0.1 N NaOH. Incubate at 70C for 10 minutes
- Neutralize by addition of 15 μ L 0.1 N HCl. □

To continue with the amino-allyl dye coupling procedure, all Tris must be removed from the reaction to prevent the monofunctional NHS-ester Cy-dyes from coupling to free amine groups in solution.

3. Add 450 μ L water to each reaction.

C. Cleanup

Add 500 μ L neutralized, diluted reaction mix to a Microcon-30 filter (Amicon).

Spin at 12g for 7 minutes.

Discard flow through.

Repeat process two more times, refilling original filter with 450 μ L water. Concentrate to 10 μ L. Samples can be stored at -20C indefinitely.

D. Coupling

Add 0.5 μ L 1M sodium bicarbonate, pH 9.0 to 50 mM final. Check 1M stock solution periodically for fluctuations in pH.

Monofunctional NHS-ester Cy3 (PA23001) and Cy5 dye (PA25001, Amersham) is supplied as a dry pellet. Each tube is sufficient to label 10 reactions under normal conditions. Dissolve dry pellet in 20 μ L DMSO. Aliquot 2 μ L into 10 single use tubes that are then dried in vacuo and store desiccated at 4C. NHS-ester conjugated Cy dye is rapidly hydrolyzed in water, therefore, do not store in DMSO or water. Decreasing the number of aliquots/dye tube may increase your signal.

If you have already made aliquots of dye, simply transfer your cDNA in bicarbonate buffer (10.5 μ L) to the aliquot of dye. Alternatively, dissolve Cy dye in 10 μ L DMSO and add 1 μ L of dye to 10.5 μ L of the cDNA reaction. 10% DMSO in the coupling reaction will not affect the chemical reaction. Aliquot unused dye and dry immediately. □

Incubate 1 hour at RT in the dark. Mix every 15 minutes.

E. Quenching and Cleanup

Before combining Cy3 and Cy5 samples for hybridization, unreactive NHS-ester Cy dye must be quenched to prevent cross coupling.

Add 4.5 μ L 4M hydroxylamine (Sigma).

Let reaction incubate 15 minutes in the dark.

To remove unincorporated/quenched Cy dyes, proceed with Qia-Quick PCR purification kit (QIAGEN). Method described below is as specified by manufacturer.

Combine Cy3 and Cy5 reactions.

Add 70 μ L water.

Add 500 μ L Buffer PB.

Apply to Qia-quick column and spin at 13K for 30-60 seconds. (optional: Reapply flow-through for optimal binding).

Decant flow-through.

Add 750 μ L Buffer PE and spin 30-60 seconds.
Decant flow-through.
Repeat PE wash two more times
Spin at high speed to dry column.
Transfer spin unit to fresh eppendorf tube.
Add 30 μ L Buffer EB to center of filter and allow to sit 3 minutes at RT.
Spin at 13K rpm for 1 minute.
Repeat elution step again with another 30 μ L of Buffer EB.
Pool eluates.

Add 420 TE and apply to fresh Microcon-30 filter.
Spin 12,000g to a volume of 29 μ L or less.

For 38 μ L array hybridization:

29 μ L cDNA probe in TE
1 μ L polyA (10 μ g; Sigma P9403)
1 μ L tRNA (10 μ g; Gibco #15401-029)
7 μ L 20X SSC
1.2 μ L SDS 10%

Heat to 100C for 2 minutes. Let stand 15 minutes RT.

Apply 38 μ L to 40K array.

*Slight modifications to original protocol by Mitch Garber and Anatoly Urisman.

Preparation of Fluorescent DNA Probe from HUMAN mRNA or Total RNA using Direct Incorporation (Max Diehn/Ash Alizadeh protocol; 3/15/01) Modified for Yeast Hybridization

I. Preparing fluorescently labeled cDNA (probe):

To anneal primer, mix 2ug of mRNA or 50-100 µg total RNA with 4ug of a regular or anchored oligo-dT primer in a total volume of 15.4 ul:

	Cy3	Cy5
mRNA (1 µg)	x µg	y µg
Oligo-dT (4 g/l)	1 µg	1 µg
ddH ₂ O (DEPC)	to 15.4 µl	to 15.4 µl
Total volume:	15.4 µl	15.4 µl

(2 µg of each if mRNA, 50-100 µg if total RNA)
(Anchored: 5'-TTT TTT TTT TTT TTT TTT TTV N-3')

- Heat to 65 oC for 10 min and cool on ice.
- Add 14.6 mL of reaction mixture each to Cy3 and Cy5 reactions:

Reaction mixture	Microliters	Unlabelled dNTPs	Vol.	Final conc.
5X first-strand buffer*	6.0	dATP (100 mM)	25 uL	25 mM
0.1M DTT	3.0	dCTP (100 mM)	25 uL	25 mM
Unlabeled dNTPs	0.6	dGTP (100 mM)	25 uL	25 mM
Cy3 or Cy5 (1 mM, Amersham)	3.0	dTTP (100 mM)	10 uL	10 mM
Superscript II (200 U/uL, Gibco BRL)	2.0	ddH ₂ O	15 uL	
Total volume:	14.6	Total volume:	100 uL	

* 5X first-strand buffer: 250 mM Tris-HCL (pH 8.3), 375mM KCl, 15mM MgCl₂)

- Incubate at 42 oC for 1 hr.
- Add 1 ISSII (RT booster) to each sample. Incubate for an additional 0.5-1 hrs.
- Degrade RNA and stop reaction by addition 15 ml of 0.1N NaOH, 2mM EDTA and incubate at 65-70 oC for 10 min. If starting with total RNA, degrade for 30 min instead of 10 min.
- Neutralize by addition of 15 ml of 0.1N HCl.
- Add 380 ml of TE (10mM Tris, 1mM EDTA) to a Microcon YM-30 column (Millipore). Next add the 60 ml of Cy5 probe and the 60 ml of Cy3 probe to the same microcon. (Note: If re-purification of cy dye flow-through is desired, do not combine probes until Wash 2.)
- WASH 1:** Spin column for 7-8 min. at 14,000 x g.
- WASH 2:** Remove flow-through and add 450 ul TE and spin for 7-8 min. at 14,000 x g. It is a good idea to save the flow trough for each set of reactions in a separate microcentrifuge tube in case Microcon membrane ruptures.
- WASH 3:** Remove flow-through and add 450 ul 1X TE and 20 µg polyA RNA (10 µg/ µl, Sigma, #P9403). Spin 7-10 min. at 14,000 x g. Look for concentration of the probe in the microcon. The probe usually has a purple color at this point. Concentrate to a volume of less than or equal to the volume listed in the "Probe & TE" column in the table below. These low volumes are attained after the center of the membrane is dry and the probe forms a ring of liquid at the edges of the membrane. Make sure not to dry the membrane completely!
- Invert the microcon into a clean tube and spin briefly at 14,000 RPM to recover the probe.

Cover Slip Size (mm)	Total Hyb Volume (ul)	Probe & TE (ul)	20x SSC (ul)	10% SDS (ul)
22 x 22□	15	12	2.55	0.45
22 x 40	25	20	4.25	0.75
22 x 60	35	28	5.95	1.05

*20x SSC: 3.0 M NaCl, 300 mM NaCitrate (pH 7.0)

13. Adjust the probe volume to the value□indicated in the "Probe & TE" column above.
14. For final probe preparation add 4.25 □20XSSC and 0.75 □10%SDS. When adding the SDS, be sure to wipe the pipette tip with clean, gloved fingers to rid of excess SDS.□ Avoid introducing bubbles and never vortex after adding SDS.
15. Denature probe by heating for 2 min at 100 oC, leave at 42C for 15-20 min and spin at 14,000 RPM.
16. Place the entire probe volume on the array under a the appropriately sized glass cover slip.
17. Hybridize at 65 oC for 14 to 18 hours in a custom slide chamber with humidity maintained by a small reservoir of 3X SSC (spot around 3-6 □3X SSC at each corner of the slide, as far away from the array as possible).

II. Washing and Scanning Arrays:

1. Ready washes in 250 ml chambers to 200 ml volume as indicated in the table below. Avoid adding excess SDS. The Wash 1A chamber and□the Wash 2 chambers should each have a slide rack ready.□All washes are done at room temperature.

2.

Wash	Description	Vol (ml)	SSC	SDS (10%)
1A	2x SSC, 0.03% SDS	200	200 ml 2x	0.6 ml
1B	2x SSC	200	200 ml 2x	--
2	1x SSC	200	200 ml 1x	--
3	0.2x SSC	200	200 ml 0.2x	--

3. Blot dry chamber exterior with towels and aspirate any remaining liquid from the water bath.
4. Unscrew chamber; aspirate the holes to remove last traces of water bath liquid.
5. Place arrays, singly, in rack, inside Wash I chamber (maximum 4 arrays at a time). Allow cover slip to fall, or *carefully* use forceps to aid cover slip removal if it remains stuck to the array. DO NOT AGITATE until cover slip is safely removed. Then agitate for 2 min.
6. Remove array by forceps, rinse in a Wash II chamber *without* a rack, and transfer to the Wash II chamber with the rack. This step minimizes transfer of SDS from Wash I to Wash II.
7. Wash arrays by submersion and agitation for 2 min in Wash II chamber, then for 2 min in Wash III (transfer the entire slide rack this time).
8. Spin dry by centrifugation in a slide rack in a Beckman GS-6 tabletop centrifuge at 600 RPM for 2 min
9. Scan arrays immediately.