This Protocol is from The Biomedical Image Processing Lab at the University of Minnesota (www.bipl.ahc.umn.edu)

Protocol for Reverse Transcription and Amino-Allyl Coupling

(Derived from a protocol developed at Rosetta Inpharmatics Kirkland, WA)

1) Reverse Transcription Reaction

Mix:	**Amount**	**ul**
Oligo dT / pdN_6	10 ug each	
Total RNA	At least 5 ug	

The above calculation is for total eukaryotic RNA. For poly-A RNA omit the random hexamer (pdN_6) from the priming and input at least 2 ug of RNA. Optimizations may be required.
For bacterial total RNA, we suggest using 10ug RNA primed with 30 ug of hexamers.

Incubate RNA and oligo dT at 70°C for 10 minutes
(use PCR tubes and thermocycler for these steps, if available)

Chill on ice for 10 minutes

Set-up cDNA synthesis reaction

	Concentration	**ul per one rxn**	**ul per 2 rxn**
5X buffer	supplied with SSII	6	12
50X aa-dUTP/dNTP	see below	0.6	1.2
DTT	0.1 M (supplied with SSII)	3	6
SuperScript II	200 U/ul	1.9	3.8
DEPC water		3	6
			14.5 ul aliquots

50X Recipe: 2:3**
10 ul each 100 mM dA, dG, dC
4 ul 100 mM aa-dUTP
6 ul 100 mM dT

A ratio of 2 aa-dUTPs: 3 dTTP's was optimized for yeast chips. Altering the ratio to 3:2 of 4:1 may help increase signal in other systems. Optimizations are recommended.

Mix RNA-primer mix (15.5 ul) with RT mix (14.5)

Incubate reaction mixture at 42°C for two hours

2) Hydrolysis

Add and mix: 10 ul 1N NaOH
10 ul 0.5 M EDTA

Incubate at 65°C for 15 minutes

Neutralize with addition of 25 ul 1 M Tris-HCl pH 7.4 and mix well

Samples may be stored at 4°C overnight at this point

3) Clean-up

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

Fill one Microcon 30 concentrator (Ambion) with 450 ul water.

Add neutralized reaction, mix and Spin at 12K for eight minutes. Discard flow through.

Repeat process 2X, refilling the original filter.

Elute by turning microcon filter upside down into a clean tube and spin at 12K for one minute. At this point a volume over 150 ul should be concentrated again with a second micron filter.

Dry eluate in a speed vac

Samples may be stored at -20°C indefinitely.

4) Coupling

Note- Cye dyes are light sensitive. Avoid overhead fluorescent lighting as much as possible. Also note that monofunctional Cye dyes should be stored at 4°C.

Resuspend cDNA pellet in 9 ul NaBicarbonate Buffer, pH 9.0 and let sit for 10-15 minutes at RT to ensure resuspension.

Transfer entire 9ul volume into tube containing the dried Cye dye aliquot (see below). Use Cy3 for one sample and Cy5 for the other.

Mix by pipetting up and down.

Incubate for one hour at RT in the dark.

Preparing Dye Aliquots:

a) If using a fresh tube of Cy3 or Cy5, resuspend the entire tube in 32 ul DMSO
b) Aliquot 4 ul x 8 tubes and immediately dry in speed vac. Aliquots can be stored indefinitely at 4°C.

Note- by decreasing the number of aliquots /dye tube may increase your signal strength.

5) Quenching and Clean-up

Before combining Cy3 and Cy5 samples for hybridizations, the reactions must be quenched to prevent cross-coupling!!

Add 4.5 ul 4 M Hydroxylamine. Let reaction incubate for 15 minutes at RT in the dark.

To remove unincorporated/quenched Cye dyes proceed with Qia-Quick PCR Purification Kit (Qiagen).

Combine Cy3 and Cy5 reactions. Add 70 ul water. Add 500 ul PB Buffer. Mix. Apply to Qia-quick column and spin at 13K for 30-60 seconds. Discard flow through.

Add 750 ul PE Buffer, mix and spin 30-60 seconds. Discard flow through. Repeat 1X.

Spin for one minute at 14K to dry column.

Transfer column to a fresh tube. Add 30 ul EB Buffer to the center of the filter and let stand one minute at RT.

Spin at 13K for 1 minute.

Repeat elution step again and dry down the eluate in a speed vac.

6) Hybridization Prep

Resuspend dried down labeled target in 15 ul of water.

Add 3 ul 20X SSC
1.5 ul polyA (10 mg/ml)

Note- if using total bacterial RNA an alternative blocker such as salmon sperm DNA should be used!! Also, hybridization volumes may be increased to accommodate for larger surface area microarrays.

Optional- Filter target/hyb mix in Millipore 0.45 um membrane: prewet with 10 ul water and spin through at 8K. Discard flow through. Add target/hyb mix to side of the tube, not directly onto the membrane, and spin at 10k. Transfer the eluate target/hyb mix to a clean 0.5 ml tube.

Add 0.45 ul 10% SDS.

Boil target/hyb mix for two minutes (Boiling denatures the sample and makes it accessible for hybridization)**. Let cool 5-10 minutes at RT.**

Target/hyb mix is ready for use.