

Protocol for Reverse Transcription with Amino-allyl dUTP and Coupling to NHS-Cyanine Dye

Based on the protocol at www.microarrays.org

This is a modification of the procedure in use at the Schreiber Lab, which itself is slightly modified from the protocol at www.microarrays.org.

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This protocol has been modified to work with amplification product from TLAD (T7-based Linear Amplification of DNA).

Materials

Item	Source	Amount	Cat #	Cost (May 2005)
pd N6	Amersham Biosci	50 Units	27-2166-01	\$149
amino-allyl dUTP	Sigma	1 mg	A0410	\$203
Superscript II RNase H ⁻	Invitrogen	4 x 10,000 U	18064-071	\$757
		10,000 U	18064-014	\$209
Microcon YM-30	Fisher Sci	500	42411	\$1,021
		100	42410	\$240
Cy 3 NHS ester (mono)	Amersham Biosci	5 x 1 mg	P23001	\$223
Cy 5 NHS ester (mono)	Amersham Biosci	5 x 1 mg	P25001	\$223
MinElute Kit	Qiagen	250 columns	28206	\$404
		50 columns	28204	\$88
Yeast tRNA	Invitrogen	50 mg	15401-029	\$99
20X SSC	Invitrogen	4 L	15557-036	\$84

Reverse Transcription

<u>Reagent</u>	<u>Volume</u>
pd N6 (random hexamer, 5 ug/ul)	1 ul
aRNA (3 µg recommended; range 2-4 µg)	14.5 ul max
<u>Nuclease-free water</u>	
Total	15.5 ul

1. Incubate at 70°C for 10 minutes to denature RNA secondary structure.
2. Incubate on ice for 10 minutes to anneal primers.
3. Add RT cocktail as follows:

<u>Reagent</u>	<u>Volume</u>
5X FS buffer	6 ul
50x aa-dUTP/dNTP mix ¹	0.8 ul
0.1 M dTT	3 ul
<u>Nuclease-free water</u>	<u>3 ul</u>
Total	12.8 ul

4. Add 2.5 ul Superscript II.
5. Incubate for 2-3 hours at 42°C.
6. Stop the reaction by adding 10 ul 0.5 M EDTA.
7. To hydrolyze the RNA, add 10 ul 1 N NaOH.
8. Incubate for 15 minutes at 65°C.
9. Neutralize the reaction by adding 25 ul 1 M HEPES pH 7.5.
10. Proceed to cleanup, or keep chilled on ice. Samples may also be stored at -20°C for up to 2 weeks.

Post-RT Cleanup

The cDNA produced by the RT reaction must be purified and all of the Tris removed before coupling to the NHS-cyanine dye.

11. Transfer each neutralized reaction to a Microcon YM-30 spin column. Volume is about 75-80 ul.
12. Add 430 ul nuclease-free water.
13. Spin at 14,000 x g for 8 minutes.
14. Remove flow-through and add 450-470 ul nuclease-free water.
15. Spin at 14,000 x g for 8 minutes
16. Repeat previous 2 steps
17. Spin until volume is reduced to about 10 ul. At this stage, liquid will appear as a wedge of liquid occupying a little less than half of the surface area of the spin column.
18. Collect liquid by inverting the column in a fresh collection tube and briefly pulse-spinning at 10,000 x g.

Dye Coupling

The amine of the amino-allyl uracil must be free-based before coupling can occur with the NHS ester. All Tris (which contains primary amines) should be gone by now, or else coupling will occur at very low efficiency to the cDNA sample. Each vial in a pack of cy dyes (listed in Materials section) must be aliquoted into 12 portions, if not all portions are going to be used immediately. Dissolve the lyophilized cy dye powder in 18 ul HPLC-grade DMSO.

¹ The aa-dUTP comes as a lyophilized powder in 1 mg quantities in a 10 ml vial. Dissolve in 20 ul of nuclease-free water, taking care to get all the crystals (some may be on the stopper septum). Then, mix with 30 ul each of 100 mM dATP, dGTP, dCTP, and 12 ul of 100 mM dTTP, giving a final ratio of 3 aa-dUTP:2 dTTP.

Ensure that all specks of powder are dissolved; some may be stuck high up on the walls of the tube and occasionally underneath the cap. Aliquot into 0.5 ml tubes and concentrate in a vacuum centrifuge for 1.5-2 hours under medium heat. Store at 4°C until use. Try to aliquot the dyes when it is NOT a humid day (dewpoint < 50°F), for the dyes are very moisture sensitive (the ester is easily hydrolyzed by ambient moisture).

19. Collect liquid by inverting the column in a fresh collection tube and briefly pulse-spinning at 10,000 x g.
20. Adjust the cDNA liquid volume to 8-10 ul by adding nuclease-free water, if needed. If more than 10 ul, the maximum volume should be no more than 20 ul.
21. Add 1 ul 1 M sodium bicarbonate (NaHCO₃) pH 9. (min. conc. is 50 mM, corresponding to 20 ul volume)
22. Transfer this solution to a pre-aliquoted, dry tube of NHS cyanine dye².
23. Incubate in the dark for 1-1.5 hours. Avoid incubating for more than 2 hours, as the weakly basic conditions will eventually degrade the dye.

Post-Coupling Cleanup

The probes are purified using the Qiagen MinElute kit. The Qiagen PCR Purification kit can also be used, though material less than 150 nt is recovered at low efficiency with this kit.

24. Add 25 ul to each labeling reaction.
25. Add 300 ul Buffer ERC. The amount of sodium bicarbonate should be sufficiently low that addition of sodium acetate should not be necessary.
26. Apply to MinElute column and spin at 14,000 x g for 1 minute.
27. Remove flow-through and add 700 ul Buffer PE.
28. Spin at 14,000 x g for 1 minute.
29. Repeat the previous 2 steps. This extra wash is necessary to remove GITC salts present in the Buffer ERC.
30. Remove flow-through and spin 1 minute at 14,000 x g to dry column.
31. Transfer column to collection tube and pipet 15-20 ul Buffer EB directly onto column.
32. Let stand for at least 1 minute, then spin for 1 minute at 14,000 x g.
33. A second elution step is usually not necessary, since the elution volume is 1.5-2X of the manufacturer's suggested elution volume. A second elution will recover approximately another 10-15% of probe, however, so if it's desired, a second elution can be performed.
34. If necessary, concentrate the volume down with a YM-30 Microcon spin column.

Hybridization Preparation

35. To the concentrated probe, add:

Reagent	<i>(48-tip array)</i>	<i>(32-tip array)</i>	<i>(16-tip array)</i>	Final Conc.
	50 ul probe	35 ul probe	16 ul probe	
Purified, dye-labeled probe	40 ul	24.5 ul	11.2 ul	(N/A)
20X SSC	8.5 ul	5.95 ul	2.72 ul	3.4X
20 ug/ul yeast tRNA	5 ul	3.5 ul	1.6 ul	2 ug/ul
10% SDS	1.5 ul	1.05 ul	0.48 ul	0.3%
Total	50 ul	35 ul	16 ul	

36. Optional – filter probe with a 0.45 um Millipore filter. This step is usually not necessary, but if there is particulate matter present in the probe (which does occur on occasion), follow these steps:
 - a. Do NOT add the SDS to the probe in the previous step.
 - b. Wet Millipore 0.45 um filter with 10 ul water.
 - c. Spin at 14,000 x g for 1 minute.
 - d. Remove flow-through.

² If using freshly aliquoted dyes dissolved in DMSO, the DMSO will not interfere with the reaction, though the fresh aliquots should be kept in a dessicator away from light until use.

- e. Apply probe to filter.
 - f. Spin at 14,000 x g for 1 minute.
 - g. Remove filter
 - h. Add 10% SDS as indicated in the table in the previous step.
37. Prepare hyb chamber:
- a. Place post-processed array, face up, in chamber.
 - b. Add the appropriate amount of 3X SSC in the hydration wells of the chamber. Note that this volume will vary based on the type of chamber used. For the Incyte Genomics hyb chambers, add 50 ul to each of the two hydration reservoirs.

Hybridization

- 38. Denature probe by boiling it for 2 minutes (100°C incubation)
- 39. Allow probe to cool to room temperature (7-10 minutes).
- 40. Briefly spin in centrifuge to bring down condensation.
- 41. Pipet probe directly onto array. Lay down VWR No. 1.5 cover slip and seal hyb chamber.
- 42. Hybridize at 65°C for 12-16 hours.

Post Hybridization Array Washes

- 43. Prepare wash solutions as follows:
 - a. Wash 1: 1X SSC, 0.03% SDS. Prepare 2 glass dishes with this wash.
 - b. Wash 2: 0.2X SSC
 - c. Wash 3: 0.05X SSC
- 44. Remove hyb chamber carefully from water bath, keeping chamber level (to prevent cover slip from sliding off of the array).
- 45. Unseal hyb chamber and carefully remove slide with forceps.
- 46. Submerge slide in first glass dish with Wash 1 solution, tilting the array side downwards. If this is an oligonucleotide array, it is essential to have the cover slip drop off quickly to prevent non-specific hybridization from occurring, so agitate quickly but carefully to remove the cover slip without scratching the array surface.
- 47. Transfer slide to second glass dish with Wash 1 solution and slide rack.
- 48. Repeat previous 4 steps until 3-5 slides have accumulated.
- 49. Plunge rack for 30 seconds.
- 50. Individually transfer each slide from the Wash 1 dish to the Wash 2 dish (containing a slide rack). Blot the bottom edge of the slide on a paper towel during the transfer to minimize SDS transfer.
- 51. Plunge rack for 30 seconds.
- 52. Briefly blot dry bottom surface of rack on a paper towel, then transfer to Wash 3 dish.
- 53. Plunge rack for 30 seconds.
- 54. Spin dry in a benchtop centrifuge with a swinging-bucket rotor at about 230 x g for 3 minutes (1000 RPM in a Sorvall Legend RT centrifuge)
- 55. Scan arrays within hours of washing, keeping slides in the dark until they are scanned. Note – ambient ozone levels should be at a maximum of 35-40 ppb. Higher levels will lead to significant bleaching of the cy 5 dye.