

# cDNA synthesis and indirect amino-allyl labeling

Although for optimal labeling we recommend 20-30  $\mu\text{g}$  of total RNA, typically, when total RNA amounts are 10  $\mu\text{g}$  or greater (5  $\mu\text{g}$  or greater when of superior quality), the RNA can be efficiently converted to cDNA and labeled by an indirect method using the protocol described below. The protocol is a slightly modified version of the Brown Lab (Stanford University) protocol available on their website (<http://cmgm.stanford.edu/pbrown/protocols/amino-allyl.htm>). The Brown lab protocol is based on a protocol developed by DeRisi (1). The original protocol is available at <http://www.microarray.org>.

## Reagents and Solutions

- Oligo(dT): 2 $\mu\text{g}/\mu\text{l}$
- 50X dNTP stock (25 mM each of dATP, dCTP, and dGTP) using 7:3 ratio of aminoallyl-dUTP to dTTP (17.5 mM aminoallyl dUTP and 7.5 mM dTTP). The 50X stock is made by adding the following.
  - 10  $\mu\text{l}$  each 100mM dATP, dCTP, dGTP (Phizer-Pharmacia)
  - 7  $\mu\text{l}$  100 mM aminoallyl-dUTP (Sigma #A0410). Dissolve 1 mg aminoallyl dUTP in 19.1 $\mu\text{l}$  0.1 M  $\text{KPO}_4$ .
  - 3  $\mu\text{l}$  100mM dTTP
- 0.1 N NaOH
- 0.1 N HCl
- Cold absolute and 80% ethanol

- Cy Dye™ Post-Labeling Reactive Dye Packs (Amersham RPN5661)
- Coupling Buffer: Sodium Carbonate Buffer 0.1 M, pH 9.0.
  - 1 M stock solution: Dissolve 8.4g NaHCO<sub>3</sub> in 70 ml water, pH to 9.0, and adjust volume to 100 ml.
  - Working solution is 0.1M, pH 9.0. Check pH before every use and discard the solution when the pH is higher than 9.15 or if older than 4 wks.

## Method

### Day 1

**Reverse Transcription (RT) Reaction:** For optimal labeling, 20-30 µg of total RNA (2-3 µg/µl) is required per reaction. As little as 5 µg of total RNA can be used if of high quality.

1. To anneal the oligo(dT) to the RNA template, add the following for each reaction:

oligo (dT)	5 µg of 2 µg/µl	2.5 µl
nuclease free-water		5.5 µl
total RNA	20 µg of 2 µg/µl	10.0 µl
	TOTAL	18.0 µl

When there are multiple reactions, prepare a master mix of oligo(dT) and RNase-free/low endonuclease water.

2. To denature, heat to 70°C for 10 minutes. Cool on ice for 5 minutes.
3. Add 11.6 µl of RT master mix to each Cy3 and Cy5 reaction. Calculate the master mix volume to accommodate an extra 1-2 reactions.

RT Master Mix (based on Superscript III RT kit, Invitrogen #18080-044):

First Strand Buffer (in Superscript III RT kit)	5X	6.0 $\mu$ l
dNTP Stock Solution (25mM of each deoxynucleotide)	50X	0.6 $\mu$ l
DTT (in Superscript III RT kit)	0.1M	3.0 $\mu$ l
Superscript III RT (Invitrogen; Cat#18080-044)	200U/ $\mu$ l	1.5 $\mu$ l
RNAsin (Fisher; Cat#FP2221)	40U/ $\mu$ l	0.5 $\mu$ l
	TOTAL	11.6 $\mu$ l

4. Incubate reaction at 50°C for 1 hr.

#### **RNA hydrolysis:**

5. Degrade RNA by addition of 15  $\mu$ l of 0.1N NaOH. Incubate at 70°C for 10min.
6. Cool to room temperature. Neutralize by addition of 15  $\mu$ l 0.1N HCl.

#### **Ethanol Precipitation:**

7. Transfer reaction mixtures to a 1.5-ml tube.
8. Add 6  $\mu$ l 3M sodium acetate, pH 5.2.
9. Add 150  $\mu$ l cold absolute ethanol.
10. Store at -20°C overnight.

#### **Day 2**

1. Centrifuge tubes at 12,000 x g for 15 min at 4°C.
2. Pour off supernatant. Add 750  $\mu$ l cold 80% ethanol; flick the tube.
3. Centrifuge tubes at 12,000 x g for 10 min at 4°C.
4. Repeat steps 2 and 3.

5. Vacuum dry samples for 10 min.

**Dye coupling:**

6. Add 5 $\mu$ l of coupling buffer and 5 $\mu$ l water.
7. Mix well by vortexing. Incubate at 42°C for 10 min.
8. Mix well by vortexing. Centrifuge to collect the sample.
9. Mix the sample by pipetting and dissolve Cy3/Cy5 dye (Amersham #RPN5661) with this solution. Mix well by pipetting.
10. Incubate 1 hr at RT in the dark. Mix by vortexing every 15-30 min.

**Cleanup with Qia-Quick PCR purification kit (Qiagen #28104 or #28106):**

11. Add 250  $\mu$ l Buffer PB, add 35  $\mu$ l of 0.1 M sodium acetate, pH 5.2, to each reaction tube. Vortex and centrifuge.
12. Apply the mixture to Qia-quick column and centrifuge at 12,000 x g for 1min. Decant flow-through.
13. Add 750 $\mu$ l Buffer PE and centrifuge at 12,000 x g for 1 min. Decant flow-through. Centrifuge at 12,000 x g for 1 min.
14. Transfer spin unit to new 1.5-ml microfuge tube.
15. Add 35  $\mu$ l Buffer EB to center of filter and incubate for 5 min at room temperature. Centrifuge at 12,000 x g for 1 min.
16. Apply the samples to the Nanodrop ND-1000 to measure the dye incorporation.
17. Mix the corresponding Cy3 labeled and Cy5 labeled samples.
18. Vacuum-dry the samples. Samples are ready for hybridization.

## Notes

Total RNA samples that are degraded and/or in amounts of less than 5  $\mu\text{g}$  require one to two rounds of amplification in order to obtain the required amount of labeled target. We follow the protocol described in the accompanying literature with the Amino Allyl MessageAmp<sup>™</sup> kit (catalog #1753) from Ambion (Austin, TX) for target amplification. In our hands, the kit produces approximately 50-120  $\mu\text{g}$  of amplified RNA (aRNA) starting with 2  $\mu\text{g}$  of total RNA (10 $\mu\text{g}$  of each labeled aRNA is used per slide). As little as 100 ng of total RNA can be amplified with two rounds. The kit is based on a modified Eberwine procedure (2), in which double stranded DNA is generated from the RNA template by reverse transcriptase and DNA polymerase using an oligo(dT) primer fused to a T7 promoter. Amino allyl UTP is incorporated into aRNA during the amplification process by T7 polymerase. The cyanine dye coupling is carried out as described above for cDNA dye coupling, except that the 5  $\mu\text{l}$  volume of water is not added to the reaction. The aRNA is added to the coupling buffer at a 2  $\mu\text{g}/\mu\text{l}$  concentration and then directly used to suspend the cyanine dye. It has been shown that the aRNA is an accurate representation of the original total cellular RNA (3).

## References

1. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. (1996) *Nat Genet* **14**, 457-460
2. Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas, J. D., and Eberwine, J. H. (1990) *Proc Natl Acad Sci U S A* **87**, 1663-1667
3. Iscove, N. N., Barbara, M., Gu, M., Gibson, M., Modi, C., and Winegarden, N. (2002) *Nat Biotechnol* **20**, 940-943