Amino-allyl Dye Coupling Protocol

Joseph DeRisi, June 2001

Typically, fluorescently labeled cDNA is generated by incorporation of dye-conjugated nucleotide analogs during the reverse transcription process. One limitation of this method is that these fluorescent nucleotides are not the normal substrates of any polymerase. The fluorescent moieties associated with these nucleotides are often quite bulky and therefore the efficiency of incorporation of such nucleotides by polymerase tends to be much lower than the natural substrates. Another consideration is that fluorescent nucleotide analogs tend to be quite expensive. Thus, the main motivation for using an alternative labeling method is to increase the fluorescent intensity of the final product and to decrease the cost.

One such alternative is to incorporate, either by synthesis or by enzyme, a less offensive nucleotide analog featuring a chemically reactive group to which a fluorescent dye may then be attached. Linkage of a primary amine to a N-hydroxysuccinimidyl ester group attached to a dye provides an easy means to accomplish these ends. The labeling of DNA with a succinimidyl ester of Cy3 for the purpose of making probes was thoroughly described by Randolph et al 1997. They investigated the effects of this labeling method on melting temperature, extinction coefficient, quantum yield, as a function of fluor density and linker arm length. The authors found that an optimal density of fluorophors to be at a spacing of six bases. Higher densities were found to decrease both the fluorescence intensity and the quantum yield of the labeled probe. This is likely due to non-productive fluor-fluor and fluor-nucleotide base interactions, resulting in quenching.

Compared to enzymatic incorporation of dye, the procedure for post-coupling of reactive dyes is a longer process both in terms of time and steps. However, the added advantage of brightness, lack of enzymatic bias, and low cost is likely to be worth the extra effort.

In the past, chemical coupling of dyes to nucleic acid has been used for various techniques such as sequencing and in situ hybridization (Brumbaugh et al. 1988). Only recently has this technology emerged as a popular approach for the field of DNA microarrays (Hughes et al. 2001). Using the same approach of Randolph et al., N-hydroxysuccinimdyl ester of Cy3 and Cy5. Several other dyes are now available in this form (Molecular Probes, Inc.). The reactive amine derivative of dUTP, 5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate, can be incorporated by a variety of RNA-dependent and DNA-dependent DNA polymerases. After removing free nucleotides, the amino-allyl labeled samples can coupled to dye, purified again, and then applied to a microarray.

The conversion of RNA into cDNA by reverse transcription should be carried out as in standard protocols except that the dNTP formulation contains aa-dUTP. As described by Randolph, a density of labeling which exceeds one dye per six bases will result in a decrease in fluorescent intensity and quantum yield. For the labeling of a complex mixture of samples, as is the case with mRNA, one can only roughly control the labeling density by limiting the amount of aa-dUTP in relation to the amount of dTTP. This choice will depend on the overall A/T content of the genome in question. For yeast, which averages around 60% A/T, I recommend a ratio of 2 aa-dUTP molecules to 3 dTTP molecules. After synthesis of cDNA, RNA may be hydrolyzed by addition of sodium hydroxide and EDTA to a final concentration of 100mM and 10mM

respectively and incubation at 65C for 10 minutes. The hydrolysis reaction should then be neutralized with a non-primary amine containing buffer, such as HEPES, or with free acid.

The following protocol is mainly derived from Randolph 1997 and Hughes 2001, with modifications and comments generated by my own lab.

Materials List

5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate sodium salt (Sigma A0410)
Fluorolink Cy5 Monofunctional Dye 5-Pack (Amersham PA25001)
Fluorolink Cy3 Monofunctional Dye 5-Pack (Amersham PA23001)
Oligo dT Primer (Operon)
Random 6-9mer (Operon)
100mM dNTPs (Amersham 27-2035-02)
MicroCon-30 (Millipore 42410)
Reverse Transcriptase: (your choice)
- StrataScript Reverse Transcriptase (StrataGene 600085)
- PowerScript Reverse Transcriptase (CloneTech 8460-2)
- SuperScript II Reverse Transcriptase (Invitrogen 18064-014)

QIAquick PCR Purification Kit(50) (Qiagen UB300)

Protocol

Incorporation of aa-dUTP by Reverse Transcription

1. Make the dNTP + aa-dUTP mixture:

50x aa-dNTP Mix	
	µL
100mM dATP	10
100mM dCTP	10
100mM dGTP	10
100mM dTTP	6
100mM aa-dUTP	4
40µL	

2. Set up the Priming Reaction:

Priming Reaction		
	[concentration]	µL
Oligo dT / Random Primer	2µg/µL each	1
poly(A)+ RNA	2µg total	14.5
		15.5µL

3. Incubate the priming reaction at 70˚C for 8 minutes. Remove and put on ice.
4. Set up the cDNA synthesis reaction master mix:

cDNA Synthesis Reaction		per rxn
	[concentration]	µL
RT Buffer	10x	3
aa-dNTP	50x	0.6
DTT	0.1M	3
Reverse Transcriptase	50U/µL	2
Water		5.9
		14.5 µL

5. Add 14.5µL of master mix to each Priming Reaction.
6. Incubate reactions at 42˚c for 2 hours.

Hydrolysis and Cleanup

1. Bring cDNA synthesis reactions to a final concentration of 100mM NaOH and 10mM EDTA. Incubate at 65C for 10 minutes.
2. Neutralize the hydrolysis reaction by the addition of HEPES, pH 7.0, to a final concentration of 500mM. Other non-primary amine containing buffers may also be used. Note that Tris buffer carries a free amine and should be avoided since this could possibly interfere with the subsequent coupling reaction.
3. Bring the reaction volume to 500µL with water. Concentrate the cDNA product by filtering through a Microcon-30. Try to get the final volume of the sample down to below 10µL. This can usually be accomplished by spinning at full speed for 6-10 minutes in a typical microcentrifuge. Do not spin to dryness as this can make the cDNA

difficult to recover.
4. Bring the concentrated product to 500µL and repeat the concentration at least twice. The net effect of this process is to remove the hydrolyzed RNA, NaOH, and buffer components.
5. The amino-allyl labeled cDNA may now be stored indefinitely at -20C.

Aliquoting Cy-dye esters
1. Amersham typically ships the succinimidyl esters of the Cy dyes as dried pellets sealed in a foil bag with a small amount of desiccant. If the desiccant material has turned from dark blue to a light pink, this is an indication that moisture has contaminated the sample. Contaminated dye packs should be returned to Amersham for a refund.
2. Resuspend the solid pellet in 12µL of water free DMF or DMSO.
3. Since a single tube of dye usually provides sufficient material to label at least 12 samples, aliquot 1µL volumes of the resuspended dye into separate screw cap tubes. Dry down the aliquots using a speed-vac, without heat.
4. Store the dye aliquots at 4C, in a light-sealed box, preferably under vacuum and in the presence of a large amount of desiccant; This will help ensure the dyes remain uncontaminated with moisture.

Coupling to N-hydroxysuccinimidyl ester dyes
1. Bring the cDNA solution to a final volume of 10µL with water. Add XXµL of 1M sodium bicarbonate buffer, pH 9.0,
2. Remove a dye aliquot from storage and use the bicarbonate buffered cDNA solution to vigorously resuspend the pellet by pipetting up and down.
3. Incubate the coupling reaction in the dark for at least 60 minutes at room temperature.

Removal of uncoupled dye material
The QiaQuick (Qiagen) PCR Purification columns work well for removal of uncoupled dye. Purify each dye labeled sample separately by following the manufacturers directions with the following modifications:
1. Mix in Buffer B (500µL) with the coupling reaction before application to the DNA binding column.
2. Rinse the column with 600µL of Buffer PE at least two times.
3. After the final rinse, spin the column one more additional time to remove any traces of the rinse buffer.
4. Add 60µL of elution Buffer EB to the column and let incubate for 5 minutes at room temperature. Spin eluate through to a collection tube.
5. Repeat the elution with another 60µL of Buffer EB.
6. Concentrate the eluate to desired volume by vacuum drying or by concentration using a Microcon-30 spin filter.

References for Amino allyl Coupling Protocol

1. Randolph, J. B., and Waggoner, A. S. Stability, specificity and fluorescence brightness of multiply-labeled fluorescent DNA probes. (1997) *Nucleic Acids Res* **25**(14)**,** 2923-9.
2. Brumbaugh, J. A., Middendorf, L. R., Grone, D. L., and Ruth, J. L. Continuous, on-line DNA sequencing using oligodeoxynucleotide primers with multiple fluorophores. (1988) *Proc Natl Acad Sci U S A* **85**(15)**,** 5610-4.
3. Hughes, T. R. et al. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. (2001) *Nat Biotechnol* **19**(4)**,** 342-7.