

aRNA Amino-allyl reverse transcription and NHS-Cy dye coupling

Reverse transcription

- Mix together:
 - aRNA 2000 ng (max. vol.: 10.9 ul)
 - Nonamer (1 ug/ul) 2 ul
 - Superas-In RNase inhibitor 0.5
- Incubate at 70 for 10', and chill on ice 30''
- Add:
 - 5X first strand buffer 6 ul
 - 0.1 M DTT 3 ul
 - dNTP mix~
 - (20 mM G/A/C, 4mM T, 16mM AA-dUTP) 2.8 ul
 - PowerScript II RT (200 u/ul) 1 ul
 - Superas-In RNase inhibitor 0.5 ul
- Incubate at 42°G for 1 hour
- Add
 - SuperScript II RT (200 u/ul) 1 ul
- Incubate at 42°G for 1 hour
- Cool reaction to 4°G for 2 min.

~dNTP mix

Add

dATP 100 mM	8 ul
dCTP 100 mM	8 ul
dGTP 100 mM	8 ul
dTTP 100 mM	1.6 ul
aa-dUTP 50 mM*	12,8 ul
PCR grade water	1.6 ul

RNA hydrolysis and purification of cDNA

- Add
 - 0.1 N NaOH. 15 ul
- Incubate at 70 for 10'
- Add to neutralize
 - 0.1 N Hcl 15 ul

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.

Clean up amino-allyl cDNA

- Purify amino-allyl single stranded cDNA with Wizard® SV Gel and PCR Clean-Up system*.
- Reduce volume to less than 10 ul with Speed Vac. Do not let completely dry!! Samples can be stored at -20 for a month.

Coupling

In the coupling reaction the terminal amino group on the aa-dUTP attacks the NHS-monoester dye to form a Cy-aa-UTP covalent bond. The reactions are performed at pH 8.5-9, which is an optimal balance between deprotonation of the amino group and nucleophilic attack and hydrolysis reactions of the NHS ester with water. The dyes should not be exposed to water prior to coupling and should be used immediately after they are resuspended in DMSO. Reactions should be carried out in the dark as the Cy dyes are light sensitive.

- Add
 - Sodium bicarbonate 1M pH 9.0 0.5 ul
- Add the amino-allyl cDNA to an aliquote of NHS-ester Cy Dye*
- Incubate 2 hours at RT in the dark.

Mono functional NHS-ester Cy3 and Cy5 dye is supplied as dry pellet (Amersham PA23001 and PA25001). Each tube is sufficient to label 10 reactions under normal conditions. Dissolve dry pellet in 20 ul DMSO. Aliquot 2 ul into single use tubes that are then dried in vacuo and stored 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. 10% of DMSO won't affect the coupling reaction.

Cleanup

Purify Cy dye amino-allyl single stranded cDNA with Wizard® SV Gel and PCR Clean-Up system (Promega).

- Add
 - Poly A (10 ug/ul) 2 ul
 - Salmon sperm DNA (20 ug/ul) 2 ul

Seep vac dry to less than 25 ul

Labeled samples are ready for hybridization

*Reagents specification

Sodium Carbonate Buffer (Na_2CO_3): 1M, pH 9.0

Dissolve 10.8 g Na_2CO_3 in 80 mL of MilliQ water and adjust pH to 9.0 with 12 N HCl; bring volume up to 100 mL with MilliQ water. Check periodically the pH because it might change over time

RNA quantification reagent manual (Invitrogen No. R11491).

Supers-In RNase inhibitor (Ambion No. 2692)

Wizard® SV Gel and PCR Clean-Up system (Promega No. A9281).

5-(3-aminoallyl)-UTP (AMBION No. #AM8439)

Cy3 mono-Reactive Dye Pack (Amersham No. PA23001)

Cy5 mono-Reactive Dye Pack (Amersham No. PA25001)