Ⅱ -Aminoallyl Labeling of cDNA for Array Hybridization

◎Material and reagents

Oligo dT-V (2 μg/μl) desalted

5X Superscript II reaction buffer – Invitrogen Cat. 10864014

0.1 M DTT– Invitrogen Cat. 10864014

Superscript II reverse transcriptase (200 U/μl) Invitrogen Cat. 10864014

100 mM dNTPs – Amersham Cat. 27-2035-03

aminoallyl-dUTP - Sigma Cat. A0410

Monofunctional NHS-ester Cy3 - Amersham Cat. PA23001

Monofunctional NHS-ester Cy5 - Amersham Cat. PA25001

Sodium bicarbonate – Sigma Cat. S5761

Hydroxylamine hydrochloride– Sigma Cat. H9876

Microcon YM 30 – Millipore Cat. 42410

PCR clean up kit – Qiagen Cat. 28106

Random primer (3 mg/ml) – Invitrogen Cat. 48190-011

Step-1 Annealing primer to mRNA template

In two 0.2 ml RNase free PCR tubes add the following

	Cy5 mRNA	Cy3 mRNA
Oligo dTV primer	0.5 μl (1.25 μl)	0.5 μl (1.25 μl)
Random Primer	0.33 μl (1.2 μl)	0.33 μl (1.2 μl)
mRNA 1 μg (2.5~3 μg)	X μl	X μl
RNase free water	To a final volume of 15 μl	
( ) for 2.5~3 μg mRNA use		

Mix and Spin

Note: When using total RNA do not use random primer. Use only Oligo-dTV primer

Incubate at 70℃ for 10 min. Spin tube and chill on ice for 10 min.

Step-2 Reverse transcription (first strand cDNA synthesis)

In a separate tube add. (Make cocktail for the desired number of reactions, except SuperScript II RTase)

Reagent	Amount
Water	3.4 µl
5X superscript buffer (Invitrogen)	6.0 µl
50X aa dUTP/dNTPs (4:1)	0.6 µl
0.1 M DTT	3.0 µl

1. Aliquot 13 µl of the cocktail in each tube containing the primer template mix.
2. Mix well and spin.
3. Incubate tubes at 65℃ for 2 min only.
4. Immediately transfer tubes to 42℃. Incubate 3 min.
5. Add 1 µl Superscript II (2 µl when use 2.5~3 µg mRNA) in each tube. Mix by flicking the tube. Spin. (Do not vortex)
6. Incubate at 42℃ for 1 hour.
7. Add 1 µl of Superscript II.
8. Mix and Spin.
9. Incubate another 1 hour at 42℃.

Note:

50X dNTP stock solution using a 4:1 ratio aminoallyl-dUTP to dTTP***.

5 µl each 100 mM dATP, dGTP, dCTP (Amersham)

4 µl 100 mM aminoallyl-dUTP** (Sigma, #A0410)

1 µl 100 mM dTTP

** Dissolve 1 mg aminoallyl-dUTP in 18 µl DEPC water, add approximately 0.68 µl 1 N NaOH, Final pH is roughly 7.0 (using pH paper).

***Altering the ratio of aminoallyl-dUTP to dTTP will affect the incorporation of Cy dye.

Step-3 Hydrolysis of RNA

Add

Reagent	Amount
0.5 M EDTA	10 µl
1.0 N NaOH	10 µl

Mix, Spin.

Incubate: 15 min. at 65℃.

Neutralize with 25 µl 1 M HEPES pH 7.4.

Step-4 Purification of Aminoallyl labeled cDNA

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.

1. Fill one Microcon-30 concentrator with 375 µl of ddH_2O.

2. Add neutralized reaction (75 µl) from step 3.

3. Spin at 12,000 x g for 10 min – Wash I.

4. Discard flow through.

5. Add 450 µl water to the column- Wash II.

6. Spin at 12,000 x g for 10 min.

7. Discard flow through.

8. Wash again adding 450 µl of water to the column – Wash III.

9. Spin at 12,000 x g for 10 min.

10. Discard flow through.

11. For elution, invert and insert column into a clean tube.

12. Spin for 3 min at 1000 x g.

13. Collect solution.

14. Concentrate to 9 µl in SpeedVac (Samples may be transferred to -20℃ for storage here).

Step-5 Coupling reaction

1. Add 1 µl 0.5 M sodium bicarbonate, pH 9.0 to Aminoallyl-cDNA solution to make 50 mM final concentration.

2. Add DNA- buffer solution to microfuge tube containing dried down dye (see below for instructions to prepare the monofunctional NHS-ester Cy dyes).

3. Pipet up and down to mix completely.

4. Incubate 1 hour at RT in the dark. Mix and Spin every 15 minutes.

Note: Check 0.5 M stock solution periodically for fluctuations in pH. Carbonate buffer changes composition over time; make it fresh every couple of week or keep at -20℃. Monofunctional NHS-ester Cy3 (PA23001) and Cy5 (PA25001, Amersham) is supplied as a dry pellet. Each tube is sufficient to label 10 reactions under normal conditions. Dissolve dry pellet in 10 µl (20 µl) DMSO. Aliquot 1 µl (2 µl) into 10 single use tubes that are then dried in SpeedVac without heating. Dried dye are stored desiccated in the dark at 4℃. NHS-ester conjugated Cy dye is rapidly hydrolyzed in water. Don't store in DMSO or water. Decreasing the number of aliquots/dye tube may increase your signal.

Step-6 Quenching and cleanup

Before combining Cy3 and Cy5 samples for hybridization, unreacted NHS-ester Cy dye must be quenched to prevent cross coupling.

1. Add 4.5 µl 4 M hydroxylamine.
2. Let reaction incubate for 15 minutes in the dark.
3. To remove unincorporated/quenched Cy dyes, proceed with the use of PCR clean up kit (QIAGEN).
4. Combine Cy3 and Cy5 reactions.
5. Add 70 µl water.
6. Add 500 µl Buffer PB.
7. Apply to QIAquick column and spin at 13 K for 1 minute. (optional: reapply flow-through for optimal binding).
8. Decant flow-through.
9. Add 750 µl Buffer PE and spin 1 minute.
10. Decant flow-through.
11. Repeat washing again (for 3 times).
12. Decant flow-through.
13. Spin at 13 K rpm for 1 minute to dry the column.
14. Transfer spin unit to fresh microfuge tube.
15. Add 50 µl Buffer EB to the center of the membrane. Let sit for 1 minute.
16. Spin and collect eluatant.
17. Dry eluatant in a SpeedVac to concentrate the volume and continue with cDNA

Step-7 Hybridization reaction

To 44.28 µl probe (for 48 pins)

yeast tRNA (2 µg/µl)	6.23 µl
20X SSC	12.60 µl
2% SDS	11.90 µl

Mix well, 90 sec, 100^{o}C; 13.2 K, 2min