Protocol for amino allyl labeling

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RT Reaction:

After TRIzole purification and RNeasy, RNA is re-suspend in H_2O. Take 1 µl for RNA concentration measurement, and make sure the total amount of RNA for labeling is > 15 µg, usually 20 µg. For amplified RNA, use 2 µg amplified RNA per labeling per slide.

Concentrate RNA to 23.2µl with Microcon-30.

	Total RNA	**Amplified RNA**
RNA	23.2µl	22.2 µl
Anchored primer (1µg/µl)	1 µl	-
(or Random Hexamer for amplified RNA, 3µg/µl)	-	2 µl
Total volume	24.2µl	24.2µl

70°C for 5 minutes and cool to 42 °C

RNAse Inhibitor RNAsin	1 µl
5X First Strand Buffer	8 µl
0.1 M DTT	4 µl
50X aa-dNTP mix	0.8 µl
SSII Reverse Transcriptase	2 µl
Total volume	40 µl

42 °C for 30 minutes
Additional 1 µl SSII RT enzyme
42 °C for 30 minutes and cool to R/T

Labeling Reaction:

1. To each labeling reaction, add 5 µl of 500mM EDTA (pH8.0), mix well before adding NaOH
2. Add 10 µl 1 M NaOH, mix well
3. 65 °C for 20 minutes to hydrolyze RNA, then cool to R/T
4. Add 10 µl 1 M HCl to neutralize NaOH
5. Probe purification with Qiagen PCR spin column protocol.
   a. Add 350 µl PB buffer to RT reaction and transfer to QIAquick column.
   b. Spin at maximum speed for 1 minute.
   c. Wash with 750 µl phosphate wash buffer (5 mM KPO4, pH 8.0 + 80% EtOH – use this home-made buffer to avoid Tris).
   d. Spin at maximum speed for 1 minute.
   e. Repeat wash steps c and d.
   f. Empty collection tube and spin at maximum speed for 1 minute.

g. Transfer column to a fresh tube, Elute with 30 µl elution buffer (4 mM KPO4, pH 8.5) with 1 minute incubation followed by spin at maximum speed for 1 minute.
h. Repeat elution step g.
i. Speedvac dry (~40 minutes)

6. Cy Dye coupling
    a. Re-suspend the cDNA in 4.5 µl 0.1 M carbonate buffer, pH 9.0 (freshly made, every two weeks, store at room temperature).
    b. Add 4.5µl NHS-Cy dyes (re-suspended in DMSO. AVOID ANY MOISTURE!!)
    c. Incubate the reactions in dark for 1 hour in room temperature.

Purification

1. Add 35 µl 100 mM NaOAc pH5.2 to the reaction.
2. Add 250 µl PB buffer and follow QIAquick PCR purification protocol.
3. Elute 2X in 30µl H2O.
   At this step, 1 µl of sample can be taken for dye incorporation analysis using NanoDrop Cy3/Cy5 program.
4. Mix the probes of Cy3 and Cy5 in equal amount. Speedvac dry (~40 minutes).

Pre-hybridization:

1. Prepare pre-hybridization buffer (5xSSC, 0.1% SDS and 1% bovine serum albumin, BSA Sigma Cat# A-9418)
2. Place slides to be analyzed into a staining dish, fill with pre-hybridization buffer, and incubate at 42 °C for 45 minutes
3. Wash the slides in a Wheaton slide rack at room temperature with DIH_2O with agitation for 2 min.
4. Dip the slides in room temperature isopropanol and dry by centrifugation at 700 rpm for 5 min. Inspect slides to make sure there is no protein marks on slides. If there is, repeat step 3 and 4.

Slides should be used immediately following pre-hybridization, at least within one hour.

Hybridization:

1. Re-disolve the combined probes in 27 µl 1X hybridization solution (240µl H2O, 250µl 20XSSC, 10µl 10%SDS, 500µl Formamide = 1ml) and combine with COT1-DNA and Poly(A)-DNA to block nonspecific hybridization

Cy5/Cy3 labeled probes	27 µl
Poly dA (8mg/ml)	1.3 µl
Yeast tRNA (4mg/ml)	1.3 µl
CoT-1 DNA (10mg/ml, concentrated)	1.3 µl
Total	30.9 µl

2. Denature at 95 °C for 2 minutes
3. Apply 14µl 5X SSC onto each modified hybridization chamber to prevent drying.

4. Apply probes to the cover slide that is big enough for the printed area (e.g. 60mm cover slip for 22K array). Then put the pre-hybridized microarray slide on the top of the cover slip, invert, and hybridize in a humidified chamber at 42 °C for 16-24 hours.

Wash:

Place slide into a staining dish with Wash #1 at R/T until cover slip falls off.

Wash #1:	at R/T in 0.2% SDS + 1 X SSC until cover slip falls off
Wash #1:	4 minutes at 42 °C in 0.2% SDS + 1 X SSC
Wash #2:	4 minutes at R/T in 0.2% SDS + 0.1 X SSC
Wash #3:	4 minutes at R/T in 0.06 X SSC

Spin immediately (700 rpm for 5 minutes at R/T)

Appendix:

Master mix for labeling reaction (4):

5X first strand buffer	8 µl X 4.5 = 36 µl
0.1 M DTT	4 µl X 4.5 = 18 µl
50X aa-dNTP mix	0.8 µl X 4.5 = 3.6 µl
RNAse inhibitor RNAsin	1 µl X 4.5 = 4.5 µl
Superscript II RTase	2 µl X 4.5 = 9 µl
Total:	15.8 µl/reaction

Calculation for each 1000ml washing buffer:

0.2% SDS:	20 ml 10%SDS in 1000 ml.
1X SSC:	50 ml 20XSSC in 1000 ml.
0.1XSSC:	5 ml 20XSSC in 1000 ml.
0.06XSSC:	3 ml 20XSSC in 1000 ml.

Solutions:

100 mM aa-dUTP (Sigma, cat # A0410)

Dissolve 10 mg in 191 ul 0.1 M KPO4 buffer, pH 7.5. Quantitate this stock solution by diluting an aliquot 1:5000 in the same buffer and measuring A289. Stock concentration in mM = OD_{289nm} x 704.

Make 10 ml 0.1 M KPO_4 buffer, pH7.5

1. Prepare 2 solutions: 1 M KH2PO4 and 1M K2HPO4.
2. Mix:
   0.802 ml 1M K2HPO4
   0.198 ml 1 M KH2PO4
3. Bring to 10 ml with H_2O. The pH of this KPO4 buffer should be at 7.5.

50X aa-dNTP mix

Nucleotide/dye ratio with the ratio of aa-dUTP to dTTP. We use a 2:3 ratio. Prep are a 50X stock:

Start Concentration	Volume	Final Concentration
100 mM dATP	25 µl	25 mM dATP
100 mM dCTP	25 µl	25 mM dCTP
100 mM dGTP	25 µl	25 mM dGTP
100 mM dTTP	15 µl	15 mM dTTP
100 mM dUTP	10 µl	10 mM aa-dUTP

Phosphate wash buffer

1. Prepare 2 solutions: 1 M KH2PO4 and 1M K2HPO4.
2. Make 1 M KPO4 (the pH should be 8.5-8.7) mix:
   9.5 ml 1M K2HPO4
   0.5 ml 1 M KH2PO4
3. Make 100 ml phosphate washing buffer, Mix in the following order:
   15.25 ml H2O
   84.25 ml 95% EtOH
   0.5 ml 1 M KPO4, pH 8.5
   (This solution will be slightly cloudy)

Phosphate elution buffer

Dilute 1 M KPO4 pH 8.5 to 4 mM for Phosphate elution buffer:

1 M KPO4 pH 8.5	0.4 ml
H2O	99.6ml
Total	100ml

Carbonate buffer 0.1 M Na2CO3, pH 9.0:

Na2CO3	0.27g
H2O	20ml
Adjust pH to 9.0 with	6 N HCl
Total with H2O	25 ml

NHS-Cy dyes

NHS-Cy3: AmershamPharmacia Cat# PA23001
NHS-Cy5: AmershamPharmacia Cat#PA25001

1. Resuspend one tube in 73 ul DMSO.
2. Use immediately, or aliquot 4.5 ul into 0.5 ml tubes and store at -80°C. Avoid moistures!!!