TITLE: **HIGH THROUGHPUT AMINOALLYL LABELING OF RNA FOR MICROARRAYS**		PAGE: 1 of 7
SOP #: M018	REVISION LEVEL: 1	EFFECTIVE DATE: 10/07/03
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1. PURPOSE

This protocol describes the labeling of eukaryotic RNA with aminoallyl labeled nucleotides via first strand cDNA synthesis on a large-scale volume (up to 96 samples at a time). This SOP is based off of SOP M004, which is for aminoallyl labeling of RNA for smaller numbers of samples.

2. SCOPE

This procedural format is utilized by Human Colon Cancer and Mouse microarray projects under the supervision of John Quackenbush within the Eukaryotic Genomics Dept.

3. MATERIALS

3.1 Hard-Shell Skirted 96 well Microplate (MJ Research; Cat # HSP-9601)
3.2 RNase free Water (ResGen; Cat # 750023)
3.3 Random Hexamer primers (3mg/mL) (Invitrogen; Cat # 48190-011)
3.4 Arabidopsis Spike-In Control cRNA
3.5 100 mM dNTP Set PCR grade (Invitrogen; Cat # 10297-018)
3.6 5-(3-aminoallyl)-2'deoxyuridine-5'triphosphate 50mM (aadUTP) (Ambion; Cat # 8439)
3.7 SuperScript II RT (200U/µl) (Invitrogen; Cat # 18064-014)
3.8 QIAquick® 96 PCR Purification Kit (Qiagen Cat # 28181)
3.9 Cy-3 ester (AmershamPharmacia; Cat # PA23001)
3.10 Cy-5 ester (AmershamPharmacia; Cat # PA25001)
3.11 BD Falcon U-Bottom Tissue Culture Plates (VWR; Cat # 62406-121)
3.12 Corning 96 well clear round bottom 1mL polypropylene block (Corning Cat # 3958)
3.13 Beckman P20 Sterile tips, (VWR; Cat # 717255)
3.14 Beckman P200 Sterile tips, (VWR; Cat # 717252)

4. REAGENT PREPARATION

4.1 Phosphate Buffers

4.1.1 Prepare 2 solutions: 1M K_2HPO_4 and 1M KH_2PO_4

4.1.2 To make a 1M Phosphate buffer (KPO_4, pH 8.5-8.7) combine:

1M K_2HPO_4……..9.5 mL
1M KH_2PO_4……..0.5 mL

4.1.3 For 250mL Phosphate wash buffer (5 mM KPO_4, pH 5-5.5, 80% EtOH) mix:

1 M KPO_4 pH 8.5…. 1.25 mL
MilliQ water……..38.13 mL
95% ethanol……...210.63 mL

Note: Phosphate wash buffer should be made fresh daily. The wash buffer will appear slightly cloudy.

4.1.4 Phosphate elution buffer is made by diluting 1 M KPO_4, pH 8.5 to 4 mM with MilliQ water.

4.2 Labeling Mix (25X) with 2:3 aa-dUTP: dTTP ratio

4.2.1 Mix the following reagents:

	Final concentration
dATP (100 mM)……..5µL…..	(12.5 mM)
dCTP (100 mM)……..5µL…..	(12.5 mM)
dGTP (100 mM)……..5µL…..	(12.5 mM)
dTTP (100 mM)……...3µL……	(7.5 mM)
Ambion aa-dUTP (50 mM)……..4µL……	(5mM)
RNase-free water……18µL	
Total Volume: 40µL	

4.2.2 Store unused solution at –20°C.

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

4.3.1 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.

4.3.2 To make a 0.1 M solution for the dye coupling reaction dilute 1:10

with water.

Note: Carbonate buffer changes composition over time; make it fresh every couple of weeks to a month.

4.4 Cy-dye esters

4.4.1 Cy3-ester and Cy5-ester are provided as a dried product in 2 tubes. Resuspend a tube of dye ester in 73 μL of DMSO before use.

4.4.2 Wrap all reaction tubes with foil and keep covered as much as possible in order to prevent photobleaching of the dyes.

Note: Dye esters must either be used immediately or aliquotted and stored at –80°C. Any introduced water to the dye esters will result in a lower coupling efficiency due to the hydrolysis of the dye esters. Since DMSO is hygroscopic (absorbs water from the atmosphere) store it well sealed in desiccant.

5. PROCEDURE

5.1 First Strand cDNA Synthesis

5.1.1 In a Hard-Shell Skirted 96 well Microplate add: 10 μg of total RNA (or 2 μg poly(A^+) RNA), 2 μL Random Hexamer primers (3mg/mL), 1.3μL Arabidopsis Spike-In Control RNA and bring the final reaction volume up to 17.9 μL with Rnase-free water. (See example below)

Example:

"X" μL…….10 μg Total RNA (or 2 μg poly(A^+) RNA)
2 μL…… Random Hexamer primers (3μg/μL)
1.3 μL ……. Arabidopsis Spike-In Control RNA (Cy3 or Cy5)
+ (14.6 – X) μL... RNase-free water
17.9 μL Total Volume

Note: Total RNA obtained from tissue or tumor samples may need to be further purified using DNase I treatment and Qiagen RNeasy spin columns.

Option: (If using reference RNA and there is an abundance: pool all reagents and RNA, according to number of samples, in the smaller side of a 25mL reservoir (add the spiking control to the mixture lastly). Dispense 17.9µL of mixture into a 96 well Microplate using a multi-channel pipette, four samples at a time and continue as normal. You will need to make enough master mix for approximately 10 extra samples to accommodate for volume lost in reservoir. Note: When volume of mastermix becomes too low to dispense correct volume, finish dispensing with a single channel pipette.)

5.1.2 Cover plate with a tape pad. Mix well by pipetting and shaking plate multiple times on bench. Centrifuge briefly. Incubate in a thermocycler using a heated lid at 70°C for 10 minutes. If condensation is present on the tape pad, centrifuge briefly after incubation and continue to next step.

5.1.3 Snap-freeze in dry ice/ethanol bath for 30 seconds, centrifuge briefly and continue at room temperature.

5.1.4 Prepare the following master mix for each sample in a tube and transfer to the smaller side of a 25mL reservoir.

5X First Strand buffer……6 µL
0.1 M DTT…….3 µL
50X aminoallyl-dNTP mix……1.2 µL
SuperScript II RT (200U/µL)….2 µL

Note: Add SuperScript II RT to the master mix last, keeping it in the -80°C freezer until use.

Dispense 12.2 µL of mixture into each sample well using a multi-channel pipette, four samples at a time in the same manner that was used in 5.1.1.

5.1.5 Mix by pipetting, cover with a tape pad, centrifuge briefly, and incubate in a thermocycler at 42°C using a heated lid, for 3 hours to overnight.

5.2 Hydrolization and Reaction Purification I: Removal of unincorporated aa-dUTP and free amines.

5.2.1 Preparations for Reaction Purification I using the Biomek FX robot:

5.2.1.1 Dispense 50µl of 1 M NaOH to each well of a 96-well BD Falcon U-Bottom Tissue Culture Plate. Dispense from a 25mL reservoir using a multi-channel pipette.

5.2.1.2 Dispense 50µl of 0.5 M EDTA to each well of a 96-well BD Falcon U-Bottom Tissue Culture Plate. Dispense from a 25mL reservoir using a multi-channel pipette.

5.2.1.2 Dispense 50µl of 1 M HCl to each well of a 96-well BD Falcon U-Bottom Tissue Culture Plate. Dispense from a 25mL reservoir using a multi-channel pipette.

5.2.1.3 Prepare 250mL of Phosphate wash buffer and split into Beckman Coulter upside down tip box lids.

5.2.1.4 Prepare 8mL of Phosphate elution buffer. Dispense 150µl of the elution buffer to each well of a 96-well BD Falcon U-Bottom Tissue Culture Plate. Dispense from a 25mL reservoir using a multi-channel pipette.

5.2.1.5 Dispense 50ml of PM buffer (provided in the QIAquick Purification Kit) into a Beckman Coulter upside down tip box lid.

5.2.1.6 Set up all plates and reagents according to Figure 1.

5.2.2 Hydrolyzation and Purification 1 using the Biomek robot

5.2.2.1 Begin Biomek program “Hydrolyzation and Purification_1”, which is located on the desktop.

5.2.2.2 After EDTA and NaOH have been added to RNA samples, remove plate, centrifuge briefly if necessary, cover with tape pad, and incubate at 65°C for 15 minutes

using thermocycler with a heated lid. After incubation, centrifuge briefly, remove tape pad, and return plate to the same position on the Biomek. Continue with the program.

5.2.2.3 After the HCl has been added to the samples and the samples have been transferred to a falcon plate, stop the robot and manually add any volume of sample that was not added by the Biomek to the falcon plate. Continue with the program.

5.2.2.4 After Biomek has added Buffer PM to each well and transferred samples to a QIAquick plate, place QIAquick plate on vacuum source for approximately 1 minute. After all liquid has been pulled through, switch off the vacuum source, empty flow-through, and continue with Biomek program.

5.2.2.5 After Biomek has added Buffer PE to each well, place plate on vacuum source for approximately 1 minute. After all liquid has been pulled through, switch off the vacuum source, empty flow-through, and continue with Biomek program.

5.2.2.6 Return plate to position on the Biomek and repeat step 5.2.1.12. After Buffer PE in all wells has been drawn through, apply maximum vacuum for an additional 10 minutes to dry the membrane.

5.2.2.7 Switch off vacuum source, ventilate QIAvac 96 slowly. Lift the top plate from the base (not the QIAquick 96 plate from the top plate), vigorously rap the top plate on a stack of absorbent paper until no drops come out, and blot the nozzles of the QIAquick 96 plate with clean absorbent paper. Return QIAquick 96 plate to position on Biomek and continue with Biomek program.

5.2.2.8 After Biomek has applied elution buffer to each sample, let stand for a minute. Replace waste tray underneath QIAquick 96 plate with empty blue collection microtube rack and apply vacuum source for 5 minutes. Switch off

vacuum source and ventilate QIAvac 96 slowly. Centrifuge blue collection microtube rack and continue with Biomek program.

Note: After the Biomek has transferred eluted cDNA to final falcon plate, stop the robot and manually add any cDNA that was not transferred to the falcon plate.

5.2.2.9 Transfer samples from falcon plate into individual eppendorf tubes, spec if desired, and dry samples in a speed vac.

5.2.2.10 Tubes with purified cDNA can be frozen at -80°C for future use or continue with step 5.3 of SOP M004.

Figure 1. Biomek setup for Hydrolization and Purification I