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1. PURPOSE

This protocol describes the labeling of eukaryotic RNA with aminoallyl labeled nucleotides via first strand cDNA synthesis followed by a coupling of the aminoallyl groups to either Cyanine 3 or 5 (Cy 3/Cy5) fluorescent molecules.

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 5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (aa-dUTP) (Ambion; Cat # 8439)
3.2 100 mM dNTP Set PCR grade (Life Technologies; Cat # 10297-018)
3.3 Random Hexamer primers (3mg/mL) (Life Technologies; Cat # 48190-011)
3.4 SuperScript II RT (200U/μL) (Life Technologies; Cat # 18064-014)
3.5 Cy-3 ester (AmershamPharmacia; Cat # PA23001)
3.6 Cy-5 ester (AmershamPharmacia; Cat # PA25001)
3.7 QIAquick PCR Purification Kit (Qiagen; Cat # 28106)
3.8 RNeasy® Mini Kit (Qiagen; Cat # 74106)
3.9 Microcon YM-30 Centrifugal Filter Devices (Millipore; Cat # 42410)

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 100 mL Phosphate wash buffer (5 mM KPO_4, 80% EtOH) mix:

1 M KPO_4 pH 8.5…. 0.5 mL
MilliQ water……...15.25 mL
95% ethanol……..... 84.25 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 Aminoallyl-dNTP 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……	(5 mM)
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 Cyanine dye esters

4.4.1 Cy3-ester and Cy5-ester are provided as a dried product in 5 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

Note: Total RNA obtained from tissue or tumor samples may need to be further purified using DN*ase* I treatment and Qiagen RNeasy spin columns before beginning the cDNA synthesis.

5.1.1 To 10 µg of total RNA (or 2 µg poly(A^+) RNA) add 2 µL Random Hexamer primers (3mg/mL), 1.3 µL Arabidopsis Spike-In Control RNA (Cy3 or Cy5) (optional) 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 (3mg/mL)
1.3 µL ……. Arabidopsis Spike-In Control RNA (Cy3 or Cy5)
+ (14.6 – X) µL... RNase-free water
17.9 µL Total Volume

5.1.2 Mix well with pipette (do not vortex) and incubate at 70°C for 10 minutes.

5.1.3 Snap-freeze in dry ice/ethanol bath for 30 seconds, centrifuge briefly at >10,000 rpm and continue at room temperature.

5.1.4 Add:

5X First Strand buffer…….. 6 µL
0.1 M DTT…….. 3 µL

25X aminoallyl-dNTP mix…….. 1.2 µL
SuperScript II RT (200U/µL)….. 2 µL

5.1.5 Mix with pipette (do not vortex) and incubate at 42°C for 3 hours to overnight.

5.1.6 To hydrolyze RNA, add:

1 M NaOH 10 µL
0.5 M EDTA 10 µL

mix and incubate at 65°C for 15 minutes.

5.1.7 Add 10 µL of 1 M HCl to neutralize pH. (Alternatively, one can add 25 µL 1 M HEPES pH 7.0 or 25 µL 1 M Tris pH 7.4)

5.2 Reaction Purification I: Removal of unincorporated aa-dUTP and free amines (use either the Qiagen ***or*** the Microcon method)

Qiagen Cleanup Method:

Note: This purification protocol is modified from the Qiagen QIAquick PCR purification kit protocol. The phosphate wash and elution buffers (prepared in 4.1.3 & 4.1.4) are substituted for the Qiagen supplied buffers because the Qiagen buffers contain free amines which compete with the Cy dye coupling reaction.

5.2.1 Mix cDNA reaction with 300 µL (5X reaction volume) buffer PB (Qiagen supplied) and transfer to QIAquick column.

5.2.2 Place the column in a 2 ml collection tube (Qiagen supplied) and centrifuge at ~13,000 rpm for 1 minute. Empty collection tube.

5.2.3 To wash, add 750 µL phosphate wash buffer to the column and centrifuge at ~13,000 rpm for 1 minute.

Note: Phosphate wash buffer should be made fresh daily.

5.2.4 Empty the collection tube and repeat the wash and centrifugation step (5.2.3).

5.2.5 Empty the collection tube and centrifuge column an additional 1 minute at maximum speed.

5.2.6 Transfer column to a new 1.5 mL microfuge tube and carefully add 30 µL phosphate elution buffer (*see 4.1.4*) to the center of the column membrane.

5.2.7 Incubate for 1 minute at room temperature.

5.2.8 Elute by centrifugation at ~13,000 rpm for 1 minute.

5.2.9 Elute a second time into the same tube by repeating steps 5.2.6-5.2.8. The final elution volume should be ~60 µL.

Note: If desired the cDNA yield can be measured via spectrophotometry (1 OD_{260} = 37 ng/µL for cDNA).

5.2.10 Dry sample in a speed vac.

or~ Microcon YM-30 Cleanup Method:

5.2.1 Place the Microcon sample reservoir into a collection microfuge tube. Add 375 µL of water to the cDNA reaction tube and then pipette the sample into the Microcon sample reservoir/collection microfuge tube without touching the membrane.

5.2.2 Centrifuge at 12,000 rpm for 4-6 min.

Note: Never centrifuge column to dryness as this will decrease product recovery. Adjust spin time to allow for optimal filtration while allowing enough solution to remain for sufficient recovery.

5.2.3 Empty collection microfuge tube.

5.2.4 To wash add 450 µL of water to the sample reservoir/collection microfuge tube and centrifuge at 12,000 rpm for 4-6 minutes.

5.2.5 Empty collection microfuge tube and repeat previous wash step.

5.2.6 Invert Microcon sample reservoir into a new collection microfuge tube and centrifuge at 12,000 rpm for 1 minute to collect purified sample.

Note: If desired the cDNA yield can be measured via spectrophotometry (1 OD_{260} = 37 ng/μL for cDNA).

5.2.7 Dry the sample in a speed vac.

5.3 Coupling Cyanine Dye Ester to aa-cDNA.

5.3.1 Resuspend aminoallyl-labeled cDNA in 4.5 μL 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0.

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

5.3.2 Add 4.5 μL of the appropriate NHS-ester Cy dye (prepared in DMSO: *see 4.5*)

Note: To prevent photobleaching of the Cy dyes wrap all reaction tubes in foil and keep them sequestered from light as much as possible.

5.3.3 Incubate the reaction for 1 hour in the dark at room temperature.

5.4 Reaction Purification II: Removal of uncoupled dye (Qiagen PCR Purification Kit)

5.4.1 To the reaction add 35 μL 100 mM NaOAc pH 5.2.

5.4.2 Add 250 μL (5X reaction volume) Buffer PB (Qiagen supplied).

5.4.3 Place a QIAquick spin column in a 2 mL collection tube (Qiagen supplied), apply the sample to the column, and centrifuge at ~13,000 rpm for 1 minute. Empty collection tube.

5.4.4 To wash, add 0.75 mL Buffer PE (Qiagen supplied) to the column and centrifuge at ~13,000 rpm for 1 minute.

Note: Make sure Buffer PE has added ethanol before using (see label for correct volume).

5.4.5 Empty collection tube and centrifuge column for an additional 1 minute at maximum speed.

5.4.6 Place column in a clean 1.5 mL microfuge tube and carefully add 30 µL Buffer EB (Qiagen supplied) to the center of the column membrane.

5.4.7 Incubate for 1 minute at room temperature.

5.4.8 Elute by centrifugation at ~13,000 rpm for 1 minute.

5.4.9 Elute a second time into the same tube by repeating steps 5.4.6-5.4.8. The final elution volume should be ~60 µL.

Note: This protocol is modified from the Qiagen QIAquick Spin Handbook (04/2000, pg. 18).

5.5 Analysis of Labeling Reaction

5.5.1 Use a 50 µL Beckman quartz MicroCuvette to analyze the entire undiluted sample in a spectrophotometer.

5.5.2 Wash the cuvette with water and blow dry with compressed air duster.

5.5.3 Pipette sample into cuvette and place cuvette in spectrophotometer.

5.5.4 For each sample measure absorbance at 260 nm and either 550 nm for Cy3 or 650 nm for Cy5, as appropriate.

5.5.5 Pipette sample from cuvette back into the original sample tube.

5.5.6 For each sample calculate the total picomoles of cDNA synthesized using:

$$\text{pmol nucleotides} = \frac{[OD_{260} * \text{volume } (\mu L) * 37 \text{ ng}/\mu L * 1000 \text{ pg/ng}]}{324.5 \text{ pg/pmol}}$$

Note: 1 OD_{260} = 37 ng/µL for cDNA; 324.5 pg/pmol average molecular weight of a dNTP)

5.5.7 For each sample calculate the total picomoles of dye incorporation (Cy3 or Cy5 accordingly) using:

$$\text{pmol Cy3} = \frac{OD_{550} * \text{volume (µL)}}{0.15}$$

$$\text{pmol Cy5} = \frac{OD_{650} * \text{volume (µL)}}{0.25}$$

$$\text{nucleotides/dye ratio} = \frac{\text{pmol cDNA}}{\text{pmol Cy dye}}$$

Note: >200 pmol of dye incorporation per sample and a ratio of less than 50 nucleotides/dye molecules is optimal for hybridizations (see Microarray Cookbook II)

5.5.8 After analysis mix together the two differentially labeled probes (Cy3 vs. Cy5) which will be hybridized to the same microarray slide.

5.5.9 Dry the Cy3/Cy5 probe mixture to completion in a speed vac and continue with SOP: M005 for the hybridization of the probe to a microarray slide.