This protocol is from The Microarray Centre at the Ontario Cancer Institute (www.uhnres.utoronto.ca/services/microarray)

Generating Labeled cDNA with Amino Allyl dUTP and Monofunctional Reactive Cyanine Dyes

This is a two-step method used to generate labeled cDNA from as little as 2 µg total RNA. In the first step amino allyl dUTP (AA-dUTP), an amine-modified nucleotide, is incorporated during reverse transcription. Subsequently, monofunctional forms of Cyanine 3 and Cyanine 5 dyes are reacted with the amine-modified cDNA

Reverse Transcription

1) Combine the following on ice:

   8.0 µl 5X First Strand Buffer (Superscript II, Life Technologies)
   1.5 µl AncT mRNA primer (5'-T_{20} VN, 100 pmol/µl)
   3.0 µl 20 mM dNTP-dTTP (6.7 mM each of dATP, dCTP, dGTP)
   3.0 µl 2 mM d TTP
   3.0 µl 2 mM AA-dUTP (Sigma)
   4.0 µl 0.1 M DTT
   1.0 ng Control RNA (we use yeast ORF transcripts)
   0.1-10 µg RNA (mRNA or total RNA)
   to 40 µl dH_2O (Sigma)

2) Incubate the labeling reaction at 65 °C for 5 minutes and then at 42 °C for 5 minutes. It is not necessary for incubation to occur in the dark.
3) Add 2 µl reverse transcriptase (Superscript II, Life Technologies) and incubate at 42°C for 2 hours.
4) To inactivate the enzyme, heat reactions at 95 °C for 5 minutes and then place on ice.
5) Add 8 µl 1 M NaOH and heat at 65 °C for 15 minutes to hydrolyze remaining RNA.
6) Add 8 µl 1 M HCl and 4 µl 1 M Tris-Cl, pH 7.5 to neutralize the solution.

Probe Purification and Precipitation

At this point reactions are purified using the Qiagen PCR Purification Kit. All traces of Tris must be removed to prevent reaction of the amine groups on Tris with the monofunctional NHS-ester of the Cyanine dye.

1) Add 38 µl dH_2O to bring each reaction volume to 100 µl.
2) Add 500 µl PB buffer to each 100 µl reaction and mix.
3) Apply solution to the column included with the kit and spin at top speed for 30 seconds. Discard flow-through.
4) Wash with 750 µl 75 % EtOH and spin at top speed for 30 seconds. Discard flow-through and repeat this wash step once.
5) Spin the column for one additional minute to ensure the membrane is dry.
6) Use 50 µl dH_2O to elute the cDNA. Sit for 5 minutes before spinning. Repeat this once.
7) Add 10 µl 3 M sodium acetate.
8) Add 1 µl glycogen (20 µg/µl, Life Technologies.
9) Add one volume 95 % EtOH (alternatively, use isopropanol) and precipitate at -20 °C for 30 minutes.
10) Spin at top speed for 5 minutes.
11) Wash the pellet with 80 % EtOH. Make sure that all ethanol is removed, however do not allow the pellet to dry completely.

Labeling Reaction with Monofunctional Reactive Cyanine Dye

1) Resuspend cDNA in 5 µl dH_2O.
2) Add 3 µl 0.3 M sodium bicarbonate, pH 9.0.
3) Dissolve one aliquot of dye in 2 µl 100 % DMSO. Mix by pipetting up and down. Please see notes on aliquoting dye at the end of this protocol.
4) Add 2 µl dye to the reaction and incubate in the dark at room temperature for 40 minutes to 1 hour.

Purification of Fluorescently Labeled Probe Using Qiagen PCR Purification Kit

1) Add 90 µl dH_2O to bring each reaction volume to 100 µl.
2) Follow the protocol as written above but perform three washes with 75 % EtOH and three elutions with buffer EB provided in the kit.
3) Add 15 µl 3 M sodium acetate. .

1) Add 1.5 µl glycon.
2) Add one volume 95 % EtOH (alternatively, use isopropanol) and precipitate at -20 °C for 30 minutes.
3) Spin at top speed for 5 minutes.
4) Wash the pellet with 70 % EtOH. Make sure that all ethanol is removed, however do not allow the pellet to dry completely.
5) Resuspend the pellet in 2.5 µl dH_2O. Prepare the hybridization solution as usual.

Aliquoting Cyanine Dyes

Cyanine 3 and Cyanine 5 monofunctional reactive dyes can be purchased from Amersham (cat. #PA 23001 and #PA 25001). Each pack contains 5 vials of dye. Dissolve one vial of dye in 72 µl dH 2 O. Aliquot 4.5 µl to each of 16 tubes. Dry dye in the speed vac and store in the dark at 2-8 °C.

NOTE: If you wish to stop the protocol at any point and resume the next day, try to do so after either of the probe purification steps. Simply freeze the eluate from the Qiagen columns at -20 °C.