**Amino-allyl Reverse Transcription**

**RNA ReverseTranscriptase Labeling with Aminoallyl – dUTP**

**REAGENTS**

**FluoroLink Cy3 and Cy5 monofunctional dyes**

(Amersham catalog # PA23001, and PA25001)  NHS-ester = 1[episil-carboxy-pentyl]1’ethyl 3, 3, 3’,3’ Bring up in 14 ul of High quality DMSO and use 1 ul

**OR**

Re-suspend one dye pack in 14 ul DMSO, dry 1 ul aliquots and store at –80C in foil (dark)

**Reverse Transcriptase** SuperScript II RNase H- Reverse Transcriptase (Gibco BRL #18064-014)

**aa\_dUTP**  5-(3-aminoallyl)-2’-deoxyuridine 5’-triphosphate (AA-dUTP) (Sigma A0410)  1 mg = 1.91 10-6 Mole   bring up in 19.1 ul (19 \*10-6 l) = 100mM.  Store stock at –80C.

**50X aa dUTP/dNTPs**:  10 ul dATP (100 mM)  10 ul dGTP (100mM) 10 ul dCTP (100mM)   6 ul dTTP (100mM)

4 ul of aa dUTP (100 mM)

**dTV** **(25-mer) primer** 50 pmol/ul

5’ - TTT TTT TTT TTT TTT TTT TTT TTT V – 3’

V = A, C, or G

**Sodium bicarbonate** 0.33 M pH 9.2 carbonate buffer= 1.36 g sodium carbonate 7.35 g sodium bicarbonate 950 ml H2O,  adjust pH to 9.2 with 1M HCl or 1 M NaOH if necessary H2O to 1 liter

**OR**

0.2 M anhydrous sodium carbonate ( 21.2 g in 1 liter)

0.1 M solution of sodium bicarbonate (16.8 g in 1 liter)

For pH 9.3 mix 7.5 ml of anhydrous with 42.5 ml of bicarb and dilute to a total of 200 ml.

**PROTOCOL**

**I.Reverse Transcriptase Reaction**

Oligo dTV primer (50pmol/ul) 2 ul

10 ug total RNA 13.5 ul

**Total 15.5 ul**

Before adding RNA: ethanol precipitate and bring up pellet in enough water than there will be 10ug in 13.5 ul.  Spec  on the Nanodrop.

If you have 10ug in smaller volume, add depc-H2O to 15.5ul.  If have 10ug in larger volume, subtract corresponding amount of depc-H2O from reverse transcriptase reaction.

Incubate at 92°C for 1 min.

Chill on ice.

**cDNA synthesis**

|  |  |  |
| --- | --- | --- |
| **Component** | **ul** | **For 10 rxns (by 10.5)** |
| 5X first strand buffer (from SSII RT) | 6 | 63 |
| 50X aa dUTP/dNTPs | 0.6 | 6.3 |
| DTT (0.1 M) | 3 | 31.5 |
| SuperScript II ReverseTranscriptase | 1 | 10.5 |
| DEPC water | 3.9 | 41 |
| **Total** | **14.5** | **14.5 X 10.5=152** |

**\***Note, DTT is an anti-oxidant that keeps an enzyme at max. activity

\*Note, most reagents are in the ligase/buffer box at –20 C.  SSII RT is in the polymerase box.

Incubate at 42  C for 2 hours or longer (o/n is ok) – use minicycler

Heat to 94°C for 2min

Quick cool on ice

Add 1 ul of RNase mix (Ambion) – stored in nuclease box

Incubate at 37°C for 15 minutes

**II. Hydrolysis**

Add: 1N NaOH 10 ul

0 .5M EDTA 10 ul

Incubate at 65C for 15 min

Neutralize: 1M Tris pH 7.4 25 ul

**III. Cleanup of cDNA**

To continue with the amino-allyl dye coupling procedure **all Tris must be removed** from the reaction to prevent the monofunctional NHS-ester Cye dyes from coupling to free amine groups in solution.

Increase volume to 200ul by adding 140 ul 1mM EDTA

Ethanol precipitate (1/10 vol 3 M NaOAC, 2.5 vol EtOH) – store in –20C for 20 min, spin at 4C for 30 min

Bring up in 50 ul dH2O

Run through a G-50 column (Amersham #37-5330-01) to remove unincorporated nucleotides.  You may prefer to make your own G-50 column.  See below for instructions.

Spec on the Beckman.  Should have 10-20% of starting yield.

Ethanol precipitate.  Can leave overnight in –20C.

 **Remember** **THE POINT of the Cleanup:** to remove all free aa-UTPs and all Tris buffer.

**IV. Coupling**

Bring dry cDNA up in 7 ul dH2O. If doing a loop design, bring cDNA up in 20ul.  Each RNA sample should be labeled with both Cy3 and Cy5.  Divide sample into Cy3 and Cy5 tubes.Heat to 95º C for 2 min, quickly chill on wet ice Add 3 ul sodium bicarbonate buffer (0.33 M pH 9.3). Reconstitute Cy dye in 1 ul DMSO and add to cDNA.

Incubate at RT for 1 hour in dark (can wrap tube rack in foil or put rack in dark drawer).

**V. Quenching and Cleanup**

Add 25ul 1 M Tris-HCl pH 7.5. Incubate at RT for 15 min in dark.

Add 10ug carrier DNA (10ug/ul sheared herring sperm DNA).  To shear DNA, vortex and run through tiny syringe multple times. Add 165 ul H2O to increase volume to 200ul

EtOH precipitate.  Bring up in 50ul H2O. Run through G50 or GeneClean columns two times or use PCR cleanup kit – to remove unincorporated Cy dyes.  Spec DNA on Beckman using Cy dye setting

Ethanol precipitate.  You can leave overnight at –20C.

Pellet will be resuspended in hybridization buffer.  See microarray hybridization protocol for this.

**Making your own G-50 columns**

Add 10g Sephadex (G-50) into approx. 250 ml DEPC water

Autoclave

Aliquot into 50 ml Falcon tubes

G50 should form sludge at bottom of tube.  Make sure that there is enough water to saturate and form an H20 layer above G-50.

Add 1.5 ml of G50 to spin column (Bio-Rad #732-6008)

Spin at max speed (3000 rpm) for 2 min.  Transfer column to new collection tube.  Save eluate to use as blank for Beckman Spec.

Add 50 ul of sample Carefully to middle of column

Spin at max speed for 4 min

Blank eluate on Beckman Spec before measuring OD of sample.