ACGT MICROARRAY FACILITY STANDARD OPERATING PROCEDURE		
TITLE: cDNA Synthesis and labelling		**PAGE: 1 of 4**
SOP#: MA008	**REVISION LEVEL: .3**	**DATE:25 June 2009**
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1. PURPOSE

This procedure describes an indirect method for labelling total RNA.

2. SCOPE

This method may be applied when probes are required for microarray hybridisations to cDNA or oligonucleotide arrays and RNA is the starting material. Random nonamers are used in this protocol so it is important the starting material is free of genomic DNA contamination.

3. MATERIALS

Oligo dT primer (Invitrogen; cat#18418-012)

Superscript III RNase H- reverse transcriptase (Invitrogen cat# 18080-044)

RNeasy® MinElute™ Cleanup kit (Qiagen Cat#74204)

Amersham CyDye Post-Labeling Reactive Dye Packs (GE Healthcare cat# RPN5661)

5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (aa-dUTP) (Ambion cat#8439)

100mM dNTP set PCR grade (Sigma cat#DNTP-100A)

Random nonamer primer 5ug/µl concentration (Inqaba Biotec)

Prepare a 20x aadNTP mix as follows:

10mM dATP, dCTP, dGTP, 4mM dTTP, 6mM aa-dUTP)

Sodium bicarbonate buffer (200mM Na_2CO_3, 200mM $NaHCO_3$) pH9.0

Dissolve 1.06g of Na_2CO_3 and 0.84g $NaHCO_3$ in 45mL of RNAse free water and adjust pH to 9.0 with 12N HCl. Adjust volume to 50mL with RNase-free water and aliquot 0.5ml into 1.5ml tubes and store at –20^{o}C for single use.

4. PROCEDURE

- Prepare 2x 1.5ml tubes, one labelled control and the other labelled experiment.

- To each tube add the following:

RNAse Inhibitor	0.5µl
Total RNA	15ug in a total volume of 22µl
Oligo dT primer (0.5ug/µl)	2.0µl
Random nonamer primer (0.5ug/µl)	1.0µl (Check the concentration with the nanodrop)

- Mix and incubate at 70^{o}C for 5min.
- Place the tube at room temperature for 10min.
- Add 15 µl of cocktail A

  Cocktail A contains:

20xaa-dNTP	2µl
5x 1st strand buffer	8µl
0.1M DTT	4µl
Reverse Transcriptase	1µl

- Mix gently by pipetting and incubate at 42^{o}C for 3-4 hours
- Add another 1µl of Reverse Transcriptase enzyme and mix gently with pipette.
- Leave overnight at 42^{o}C.

- Hydrolyse the RNA by adding 20µl of mix B.

  Mix B contains the following:

  10µl 1M NaOH

  10µl 0.5M EDTA

- Mix and incubate at 65^{o}C for 15min.
- Add 25µl 1M HEPES (pH 7.0) to neutralise the pH.

Use the RNeasy® MinElute™ Cleanup kit to purify the cDNA.

- NB you will perform two clean-up reactions separately for experiment and one control sample.
- Adjust the sample to 100µl with RNase-free water (15µl).
- Add 350µl Buffer RLT (NB: DO **NOT** ADD ß-mercaptoethanol) and mix thoroughly. Add 250µl of 96-100% ethanol and mix thoroughly by pipetting.
- Apply 700µl of the sample onto the RNeasy® MinElute™ Column in a 2ml collection tube. Remember to label the spin columns.
- Close the tube gently and centrifuge for 15sec at 12000rpm in a mini-spin. Discard the collection tube.
- Transfer the spin column to a new collection tube and pipette 500µl Buffer RPE onto the spin column.
- Centrifuge for 15sec at 12000rpm in a mini-spin. Discard the flow-through.
- Add 500µl 80% EtOH to the spin column and centrifuge for 2min at 12000rpm in a mini-spin. Discard the collection tube.
- Transfer the spin column to a new collection tube and cut off the caps. Remember to label the tubes on the side of the column.
- Centrifuge the tubes for 5min at 13.4 x1000 rpm.
- Discard the collection tube and place the spin column into a new tube.
- Add 14µl of RNase-free water and allow tube to sit for 1min.
- Centrifuge at 13.4 x 1000rpm for 1min.
- Add another 14µl of RNAse free water and allow the tube to sit for 1min.
- Centrifuge at 13.4 x 1000rpm for 1min.

Check cDNA concentration using the nanodrop.

- Place each sample into new 2ml tubes.
- Completely dry samples in the SpeediVac set at 50^{o}C (check after 5min).
- Re-dissolve the pellet in 5µl of $NaHCO_3$ buffer (pH 9) by flicking the tube several times and placing the tube at 37^{o}C for 10min.
- Add 5µl of cy3 or cy5 dye to each tube and mix by flicking the tube.
- Spin the tube at 1000xg for 30sec.

- Incubate the dye and DNA mix in the tube at room temperature for 2hrs covered in foil. Gently mix by lightly flicking the tube every 15min.
- Add 35µl NaOAc, pH 5.2.

Use the RNeasy® MinElute™ Cleanup kit to purify the probes.

- NB you will perform two clean-up reactions – one for labelled control and one for labelled experiment.
- Adjust the sample to 100µl with RNase-free water (15µl).
- Add 350µl Buffer RLT (NB: DO **NOT** ADD ß-mercaptoethanol) and mix thoroughly.
- Add 250µl of 96-100% ethanol and mix thoroughly by pipetting.
- Apply 700µl of the sample onto the RNeasy® MinElute™ Column in a 2ml collection tube. Remember to label the spin columns.
- Close the tube gently and centrifuge for 15sec at 12000rpm in a mini-spin. Discard the collection tube.
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- Centrifuge the tubes for 5min at 13.4 x1000 rpm.
- Discard the collection tube and place the spin column into a new tube.
- Add 14µl of RNase-free water and allow tube to sit for 1min.
- Centrifuge at 13.4 x 1000rpm for 1min.
- Add another 14µl of RNAse free water and allow the tube to sit for 1min.
- Centrifuge at 13.4 x 1000rpm for 1min.

Check incorporation using the nanodrop or spectrophotometer.