<table>
<tr><td colspan="3">ACGT MICROARRAY FACILITY
STANDARD OPERATING PROCEDURE</td></tr>
<tr><td colspan="2">TITLE: aRNA amplification</td><td>PAGE: 1 of 4</td></tr>
<tr><td>SOP#: MA012</td><td>REVISION LEVEL: .1</td><td>DATE: 28 July 2006</td></tr>
<tr><td>AUTHOR: S. Naidoo</td><td colspan="2">REVIEWERS: Luke Solomon, Erica Pierce</td></tr>
</table>

1. PURPOSE

This procedure describes the amplification and labelling of RNA. This procedure may be required for microarray experiments with limited amounts of starting material. This method is based on the Eberwine method (van Gelder et al., 1990). A major advantage of this method, compared to others is that amplification is linear which helps maintain the relationships between the abundances of different transcripts.

2. SCOPE

This method can be a used for most organisms. The protocol described has been used extensively in Arabidopsis and other plants. For other organisms with a high GC or TA content, the ratio of aadUTP: dTTP will have to be optimised for efficient dye coupling.

3. MATERIALS

Aminoallyl message Amp II kit (Ambion cat# 1753)
RNAse free tips, tubes
Refrigerated microcentrifuge
DEPC treated water
Thermal cycler or incubator set at 42°C
100% Ethanol

4. PREPARATION

cDNA wash buffer
Add 11.2ml 100% ethanol (ACS grade) to the cDNA Wash Buffer.
Mix well and mark the bottle (ethanol added)
aRNA wash buffer

Add 22.4ml ACS grade 100% Ethanol to the bottle labelled RNA Wash buffer. Mix well and mark the bottle (ethanol added)

5. **PROCEDURE**

First strand cDNA synthesis

Place up to 5ug of total RNA (typically 100- 1000ng) or 0.1ug of poly(A) RNA (typically 10- 100ng is sufficient) into a sterile RNAse free microfuge tube.
Add 1ul of T7 Oligo(dT) Primer
Add Nuclease free water to a final volume of 12ul.
Incubate 5min at 70°C in a thermal cycler
Remove the RNA samples from the 70°C incubator and centrifuge briefly (~5sec) to collect the sample.

Immediately transfer sample to ice.
Assemble the Reverse Transcription Master Mix at room temperature, then place on ice.

Reverse Transcription Master Mix (Per Reaction)
2ul 10X First Strand Buffer
1ul RNAse Inhibitor
4ul dNTP mix
1ul Reverse Transcriptase

Mix well by gently pipetting up and down or flicking the tube a few times.
Centrifuge the samples briefly to collect the sample at the bottom of the tube and place on ice.

Transfer 8ul of Reverse Transcription Master Mix to each RNA sample and mix by pipetting.
Incubate at 42°C in an incubator or PCR machine for 2hrs.
Centrifuge the tubes briefly to collect the contents at the bottom of the tubs and place on ice.

Second strand cDNA synthesis

Per reaction, add the following components to the 20ul 1st strand cDNA:

63ul Nuclease- free water
10ul 10x Second strand buffer
4ul dNTP mix
2ul DNA Polymerase
1ul RNase H

Gently mix by pipetting up and down or by flicking the tube a few times.
Centrifuge the samples briefly.
Incubate in a thermal cycler or a refrigerated water bath at 16°C for 2hrs.

cDNA clean up

Use the cDNA purification kit supplied with the message amp- II kit or you could also use the Qiaquick clean up kit to clean up the cDNA.

Check the cDNA concentration of the cleaned cDNA using the nanodrop spectrophotometer.
The yield should be 10- 15ng/ul from 5ug total RNA.

In vitro transcription for aRNA synthesis

Prepare the following reaction mix:

16ul	double stranded cDNA
3ul	aadUTP Solution (50mM)
12ul	ATP, CTP, GTP Mix (25mM)
1ul	UTP Solution (50mM)
4ul	T7 10X Reaction Buffer
4ul	T7 Enzyme Mix

Mix well with a pipette and centrifuge at 3000 x g for 30sec.

Incubate the tube at 37°C in a PCR machine for 4 to 14hrs (max).

Stop the reaction by adding 60ul nuclease free water to each aRNA sample to bring the final volume to 100ul.

Mix thoroughly by gentle vortexing, and either proceed to the aRNA purification or store at –20°C.

Purification of aRNA

Purify the aRNA using the aRNA purification cartridge supplied with the message AmpII Kit.

Determine the concentration of RNA using the nanodrop.

Aliquot 1-4ug of aRNA and completely dry it using a SpeedVac set at 45°C.

Store the aRNA at –80°C for further use or continue directly with dye coupling.

Indirect Dye Coupling (See MA008 for details on $NaHCO_3$ buffer and dye preparation)

Re-dissolve the pellet in 5ul of $NaHCO_3$ buffer (pH 9) by flicking the tube several times and placing the tube at 37 °C for 10min.

Add 5ul 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 35ul NaOAc, pH 5.2.

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

Determine the yield using the nanodrop.