

# mRNA AMPLIFICATION & HYBRIDIZATION (PLANT)

## SGED SOP 9.1.1

Note: This protocol is modified from a protocol established by Jack Gardiner from the University of Arizona for use with maize oligo arrays ([www.maizearray.org](http://www.maizearray.org)).

\*cRNA refers to copy RNA.

### Title: RNA Amplification - Setting Up for First Strand cDNA Synthesis

#### Materials required.

Aminoallyl Message Amp II kit (Ambion Cat# 1751)  
5-(3-aminoallyl)-UTP (Ambion Cat# 8437)  
DNA Clear Kit for extra cDNA purifications (Ambion Cat # 1756)  
MEGA Clear Kit for extra acRNA purifications (Ambion Cat # 1908)  
RNase free tips, tubes  
Refrigerated Microcentrifuge  
DEPC treated H<sub>2</sub>O or Nuclease free H<sub>2</sub>O  
Thermal cycler or incubators set at 42C  
100% EtOH

\*Prepare components of Ambion amplification kit as directed (add 100% EtOH, etc.)

#### First Strand cDNA Synthesis:

1. Place ~ 2.0 µg of total RNA into a sterile RNase-free 0.2 ml microfuge tube.
2. Add 1 µL of T7 Oligo (dT) Primer.
3. Add Nuclease-free Water to a final volume of **6 µL**.
4. Incubate 10 min at 70°C.
5. Remove the RNA samples from the 70°C incubator and centrifuge briefly (~5 sec) to collect sample at bottom of tube and immediately transfer to ice.

Assemble the Reverse Transcription Master Mix at room temperature, then place on ice.  
(To reduce pipetting, prepare enough Reverse Transcription Master Mix to synthesize first strand cDNA for all RNA samples in the experiment. It is prudent to include 5% overage to cover pipetting errors. The following recipe is for a single reaction)

<u>Amount</u>	<u>Component</u>
1 µL	10X First Strand Buffer
0.5 µL	RNase Inhibitor
2 µL	dNTP Mix
0.5 µL	<u>Array Script (Reverse Transcriptase)</u>
<u>4uL Mix</u> + <u>6uL</u> RNA/dT primer mix	
<b>10uL total</b>	

6. It is a good idea to make a master mix for several reactions, mix well by gently pipetting up and down or flicking the tube a few times. Centrifuge briefly (~5 sec) to collect the master mix at the bottom of tube and place on ice.

7. Transfer 4  $\mu\text{L}$  of *Reverse Transcription Master Mix* to each RNA sample, mix thoroughly by gently pipetting up and down or flicking the tube a few times and place the tubes in a 42°C incubator. I generally use PCR machine with lid temperature set for 48 C. If using a waterbath or heating block, quick spin the samples periodically to account for condensation.

After the 2 h incubation at 42°C, centrifuge the tubes briefly (~5 sec) to collect the reaction at the bottom of the tube. Place the tubes on ice and proceed to the second strand cDNA synthesis (below).

## **Title: RNA Amplification - Second Strand cDNA Synthesis, and Setting Up for cRNA Synthesis.**

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### **Materials**

Aminoallyl Message Amp II kit (Ambion Cat# 1751)  
5-(3-aminoallyl-UTP) (Ambion Cat# 8437)  
RNase free tips, tubes  
Refrigerated Microfuge centrifuge  
DEPC treated H<sub>2</sub>O, or Nuclease free water  
Thermal cycler or incubators set at 16C and 37C

### **Second Strand cDNA Synthesis:**

1. On ice, add the second strand cDNA synthesis reagents in the order listed to each sample from step 4 above. When processing more than one sample, it is a good idea to make a master mix of second strand cDNA synthesis reagents to avoid variability; include ~5% overage to cover pipetting error.

The following recipe is for a single reaction:

<u>Amount</u>	<u>Component</u>
10 $\mu\text{L}$	cDNA sample (from step above)
31.5 $\mu\text{L}$	Nuclease-free Water
5 $\mu\text{L}$	10X Second Strand Buffer
2 $\mu\text{L}$	dNTP Mix
1 $\mu\text{L}$	DNA Polymerase
<u>0.5 <math>\mu\text{L}</math></u>	<u>RNase H</u>

40ul Mix + 10ul RNA mix from above

**50ul total**

2. Since the reagent volumes are very small it is recommended to make a master mix for several reactions, and pipette 40 ul of second strand reaction mix to each first strand reaction tube. Gently mix by pipetting up and down or by flicking the tube a few times, then centrifuge the tubes briefly (~5 sec) to collect the reaction at the bottom of tube.
3. Incubate in a thermal cycler or in a refrigerated water bath (a heat block in a 4°C refrigerator is **not** recommended because the temperature will fluctuate too much).

After the 2 h incubation at 16°C, proceed to cDNA Purification (below), or **immediately** freeze reactions at -20°C. **Do not** leave the reactions on ice for long periods of time!!!

## **cDNA Purification**

Use the cDNA purification kit supplied with the messageamp-II kit or you can also use other PCR purification kits like Qiaquick in case of individually assembled kit.

Before beginning the cDNA purification, preheat the 10 mL bottle of Nuclease-free Water to 50°C for at least 10 min.

1. Check that the cDNA filter cartridge is firmly seated in a 2 mL wash tube and pipet 50  $\mu$ L cDNA binding buffer onto the filter in the cDNA filter cartridge.
2. Incubate at room temperature for 5 min. (DO NOT spin the cDNA binding buffer through the cDNA filter cartridge).
3. Add 250  $\mu$ L of cDNA binding buffer to each cDNA sample from the second strand cDNA synthesis and mix thoroughly by repeated pipetting.
4. Pipet the cDNA sample/cDNA Binding Buffer onto the center of an equilibrated cDNA Filter Cartridge.
5. Centrifuge for ~1 min at 10,000 x g, or until the mixture has passed through the filter.
6. Discard the flow-through and replace the cDNA filter cartridge in the 2 mL wash tube. Make sure that the ethanol has been added to the bottle of cDNA Wash Buffer before using it.
7. Apply 500  $\mu$ L cDNA wash buffer to each cDNA filter cartridge. Centrifuge for ~1 min at 10,000 x g, or until all the cDNA wash buffer is through the filter.
8. Discard the flow-through and spin the cDNA filter cartridge for an additional minute to remove trace amounts of ethanol.
9. Transfer cDNA Filter Cartridge to a cDNA Elution Tube. To the center of the filter in the cDNA Filter Cartridge, apply 6  $\mu$ L of nuclease free water that is preheated to 50°C. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until all the nuclease-free water is through the filter.
10. Repeat the previous step with additional 6  $\mu$ L of pre-heated nuclease-free water. The double-stranded cDNA will now be in the eluate (~11  $\mu$ L).
11. Discard the cDNA Filter Cartridge.

Check the cDNA concentration in the solution by applying 1.5  $\mu$ L of eluted cDNA on to the Nanodrop spectrophotometer. In general the cDNA yield should be around 8-10 ng /  $\mu$ L if you start with 1-2  $\mu$ g of total RNA. I have used yields as low as 3ng/ $\mu$ L and had success (depends on the source of RNA). The purified cDNA can be stored overnight at -20C if necessary.

## **In Vitro Transcription for cRNA synthesis**

The oligo microarrays, being printed with positive-strand DNA elements, require labeled negative-strand targets for hybridization. (Not an issue with cDNA arrays.) Since the first round of amplified cRNAs represents the negative-strand, it is recommended to label the cRNA itself. cRNA labeling can be done using two methods: (a). direct incorporation of Cy-dye modified UTP during the process of in vitro transcription, or (b). indirect labeling, by incorporating aminoallyl modified UTPs during in vitro transcription followed by monoreactive cy-dye coupling. Since the cy-dye modified nucleotides used for direct labeling are extremely expensive, we recommend the second approach.

Aminoallyl UTP (aaUTP) does not contain a bulky sidechain modification, which means that one can replace 100% of the UTP with aaUTP during RNA synthesis without loss of incorporation. We recommend using a 1:1 ratio of UTP to aaUTP.

### **In Vitro Transcription Reaction Mixture:**

1. Make the reaction mix by adding the reagents in the following order:

x $\mu$ L	double-stranded cDNA (use all from above rxn ~10ul)
1.5 $\mu$ L	aaUTP Solution (50 mM) (Ambion #8437)
6.0 $\mu$ L	ATP, CTP, GTP Mix (25 mM) (2ul/NTP)
0.5 $\mu$ L	UTP Solution (75 mM)
2.0 $\mu$ L	T7 10X Reaction Buffer
<u>2.0 <math>\mu</math>L</u>	<u>T7 Enzyme</u>
<u>~10ul cDNA + 12 ul of Mix</u>	
= ~22ul total	

2. Mix well with pipette, centrifuge at 3000 x g for 30 sec, then incubate the tube at 37°C in a PCR machine (the lid temperature should be set at 40°C). A water bath can be used if necessary- be aware of condensation (may need to quick-spin periodically).

(If you are working with more than one sample it is recommended to make a master mix without cDNA).

The minimum recommended incubation time is 4 h, and the maximum is 14 h. Try to do at least 5 hours. Stop the reaction by adding 80  $\mu$ L nuclease-free water to each cRNA sample to bring the final volume to 100  $\mu$ L. Mix thoroughly by gentle vortexing, and either proceed to the cRNA purification step (below), or store at -20°C immediately.

## **Title: RNA Amplification - cRNA Purification & Quantification**

### **Materials**

Aminoallyl Message Amp II kit (Ambion Cat# 1751)  
RNase free tips, tubes  
Refrigerated Microfuge centrifuge  
100% EtOH  
DEPC-treated H<sub>2</sub>O or nuclease free water

### **cRNA Purification**

Before proceeding to the dye coupling it is important to remove all the unincorporated nucleotides from the cRNA. Check to make sure that each IVT reaction was brought to 100  $\mu$ L with nuclease-free water.

1. Add 350  $\mu$ L of cRNA binding buffer to each cRNA sample, and proceed to the next step immediately.
2. Add 250  $\mu$ L of 100% ethanol to each cRNA sample, and mix by pipetting. **Do NOT vortex to mix and do NOT centrifuge. Proceed immediately** (to avoid cRNA semi-precipitation)
3. Pipet each sample mixture from step 2 onto the center of the filter in the cRNA filter cartridge. centrifuge for ~1 min at 10,000 X g, or continue until the mixture has passed through the filter.
4. Discard the flow-through and replace the cRNA filter cartridge back into the cRNA collection tube.
5. Apply 500  $\mu$ L wash buffer to each cRNA filter cartridge, centrifuge for ~1 min at 10,000 X g, or until all the wash buffer is through the filter.
6. Discard the flow-through and spin the cRNA filter cartridge for an additional ~3 min to remove trace amounts of wash buffer.
7. Transfer filter cartridge(s) to a fresh cRNA collection tube, to the center of the filter, add 30 $\mu$ L nuclease-free water (pre-heated to 50C).
8. Leave at room temp for 2 min and then centrifuge for ~1.5 min at 10,000 X g, or until the nuclease-free water is through the filter. Repeat with 30 $\mu$ L of nuclease-free water.

9. The cRNA will now be in the cRNA collection tube in ~60  $\mu\text{L}$  of nuclease-free water.

Determine the concentration of RNA using the Nanodrop or a conventional spectrophotometer. Aliquot 2 $\mu\text{g}$  of cRNA into several tubes and completely dry it using a Speedvac centrifuge set at medium heat (45°C). Store the remaining cRNA as well as the dried aliquots at -80C for further use. Use 2 $\mu\text{g}$  cRNA per target in a hybridization.

## **Title: Coupling aa-cRNA to Cy Dye Ester and Cleanup.**

### **Materials:**

Cy3 Monoreactive dye (Amersham Pharmacia; Cat# PA23001)

Cy5 Monoreactive dye (Amersham Pharmacia; Cat# PA25001)

a) Cy3 and Cy5 esters are provided as a dried product in 5 tubes; resuspend each tube of dye ester with 73 $\mu\text{l}$  of DMSO before use. Aliquot into 4.5 $\mu\text{l}$  each tubes for easy use. Store at -80C.

Sodium Carbonate Buffer

- a) Dissolve 10.6g  $\text{Na}_2\text{CO}_3$  in 80ml of MilliQ water and adjust pH to 9.0 with 12N HCl. Bring volume up to 100ml with MilliQ water
- b) To make a 0.1M working solution for the dye coupling reaction, dilute 1:10 with water.
- c) Note- Carbonate buffer changes composition over time; make it fresh every two weeks.

RNeasy MinElute, 50rxns (Qiagen, #74204)

Pre-hyb Buffer: 5x SSC, 0.1%SDS, 1% BSA, filter sterilized.

### **Cy-Dye Coupling:**

Keep all reactions covered with aluminum foil when not working with them.

1. Resuspend cRNA in 4.5 $\mu\text{l}$  of **0.1M** carbonate buffer; dissolve pellet at 37C for 10min.
2. Add 4.5 $\mu\text{l}$  of Cy dye (already re-suspended and aliquoted)
3. Incubate at RT for 1-2 hours in the dark.
4. Fill Coplin jar with pre-hyb solution and place in 42C water bath.

### **Post Coupling Reaction- Removal of Unincorporated Dye**

The Qiagen RNeasy MinElute column is used for this purpose.

1. Adjust sample to a volume of 100  $\mu\text{L}$  with RNAase-free water. Add 350  $\mu\text{L}$  of RLT buffer, and mix thoroughly.
2. Add 250  $\mu\text{L}$  of 96–100% ethanol to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge, continue immediately with step 3.
3. Apply 700  $\mu\text{L}$  of the sample to an RNeasy MinElute Spin Column in a 2 mL collection tube (supplied). Close the tube gently, centrifuge for 15 s at 8000 x g (8,400rpm).
4. Transfer the spin column into a new 2 ml collection tube. Pipet 500  $\mu\text{L}$  RPE buffer onto the spin column. Close the tube gently, and centrifuge for 15 s at 8000 x g to wash the column. Discard the flow-through (reuse the collection tube in step 5).

Note: RPE buffer is supplied as a concentrate; ensure that ethanol is added before use.

5. Add 500  $\mu$ L of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at 8000 x g to dry the silica-gel membrane. Discard the flow-through and collection tube.
6. Transfer the RNeasy MinElute Spin Column into a new 2 mL collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at 12000 x g for 2 min. Discard the flow-through and collection tube.
7. To elute, transfer the spin column to a new microfuge tube. Pipet 20  $\mu$ L DEPC water (or Rnase free water) and leave at RT for 2 min. Close the tube gently, and centrifuge for 1 min at 12000 x g for 1 min.
8. Repeat step 7 with an additional 20  $\mu$ L of DEPC water (or Rnase free water).
9. Measure the amount of dye incorporated into cRNA using a NanoDrop.
10. Combine respective Cy3 and Cy5 probes to same tube
11. Dry down in speed vac on medium heat for ~45 min.

## **Title: Hybridization Steps**

### **Pre-Hybridization:**

1. Place slide in pre-heated pre-hyb for 45 min. while probe is drying down.
2. At end of pre-heat time, wash once in MilliQ water in staining dish with shaking, for 5 min.
3. Repeat wash in fresh dish of MilliQ water for an additional 5 min.
4. Wash in Isopropanol with shaking for 2 min.
5. Spin dry either in Juoan centrifuge on program 33 (in plate racks while in slide holder for staining dish) or in the slide minifuge.

### **Hybridization:**

1. Prepare hybridization solution (1rxn): (50% formamide, 5X SSC, 0.1% SDS)
 

Formamide	25ul
20X SSC	12.5ul
10% SDS	0.5ul
MilliQ water	12ul
polyA-DNA	1ul
Salmon sperm DNA	0.75ul
2. Resuspend dried probe in 51ul of hybridization solution
3. Denature by heating at 95C for 3min.
4. Snap freeze on ice for 1 min.; spin down droplets for 1 min.
5. Apply to slide with a clean lifter-slip and incubate at 42C overnight, in the dark.

### **Post-Washes (next day):**

1. Heat 2x SSC, 0.1% SDS at 42C
2. Place slide in above to remove cover slip
3. Wash slide in 2X SSC, 0.1% SDS at 42C for 5min.
4. Wash slide in 0.05x SSC, 0.1% SDS for 10min. at RT
5. Wash slide in 0.05x SSC for 1 min.
6. Repeat step 5, 4 times with fresh solution.