RNA Amplification and Cy Dye Coupling

Overview

A typical microarray experiment employs 30-50 µg of total RNA, corresponding to about 1 µg of polyA$^+$ RNA. Therefore, RNA amplification techniques become essential for experiments involving limited amounts of starting materials, for example microarray analysis involving rare tissues such as the female gametophyte, the developing embryo, and other dissected tissues and cell types. Most RNA amplification techniques are based on the method of Eberwine (van Gelder et al., 1990), employing double stranded cDNA synthesis using oligo dT primers incorporating one of the T3 or T7 viral promoters, followed by *in vitro* transcription as a means to linearly increase the concentration of messenger RNA. The optimized Eberwine method is capable of amplification of mRNA up to ~10^3 fold for one round of amplification, and up to ~10^5 fold for two rounds of amplification (Wang et al., 2000; Baugh et al., 2001).

In our experience, total RNA isolated from a variety of maize tissues using our Trizol/ Qiagen RNeasy protocol works very well for the RNA amplification and dye coupling described below. The protocol uses the Ambion Message Amp II kit and reduced amounts of reagents (half reactions) for a single round of amplification starting with ~1.5 ug of total RNA. The resulting aminoallyl cRNA is then coupled to either Cy3 or Cy5 to produce the fluorescently labeled target. We have used this procedure to successfully amplify RNA isolated from pollen, seedling leaves and roots, sheath, anthers, adult leaf, callus, and developing ears, tassels, embryos, and endosperm. The use of half reactions saves on reagent costs and yields enough cRNA for multiple hybridizations should that become necessary.

Materials Required

Qiagen RNAeasy Mini elute columns and buffers (Qiagen Cat # 74204)
Aminoallyl Message Amp II kit (Ambion Cat# 1751, 8437)
DNA Clear Kit for extra cDNA purifications (Ambion Cat # 1756)
MEGA Clear Kit for extra aaRNA purifications (Ambion Cat # 1908)
50 MM 5-(3-AminoAllyl)-UTP (Ambion Cat # 8437)
RNAse free tips, tubes
Refrigerated Microcentrifuge
DEPC treated H_2O
Thermal cycler or incubators set at 16, 37, or 42°C
100% EtOH
Cy3 Monoreactive dye (Amersham Pharmacia; Cat# PA23001)
Cy5 Monoreactive dye (Amersham Pharmacia; Cat# PA25001)
DMSO (Sigma; Cat#D8418)
Hydroxylamine (Sigma Cat#159417)
Sodium Carbonate
Sodium Bicarbonate

Solution Preparation

Preparation of cDNA Wash Buffer

Add 11.2 ml 100% ethanol (ACS grade or better) to the bottle labeled cDNA Wash Buffer. Mix well and mark the label to indicate that the ethanol was added.

Preparation of aRNA Wash Buffer

Add 22.4 ml ACS grade 100% ethanol (ACS grade or better) to the bottle labeled aRNA Wash Buffer. Mix well and mark the label to indicate that the ethanol was added.

Preparation of sodium carbonate buffer (200mM Na_2CO3, 200mM NaHCO3): pH 9.0. Dissolve 1.06g Na_2CO_3, and 0.84g $NaHCO_3$ in 45 mL of RNAase-free water, and adjust pH to 9.0 with 12 N HCl; bring volume up to 50 mL with RNAase-free water. Aliquot into 0.5 mL RNAase free tubes, and store at -20^{o}C. Use one tube at a time, and discard the tube after use. OR: Dissolve 1.06g Na_2CO_3 in 50 mL of RNAase-free water and 0.84g $NaHCO_3$ in 50 mL of RNAase-free water. Use one as the acid and one as the base. Combine the two until they reach pH 9.0.

Preparation of Cy3 and Cy5 monoreactive dye. These dyes are supplied as five aliquots. The dye in each tube is sufficient for four labeling reaction. Dissolve entire contents of a single tube in 22 µL DMSO by flicking the tube several times, and leaving at RT for at least 30 min protected from light. Spin at 1000 X g for 30 sec to collect the dye at the bottom of the tube. The dye is now ready for use, but can be stored at -20^{o}C for up to one month. Always protect the dye from light by wrapping tubes with aluminum foil.

T7 Primer Annealing and First Strand cDNA Synthesis

T7 Primer Annealing

1. Place ~ 1.5 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 in a thermal cycler.
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.

If your RNA samples are all at a standard concentration, you may want to assemble your T7 primer and H2O as a bulk mix for easy dispensing and increased accuracy.

First Strand Synthesis

Assemble the Reverse Transcription Master Mix at room temperature as a bulk mix and then place on ice. It is good idea to make a master mix for several reactions as this reduces pipetting errors. Mix well by gently pipetting up and down or flicking the tube a few times. It is prudent to include 5% overage to cover pipetting errors. Use the table below for setting up multiple reactions.

First Strand cDNA	**Number of Reactions**				
Master Mix	**1**	**4**	**8**	**12**	**16**
10x First Strand Buffer	1 µL	4 µL	8 µL	12 µL	16 µL
Ribo. Inhibitor	0.5 µL	2 µL	4 µL	6 µL	8 µL
dNTP	2 µL	8 µL	16 µL	24 µL	32 µL
Array Script	0.5 µL	2 µL	4 µL	6 µL	8 µL
Total Volume	4 µL	16 µL	32 µL	48 µL	64 µL
Total/Rxn	4.0 µL/Reaction				

6. Transfer 4 µL of Master Mix to each 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. We generally use PCR machine with lid temperature set for 48 C.

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).

Second Strand cDNA Synthesis

1. The reagent volumes are very small it is therefore recommended to make a master mix for several reactions, and pipette 40 ul of second strand master mix directly into each first strand reaction tube. Prepare the Second Strand Synthesis Master Mix on ice adding each of the ingredients in the order listed in the table below. 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.

Second Strand cDNA	**Number of Reactions**				
Master Mix	**1**	**4**	**8**	**12**	**16**
DEPC H2O	31.5 µL	126 µL	252 µL	378 µL	504 µL
10x 2nd S. Buffer	5 µL	20 µL	40 µL	60 µL	80 µL
dNTP	2 µL	8 µL	16 µL	24 µL	32 µL
DNA Polymerase	1 µL	4 µL	8 µL	12 µL	16 µL
RNase H	0.5 µL	2 µL	4 µL	6 µL	8 µL
Total	40 µL	160 µL	320 µL	480 µL	640 µL
Total/Rxn	40 µL/Reaction				

2. Incubate at 16°C for two hours in a thermal cycler or a refrigerated water bath and 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 Message Amp-II kit or the Ambion DNA Clear Kit for cDNA purifications (Ambion Cat # 1756)

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 µL 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 µL 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 µL 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 µL 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 µL of pre-heated nuclease-free water. The double-stranded cDNA will now be in the eluate (~11 µL).
11. Discard the cDNA Filter Cartridge.

Check the cDNA concentration in the solution by applying 1.5 ul of eluted cDNA on to the Nanodrop spectrophotometer. In general the cDNA yield should be around 5-10 ng / µL if you start with ~1 µg of total RNA.

In Vitro Transcription to produce aminoallyl labeled cRNA

The oligo microarrays, being printed with positive-strand DNA elements, require labeled negative-strand targets for hybridization. Since the first round of amplified aRNAs represents the negative–strand, it is recommended to label the aRNA itself. aRNA 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 2:1 molar ratio of aaUTP to UTP. The ATP, CTP, GTP Mix is assembled by mixing equal parts of the three nucleotides from 75 mM stock solutions so that each nucleotide is at a 25 mM concentration. Below is table for preparing your aminoallyl labeled cRNA master mix. Prepare the master mix on ice and mix well. Add 12 ul of master mix to the ~8.0 µl of remaining double stranded cDNA

cRNA Synthesis Master Mix	**Number of Reactions**				
	1	**4**	**8**	**12**	**16**
aaUTP (50 mM)	1.5 µl	6 µl	12 µl	18 µl	24 µl
ATP, CTP, GTP Mix	6 µl	24 µl	48 µl	72 µl	96 µl
UTP Solution (75 mM)	0.5 µl	2 µl	4 µl	6 µl	8 µl
T7 10x Rxn Buffer	2 µl	8 µl	16 µl	24 µl	32 µl
T7 Enzyme	2 µl	8 µl	16 µl	24 µl	32 µl
Total Volume	12 µl	48 µl	96 µl	144 µl	192 µl
Total/Rxn	12 µl /Reaction				

Mix the master mix and sample well with pipette, centrifuge at 3000 x g for 30 seconds. Incubate the tube at 37°C in a PCR machine (the lid temperature should be set at 40°C). The minimum recommended incubation time is 4 h, and the maximum is 14 h. We generally let our reactions run overnight. Stop the reaction by adding 80 µL nuclease-free water to each cRNA sample to bring the final volume to 100 µL. Mix thoroughly by gentle vortexing, and either proceed directly to the cRNA purification step (below), or store at –20°C.

aRNA Purification

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

1. Add 350 µL of aRNA binding buffer to each aRNA sample, and proceed to the next step immediately.
2. Add 250 µL of ACS grade 100% ethanol to each aRNA sample, and mix by pipetting the mixture up and down three times. ***Do NOT vortex to mix and do NOT centrifuge.***
3. Proceed ***immediately*** to the next step as soon as you have mixed the ethanol into each sample. Any delay in proceeding could result in loss of aRNA because once the ethanol is added, the aRNA will be in a semi-precipitated state.
4. Pipet each sample mixture from step 2 onto the center of the filter in the aRNA filter cartridge. Centrifuge for ~1 min at 10,000 X g, or continue until the mixture has passed through the filter.
5. Discard the flow-through and replace the aRNA filter cartridge back into the aRNA collection tube.
6. Apply 650 µL wash buffer to each aRNA filter cartridge, centrifuge for ~1 min at 10,000 X g, or until all the wash buffer is through the filter.
7. Discard the flow-through and spin the aRNA filter cartridge for an additional ~3 min to remove trace amounts of wash buffer.
8. Transfer filter cartridge(s) to a fresh aRNA collection tube, to the center of the filter, add 30µL nuclease-free water (pre-heated to 50C).
9. 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 30µL of nuclease-free water.
10. The aRNA will now be in the aRNA collection tube in ~60 µL of nuclease-free water.

Determine the concentration of RNA using the Nanodrop or a conventional spectrophotometer. Total yield of aminoallyl labeled cRNA is generally in the range of 20 to 40 ug of cRNA. Typically we use about 3 ug of cRNA target per slide or 6 ug for the two slide set. Aliquot ~6 µg of aRNA for dye coupling and completely dry it using a Speedvac centrifuge set at room temperature. Store the remaining aRNA at -80 for further use.

Coupling AA-cRNA to Cy Dye Ester.

1. Dissolve the dried cRNA with 5 µL of $NaHCO_3$ buffer by flicking the tube several times and leaving the tube at RT for at least 20 min.
2. Add 5 µL of Cy3 or Cy5 (in DMSO) to each tube, and mix them thoroughly by flicking the tube several times.
3. Spin the tube at 1000 X g for 30 sec.

4. Incubate the dye and cRNA mix in the tube at RT for 2 h covered in aluminum foil.

Quenching Reaction This optional step involves quenching any unreacted Cy dye by adding an excess of primary amines.

1. Add 4.5 µL 4M hydroxylamine.
2. Incubate for 15 min in the dark at RT.

Removal of Unincorporated Dye The Qiagen RNeasy MinElute column is used for this purpose.

1. Adjust sample to a volume of 100 µL with RNAase-free water. Add 350 µL of RLT buffer, and mix thoroughly.
2. Add 250 µL of 96–100% ethanol to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge, continue immediately with step 3.
3. Apply 700 µL 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, and discard the flow-through.
4. Transfer the spin column into a new 2 ml collection tube. Pipet 500 µL 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 µL 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. Repeat with an additional 500 µL of 80% ethanol.
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 5 min. Discard the flow-through and collection tube.
7. To elute, transfer the spin column to a new microfuge tube. Pipet 20 µL DEPC 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 µL of DEPC water. (The labeled RNA may also be eluted using only 10 µL of DEPC water if a higher concentration is desired).

Measure the amount of dye incorporated into aRNA using a NanoDrop or conventional spectrophotometer. We generally recover enough dye labeled cRNA (~5 ug) for two slide hybridizations.