Amino Allyl MessageAmp™ Kit

RNA Amplification and Labeling for Array Analysis

Part Number AM1752

Amino Allyl MessageAmp™ Kit

(Part Number AM1752)

Protocol

P/N 1752M Revision C **Revision Date: August 20, 2010**

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I. Introduction

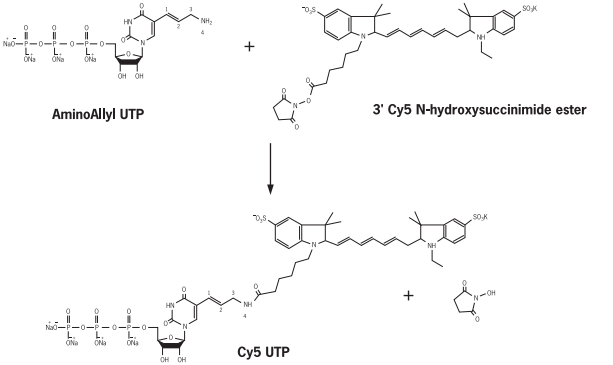
A. Background

The Amino Allyl MessageAmp™ aRNA Amplification procedure is based on the RNA amplification protocol developed in the laboratory of Dr. James Eberwine (Van Gelder et al. 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and in vitro transcription of the resulting DNA with T7 RNA Polymerase to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. The antisense RNA is referred to as ***aRNA*** and the amplification method is referred to as the ***aRNA amplification procedure***.

aRNA has been used in RT-PCR and cDNA library construction procedures, but its primary application is to prepare labeled nucleic acid for gene array analysis from very small amounts of RNA. The aRNA amplification procedure is preferred over other methods of nucleic acid amplification for global gene expression studies because the method does not significantly distort the relative abundance of individual mRNA sequences within an RNA population (Baugh et al. 2001, Pabon et al. 2001, Poirier and Erlander 1998). This is because RNA polymerase activity is generally not affected by either the concentration of individual templates in a complex mixture nor by the sequences of the template molecules being transcribed. For the few templates that are transcribed more or less efficiently than other templates, the amplification bias is typically equivalent in all samples. This means that even though the aRNA amplification procedure might not generate exactly the same number of aRNA molecules from each template, their amplification is reproducible from reaction to reaction making it possible to compare the expression profiles of different amplified RNA samples.

The Amino Allyl MessageAmp aRNA Kit is configured to incorporate the modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) into the aRNA during in vitro transcription. aaUTP contains a reactive primary amino group on the C5 position of uracil that can be chemically coupled to the desired N-hydroxysuccinimidyl ester-derivitized reactive dyes (NHS ester dyes) in a simple, efficient reaction (see Figure 1). Once purified, the dye labeled aRNA can then be used for microarray hybridization.

Figure 1. Amino Allyl Labeling Reaction



Procedure Overview

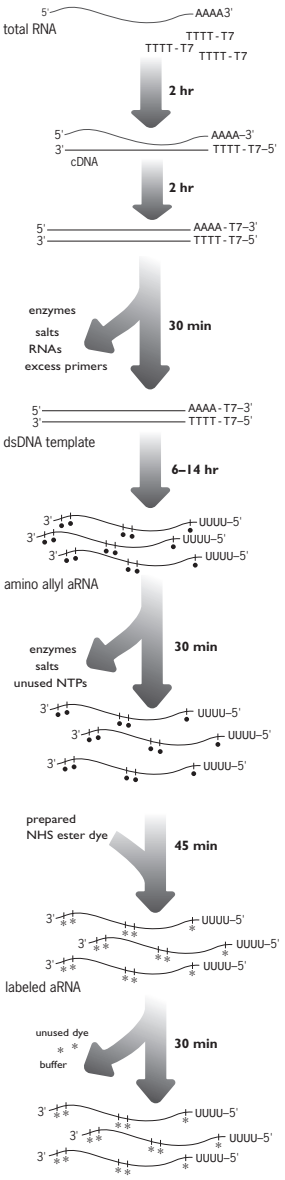
The aRNA amplification procedure is depicted in Figure 2

1. ***First strand cDNA synthesis*** is primed with the T7 Oligo(dT) Primer to synthesize cDNA with a T7 promoter sequence by reverse transcription.
2. ***Second strand cDNA synthesis*** converts the single-stranded cDNA with the T7 promoter primer into double-stranded DNA (dsDNA) template for transcription.
3. ***cDNA purification*** removes RNA, primers, enzymes, and salts from the dsDNA that inhibit in vitro transcription.
4. ***In vitro transcription*** with aaUTP generates multiple copies of amino allyl modified aRNA from the double-stranded cDNA templates; it is the amplification step.
5. ***aRNA purification*** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the aRNA and facilitate NHS ester coupling or subsequent enzymatic manipulations.
6. ***Dye coupling reaction*** takes place between the amino allyl modified UTP residues on the aRNA and amine reactive dyes.
7. ***Labeled aRNA purification*** removes free dye and exchanges the buffer with Nuclease-free Water.

Optional second round of amplification

Additional amplification of an RNA sample can be achieved by subjecting the aRNA to a second round of amplification (see Figure 3). The primers that are used in the first and second strand cDNA synthesis reactions are different for the second round of amplification, but otherwise the same reagents and methodology are used. When including two rounds of amplification it is important to synthesize the first round aRNA with the unmodified UTP Solution because amino allyl modified aRNA (transcribed with the aaUTP Solution) cannot be efficiently amplified.

Figure 2. Amino Allyl MessageAmp™ Procedure



II.*C. First Strand cDNA Synthesis*

1. Mix RNA and T7 Oligo(dT) Primer, and bring the volume to 12 µL
2. Incubate 10 min at 70°C, then place on ice
3. Add 8 µL of Reverse Transcription Master Mix and place at 42°C
4. Incubate for 2 hr at 42°C

II.*D. Second Strand cDNA Synthesis*

1. Add 80 µL Second Strand Master Mix to each sample
2. Incubate 2 hr at 16°C

II.*E. cDNA Purification*

2. Equilibrate one cDNA Filter Cartridge for each sample
3. Add 250 µL of cDNA Binding Buffer to each cDNA sample
4. Apply mixture to an equilibrated cDNA Filter Cartridge
5. Wash the cDNA Filter Cartridge with 500 µL cDNA Wash Buffer
6. Elute cDNA with 2 x 9 µL Nuclease-free Water
7. Bring samples to 14 µL with Nuclease- free Water if necessary

II.*F In Vitro Transcription to Synthesize aRNA*

1. Assemble the 40 µL transcription reaction components at room temp
2. Incubate the reaction for 6–14 hr at 37°C
3. (Optional) Add 2 µL DNase I, mix, and incubate 30 min at 37°C

II.*G. aRNA Purification*

1. Add 58 µL Nuclease-free Water to each aRNA sample
2. Add 350 µL of aRNA Binding Buffer
3. Add 250 µL of 100% ethanol
4. Pass sample through an aRNA Filter Cartridge(s)
5. Wash with 650 µL aRNA Wash Buffer
6. Elute aRNA with 2 x 50 µL 50°C Nuclease-free Water

IV.*A. Concentrate the Amino Allyl aRNA*

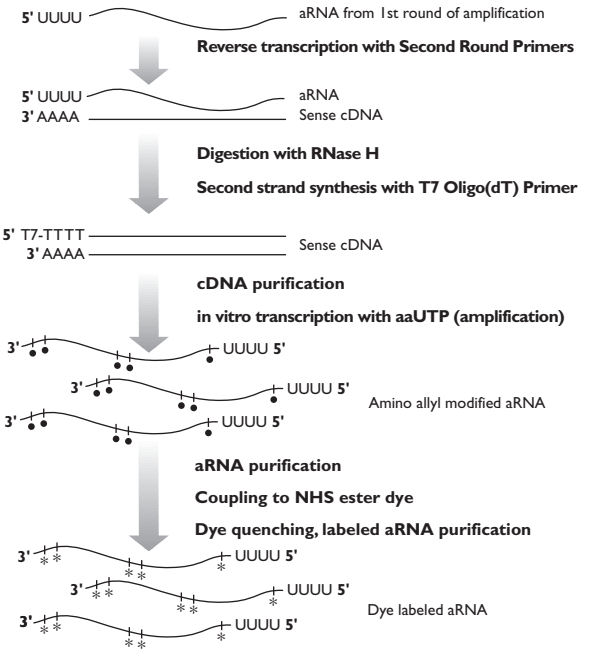
1. Determine the aRNA concentration
2. Vacuum dry 5–20 µg aRNA

IV.*C. aRNA:Dye Coupling Reaction*

1. Add 9 µL Coupling Buffer to the aRNA
2. Add 11 µL prepared dye to the aRNA and mix well
3. Incubate 30 min at room temp in the dark
4. Add 4.5 µL 4M Hydroxylamine, mix, and incubate 15 min in the dark

IV.*D. Dye Labeled aRNA Purification*

Figure 3. Second Round of Amplification with the Amino Allyl MessageAmp™ Kit



The Amino Allyl MessageAmp advantage

Our scientists have streamlined and optimized each step in the aRNA procedure to develop the Amino Allyl MessageAmp Kit. The ***first strand cDNA synthesis reaction*** is optimized to ensure that every cDNA bears a T7 promoter at its 5' end, and that even very limited amounts of mRNA are fully converted to full-length cDNA. The ***second strand cDNA synthesis*** reaction is designed for the efficient synthesis of full length, double-stranded cDNAs and the complete conversion of single-stranded cDNA into double-stranded transcription templates. The ***cDNA purification*** procedure not only removes enzymes, salts, and unincorporated dNTPs, but it efficiently removes RNA from the cDNA sample. This eliminates the heating or enzymatic digestion step that is commonly used in other procedures to degrade RNA (especially ribosomal RNA). The ***in vitro transcription*** reaction features Ambion® patented MEGAscript technology for maximal transcriptional amplification and yield of aRNA. The MEGAscript reaction used in the Amino Allyl MessageAmp Kit is optimized to ensure the efficient transcription of limited amounts of template DNA as well as the synthesis of long transcripts. The NTPs are provided in three separate vials: an ATP, CTP and GTP Mix containing 25 mM of each nucleotide, a 50 mM UTP Solution, and a 50 mM aaUTP Solution [5-(3-aminoallyl)-UTP].

Providing the UTP and the aaUTP separately provides flexibility to make unlabeled aRNA, or to incorporate aaUTP during either the first or second round of amplification. Unlike most modified nucleotides, the inclusion of aaUTP in an in vitro transcription reaction has only a minor effect on the reaction efficiency and yield. Additionally, since the incorporation of aaUTP ***by in vitro transcription*** will be virtually identical in different samples, and since the ***dye coupling reaction*** is efficient and reproducible, labeled samples will not have the biases that can result from direct incorporation of modified nucleotides. The simple, rapid ***aRNA purification*** procedure is used both to prepare the aRNA for dye coupling and to remove free dye molecules after the coupling reaction in preparation for array hybridization.

B. Materials Provided with the Kit and Storage

The Amino Allyl MessageAmp Kit includes reagents for amplification and labeling of 20 samples using a single round of amplification or up to 10 samples with 2 rounds of amplification for each.

cDNA Synthesis Reagents

Store in a non frost-free freezer

Amount	Component	Storage
60 µL	T7 Oligo(dT) Primer*	–20°C
20 µL	Reverse Transcriptase	–20°C
20 µL	RNase Inhibitor	–20°C
40 µL	10X First Strand Buffer	–20°C
160 µL	dNTP Mix	–20°C
200 µL	10X Second Strand Buffer	–20°C
40 µL	Second Round Primers	–20°C
40 µL	DNA Polymerase	–20°C
20 µL	RNase H	–20°C
10 µL	Control RNA 1 mg/mL HeLa cell total RNA	–20°C
1.75 mL	Nuclease-free Water	any temp†

* The T7 Oligo(dT) Primer is also available separately from Ambion; P/N AM5710

† Store Nuclease-free Water at –20°C, 4°.C, or room temp.

In vitro transcription reagents

Store in a non frost-free freezer

Amount	Component	Storage
80 µL	T7 Enzyme Mix	–20°C
80 µL	T7 10X Reaction Buffer	–20°C
90 µL	UTP Solution (50 mM)	–20°C
60 µL	aaUTP Solution (50 mM) 5-(3-amino allyl)-UTP	–20°C
240 µL	ATP, CTP, GTP Mix (25 mM each nucleotide)	–20°C
45 µL	DNase I	–20°C

cDNA and aRNA Purification Components

Amount	Component	Storage
14 mL	cDNA Wash Buffer Add 11.2 mL 100% ethanol before use	4°C / room temp
28 mL	aRNA Wash Buffer Add 22.4 mL 100% ethanol before use	4°C / room temp
7 mL	cDNA Binding Buffer	room temp*
20 mL	aRNA Binding Buffer	room temp
40	aRNA Filter Cartridges	room temp
80	aRNA Collection Tubes	room temp
20	cDNA Filter Cartridges + Wash Tubes	room temp
20	cDNA Elution Tubes	room temp
10 mL	Nuclease-free Water	any temp†

* The cDNA Binding Buffer may form a precipitate if stored below room temp. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.

† Store Nuclease-free Water at –20°C, 4°.C, or room temperature.

Dye Labeling Reagents

Store in a non frost-free freezer.

Amount	Component	Storage
180 µL	4M Hydroxylamine	–20°C
440 µL	DMSO	–20°C
400 µL	Coupling Buffer	–20°C

C. Materials Not Provided with the Kit

- 100% Ethanol (to prepare cDNA and aRNA Wash Buffers)
- Additional 100% DMSO may be required depending on how the dye is supplied
- Water bath(s), thermal cycler, or heating blocks set at 70°C, 42°C, 37°C and 16°C. (See *Incubator type* on page 12 for more information.)

IMPORTANT

Our scientists have observed that when using a thermal cycler for incubations of ≤42°C, the heated lid should be turned off or not used to cover the reaction tubes. The heat from the lid creates a higher temperature than desired in at least a portion of the reaction solution.

- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- (Optional) Materials and equipment for RNA analysis
- (Optional) Spectrophotometer
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes Inc.)
- NHS esters of fluorescent dyes
  This kit was developed with Cy™Dye fluorescent dyes from Amersham Biosciences, but mono-reactive NHS esters of any label moiety should be capable of coupling to the amino allyl modified aRNA generated with this kit.
  - Amersham Biosciences CyDyes™
    CyDye Post Labelling Reactive Dye Pack Cat #RPN 5661
    FluoroLink™ Cy5 mono-functional Dye 5-Pack Cat #PA 25001
    FluoroLink Cy3 mono-functional Dye 5-Pack Cat #PA 23001
  - Molecular Probes Alexa Fluor® Succinimidyl Esters
    Alexa Fluor 546, Cat #A-20002
    Alexa Fluor 555, Cat #A-20009
    Alexa Fluor 647, Cat #A-20006
    Alexa Fluor 660, Cat #A-20007
  - Pierce Biotechnology
    NHS-Fluorescein, Product #46100
    NHS-Rhodamine, Product #46102
  - Denovo Biolabels GmbH Oyster® Dyes:
    Oyster - 556, Cat #OY-556-1-N-1x0.2
    Oyster - 645, Cat #OY-645-2-N-1x0.2
    Oyster - 656, Cat #OY-656-1-N-1x0.2

D. Related Products Available from Applied Biosystems

MessageAmp™ aRNA Amplification Kits see our web or print catalog	Ambion offers a full line of MessageAmp Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion offers the MessageAmp Premier and MessageAmp III RNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.
Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452, AM8453	Ambion's biotinylated UTPs are ideal for use as substrates in vitro transcription reactions, and can be utilized by a variety of RNA polymerases, including T7, T3, and SP6 RNA polymerases. Biotinylated RNA can be used in place of radioactively labeled RNA in many applications with detection via one of a variety of streptavidin-based methods.
RNase-free Tubes & Tips see our web or print catalog	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
FirstChoice® Total and Poly(A) RNA see our web or print catalog	Ambion provides high quality total and poly(A) RNA from a variety of human, mouse and rat tissues and from human cell lines. DNA is removed with a stringent DNase treatment, and the purity and integrity of these RNAs are verified by Agilent bioanalyzer evaluation, denaturing agarose gel electrophoresis, or Northern analysis. FirstChoice Total RNA is prepared by methods that quantitatively recover small RNAs (miRNA, siRNA, and snRNA). FirstChoice Total and Poly(A) RNAs are ready for use in any application that requires highly purified, intact RNA. See the catalog or website (www.appliedbiosystems.com) for a complete listing of available FirstChoice RNAs.
RNA Isolation Kits see our web or print catalog	Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits.
DNA-*free*™ Reagents P/N AM1906	DNase treatment and removal reagents. This product contains Ambion's ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.
ArrayControl™ P/N AM1780	The ArrayControl Spikes are a set of eight control RNA transcripts designed for the normalization and validation of glass microarray experiments. The Spikes range in size from 750 to 2000 bases and each transcript has a 30-base 3' poly(A) tail. The precisely quantitated RNA Spikes are designed to be added to your RNA sample before labeling, to serve as internal controls for sample labeling and hybridization efficiency.

RNA Fragmentation Reagents P/N AM8740	Amplified RNA is commonly fragmented prior to hybridization on oligonucleotide microarrays to improve the hybridization kinetics and signal produced on oligonucleotide microarrays. Ambion's RNA Fragmentation Reagents include a 10X Fragmentation Reagent and a Stop Solution.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette see our web or print catalog	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. Ambion also offers the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.
Millennium™ Markers and BrightStar® Biotinylated Millennium™ Markers P/N AM7150 and AM7170	Ambion Millennium™ Markers are designed to provide very accurate size determination of single-stranded RNA transcripts from 0.5 to 9 kb and can be used in any Northern protocol. They are a mixture of 10 easy-to-remember sizes of in vitro transcripts: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 9 kb.
RNA 6000 Ladder P/N AM7152	The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer.

II. aRNA Amplification Procedure

A. Planning the Experiment

Input RNA requirements

RNA quantity

We recommend using ***100–2000 ng of total RNA***, or ***10–100 ng of poly(A) selected RNA*** in the Amino Allyl MessageAmp procedure.

The maximum recommended amount of RNA for this procedure is 5 µg of total RNA or up to 1 µg of poly(A) RNA.

RNA purity

The single most important factor affecting how efficiently an RNA sample will be amplified using the Amino Allyl MessageAmp Kit is the quality of the RNA. RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1.

RNA integrity

The integrity of the RNA sample, or the proportion that is full-length, is another important component of RNA quality. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that lack the sequence encoded upstream of the break in the RNA molecule.

There are currently no quantitative methods for measuring what percentage of mRNAs in a sample are full-length, however several procedures do exist for establishing the relative integrity of a sample. The most often used procedure is fractionating 2–4 µg of a total RNA sample on a denaturing agarose gel (instructions are in section III.B on page 21). Ethidium bromide staining of the RNA reveals the 18S and 28S ribosomal RNA (rRNA) bands. If these bands are discrete (i.e. no significant smearing below each band) and the 28S rRNA band is approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to this method is that microgram amounts of RNA must be sacrificed.

A method that requires only nanogram quantities of RNA is using the Agilent 2100 bioanalyzer and RNA 6000 LabChip® Kit. Like a denaturing agarose gel, the bioanalyzer fractionates RNA molecules according to size, and the amounts of 18S and 28S rRNA can be determined. The ratio of the 28S to 18S band will approach 2:1 in RNA samples comprised of primarily full-length RNA.

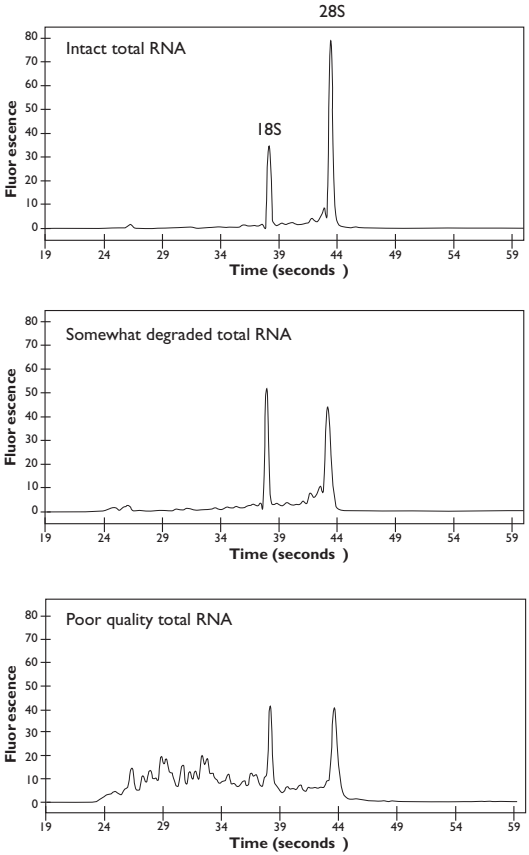


Figure 4. Bioanalyzer Images of Increasingly Degraded Total RNA

These electropherographs (from the Agilent 2100 bioanalyzer) show progressively worse RNA degradation. Notice how the ribosomal RNA peaks are at a ratio of about 2 (28S:18S) in an intact total RNA sample. Total RNA that is somewhat degraded shows nearly equal amounts of 28S and 18S rRNA. Further degradation decreases the rRNA peaks more, and the lower molecular weight degradation products (rRNA and mRNA) become apparent.

Time requirements

The Amino Allyl MessageAmp procedure requires a minimum of 13 hours to complete. Although it can be done in a single day, we recommend that the procedure be divided between 2 successive days as shown below:

Day 1

1. First strand cDNA synthesis (2 hr)
2. Second strand synthesis (2 hr)
   If desired, the procedure can be stopped after the second strand synthesis by placing the tubes immediately at –20°C.
3. cDNA purification (0.5 hr)
   If desired, the procedure can be stopped after the cDNA purification by placing the tubes at –20°C.
4. (option 1) overnight in vitro transcription (6–14 hr)

Day 2

1. (option 2) in vitro transcription (6–14 hr)
2. aRNA purification (0.5 hr)
3. aRNA quantitation (amount of time required varies with different methods, see section III.A on page 21)
4. aRNA:dye coupling reaction (0.75 hr)
5. Labeled aRNA purification (0.5 hr)

Procedural notes

Reagent handling

The frozen reagents needed for each enzymatic reaction in the aRNA procedure (i.e. primers, nucleotides and 10X buffers) should be thawed completely, mixed thoroughly, and kept on ice before use. Note that enzyme solutions should be mixed by ***gently*** flicking the tube a few times before adding them to reactions.

Once all the reagents have been added to a reaction mixture, it should be mixed thoroughly and allowed to warm to room temperature before adding enzymes. After adding enzyme(s), pipette the mixture, or flick the tube several times to mix thoroughly but gently.

Reaction incubation times

The incubation times in the procedure were optimized in conjunction with the kit reagents to ensure the maximum yield of nucleic acid product in each step. ***Slightly*** longer incubation times should not adversely affect the yield of aRNA. ***Significantly*** longer incubation times should be avoided because reduced yields have been observed. For reproducibility, incubation times should be monitored with a timer.

Incubator type

The Amino Allyl MessageAmp procedure is very sensitive to temperature, and there are several short incubations where it is especially important to use incubators that are capable of bringing the reaction solutions to the target temperature rapidly and accurately. It is also very important that condensation does not form in the reaction tubes during any of the incubations. Condensation changes the composition of reaction mixtures, and we have seen that it can greatly reduce yield.

Incubator recommendations for long incubations (>2 hr)

For incubations longer than 2 hours, air incubators such as hybridization ovens are recommended because the uniform heat completely surrounding the tubes minimizes condensation.

Incubator recommendations for first and second strand cDNA synthesis reactions

Below we list in order the incubator types we recommend for the first and second strand cDNA synthesis incubations (sections II.C on page 14 and II.D on page 15).

- Thermal cycler; if the thermal cycler has a heated lid, use it with the heat turned off. Otherwise the heat from the lid (typically 100°C) will affect the temperature of the solution in the tube, compromising the reaction. If the heated lid cannot be turned off, do not cover the reaction tubes with the thermal cycler lid.
- Air incubator, e.g. carefully monitored hybridization ovens or constant temperature incubators.
- Water bath; cover with a lid to reduce condensation in the tubes.
- Heat block; heat blocks are not optimal for this procedure because condensation typically forms inside tubes, changing the reaction mixture.

Reaction incubation temperature

Make sure the device used for reaction incubation maintains a constant temperature at each recommended temperature. Variable or inaccurate incubation temperatures can limit aRNA synthesis. Never place reactions in the incubator until the correct temperature has stabilized.

B. Before Using the Kit for the First Time

Prepare 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.

Prepare 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.

C. First Strand cDNA Synthesis

Incubators needed:

- 70°C: thermal cycler recommended
- 42°C: hybridization oven or air incubator recommended

1. Mix RNA and T7 Oligo(dT) Primer, and bring the volume to 12 µL

a. Place up to 5 µg of total RNA (typically 100–1000 ng) or up to 1 µg of poly(A) selected RNA (typically 10–100 ng) into a sterile RNase-free microfuge tube.

b. Add 1 µL of T7 Oligo(dT) Primer.

c. Add Nuclease-free Water to a final volume of 12 µL.

2. Incubate 10 min at 70°C, then place on ice

a. Incubate 10 min at 70°C in a thermal cycler.

b. Remove the RNA samples from the 70°C incubator and centrifuge briefly (~5 sec) to collect sample at bottom of tube. Place the mixture on ice while completing step 2.

3. Add 8 µL of *Reverse Transcription Master Mix* and place at 42°C

a. At room temp, prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube. Prepare enough to synthesize first strand cDNA from all the RNA samples in the experiment, including ≤5% overage to cover pipetting error.
Assemble the Reverse Transcription Master Mix in the order shown:

Reverse Transcription Master Mix (for a single 20 µL reaction)	
Amount	Component
2 µL	10X First Strand Buffer
1 µL	Ribonuclease Inhibitor
4 µL	dNTP Mix
1 µL	Reverse Transcriptase

b. 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.

c. Transfer 8 µL of Reverse Transcription Master Mix to each RNA sample from step 1.b, mix thoroughly by gently pipetting up and down or flicking the tube a few times and place the tubes in a 42°C incubator. (See *Incubator recommendations for first and second strand cDNA synthesis reactions* on page 13.)

d. Place the samples in a 42°C incubator.

4. Incubate for 2 hr at 42°C

After the 2 hr 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 immediately proceed to the second strand cDNA synthesis (below).

D. Second Strand cDNA Synthesis

Incubator needed:

- 16°C: thermal cycler recommended

1. Add 80 µL *Second Strand Master Mix* to each sample

a. On ice, prepare a ***Second Strand Master Mix*** in a nuclease-free tube in the order listed below. Assemble enough to synthesize second strand cDNA from all the samples in the experiment, including ≲% overage to cover pipetting error.
Assemble the Second Strand Master Mix in the order shown:

Second Strand Master Mix (for a single 100 µL reaction)	
Amount	Component
63 µL	Nuclease-free Water
10 µL	10X Second-strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase
1 µL	RNase H

b. Gently pipette up and down or flick the tube a few times to mix, then centrifuge the tubes briefly (~5 sec) to collect the reaction at the bottom of tube.

c. Transfer 80 µL of Second Strand Master Mix to each sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

d. Place the tubes in a 16°C thermal cycler. It is important to cool the thermal cycler block to 16°C before adding the reaction tubes because subjecting the reactions to temperatures >16°C will compromise aRNA yield.

2. Incubate 2 hr at 16°C

Incubate in a thermal cycler or in a refrigerated water bath (do not use a heat block in a 4°C refrigerator because the temperature will fluctuate too much).

STOPPING POINT

This is a potential overnight stopping point (at –20°C), but it is better to complete the cDNA purification (next section) before stopping.

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

E. cDNA Purification

The procedure described here is recommended for most users because it is rapid, efficient, and does not require organic solvents. When scaling up the Amino Allyl MessageAmp procedure, it may be more convenient to purify the cDNA with phenol as described in section VII.A on page 36.

IMPORTANT

Check the cDNA Binding Buffer for precipitation before using it. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temp.
cDNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

1. Preheat Nuclease-free Water to 50°C (≥10 min)

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

2. Equilibrate one cDNA Filter Cartridge for each sample

Immediately before starting the cDNA purification, equilibrate one cDNA Filter Cartridge per cDNA sample:

a. Check that the cDNA Filter Cartridge is firmly seated in a 2 mL Wash Tube (supplied), and pipet 50 µL cDNA Binding Buffer onto the filter in the cDNA Filter Cartridge.

b. 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

Add 250 µL of cDNA Binding Buffer to each cDNA sample from step D.2 on page 15, and mix thoroughly by repeated pipetting or gentle vortexing.

4. Apply mixture to an equilibrated cDNA Filter Cartridge

a. Pipet the cDNA sample\cDNA Binding Buffer (from step 3) onto the center of an equilibrated cDNA Filter Cartridge (from step 2).

b. Centrifuge for ~1 min at 10,000 x g, or until the mixture has passed through the filter.

c. Discard the flow-through and replace the cDNA Filter Cartridge in the 2 mL Wash Tube.

5. Wash the cDNA Filter Cartridge with 500 µL cDNA Wash Buffer

Make sure that the ethanol has been added to the bottle of cDNA Wash Buffer before using it.

a. Apply 500 µL cDNA Wash Buffer to each cDNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 x g, or until all the cDNA Wash Buffer is through the filter.

c. Discard the flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of ethanol.

d. Transfer cDNA Filter Cartridge to a cDNA Elution Tube.

6. Elute cDNA with 2 x 9 µL Nuclease-free Water

a. To the center of the filter in the cDNA Filter Cartridge, apply 9 µL of Nuclease-free Water that is preheated to 50°C.

b. 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.

c. Elute with a second 9 µL of preheated Nuclease-free Water. The double-stranded cDNA will now be in the eluate (~14 µL). Discard the cDNA Filter Cartridge.

7. Bring samples to 14 µL with Nuclease- free Water if necessary

Check the volume of each eluted cDNA sample.

- If the volume is 14–16 µL, place samples on ice or at –20°C.
- Infrequently, the volume will be less than 14 µL, if this is the case, add Nuclease-free Water to bring each sample to 14 µL. Mix well, and place on ice or at –20°C.

STOPPING POINT

The purified cDNA can be stored overnight at –20°C at this point if desired.

8. (Optional) Check the dsDNA on an agarose gel

If ≥1 µg of total RNA or ≥30 ng of poly(A) RNA was used for aRNA amplification, there may be enough dsDNA at this point in the procedure to visualize it on an agarose gel. Run 1 µL of the sample on a 1% agarose TBE gel using ethidium bromide (or another nucleic acid dye) to visualize the DNA. There should be a smear of cDNA corresponding to >500 bp. Realize that taking a sample for the gel reduces the amount of cDNA template, thus reducing the potential aRNA yield.

F. In Vitro Transcription to Synthesize aRNA

Incubator needed:

- 37°C: hybridization oven or air incubator recommended

Because the amino allyl UTP nucleotide (aaUTP) does not contain a "bulky" modification, it is possible to replace 100% of the UTP with aaUTP during RNA synthesis. The in vitro transcription reaction condition below uses a 1:1 ratio of UTP to aaUTP (i.e. 50%). We recommend this ratio for most samples, however, it is possible to adjust the level of aaUTP incorporation by using a different UTP:aaUTP ratio. Increasing the amount of aaUTP will produce aRNA that can be used to make probe with more coupled dye molecules, and in some cases will lead to brighter signal on microarrays. This kit contains enough 50 mM aaUTP to set up 20 in vitro transcription reactions at the 1:1 ratio recommended. If more aaUTP is needed it is available separately from Ambion (P/N AM8437).

1. Assemble the 40 µL transcription reaction components at room temp

a. At room temperature, prepare an ***IVT Master Mix*** by adding the following reagents to a nuclease-free microcentrifuge tube in the order listed below. Assemble enough for all the samples in the experiment, including ≤5% overage to cover pipetting error.

IMPORTANT

*If two rounds of amplification will be done, this first round transcription should be **unmodified**, containing **only** UTP (no aaUTP).*

Amino allyl	Unmodified	Component
14 µL	14 µL	double-stranded cDNA (from step 7 on page 17)
IVT Master Mix		
3 µL	– –	aaUTP Solution (50 mM)
12 µL	12 µL	ATP, CTP, GTP Mix (25 mM)
3 µL	6 µL	UTP Solution (50 mM)
4 µL	4 µL	T7 10X Reaction Buffer
4 µL	4 µL	T7 Enzyme Mix

b. Gently pipette up and down or flick the tube a few times to mix, then centrifuge the tubes briefly (~5 sec) to collect the IVT Master Mix at the bottom of tube.

c. Transfer 26 µL of IVT Master Mix to each sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube. The final reaction volume is 40 µL.

2. Incubate the reaction for 6–14 hr at 37°C

The minimum recommended incubation time is 6 hr. The maximum incubation time is 14 hr. Be sure that the incubation temperature remains constant at 37°C. We recommend doing this incubation in an air incubator such as a hybridization oven because it is extremely important that condensation does not form inside the tubes; this would change the concentrations of the reactants and reduce yield.

3. (Optional) Add 2 µL DNase I, mix, and incubate 30 min at 37°C

This optional DNase treatment removes template cDNA from the aRNA.

a. Add 2 µL DNase I to each reaction. Gently pipette up and down or flick the tube to mix, then centrifuge the tubes briefly (~5 sec) to collect the reaction at the bottom of tube.

b. Incubate for 30 min at 37°C.

c. Proceed to aRNA purification step (below) or store at –20 °C.

STOPPING POINT

The aRNA can be stored overnight at –20°C at this point if desired.

G. aRNA Purification

This purification removes unincorporated aaUTP and Tris from in vitro transcription that would compete with the aRNA for dye coupling if they were not removed; it also removes enzymes, salts, and other unincorporated nucleotides. After the dye coupling step, this purification will be repeated to remove excess dye from the aRNA.

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temp.
aRNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Preheat Nuclease-free Water to 50–60°C (≥10 min)

Before beginning the aRNA purification preheat the 10 mL bottle of Nuclease-free Water to 50–60°C for ≥10 min.

1. Add 58 µL Nuclease-free Water to each aRNA sample

a. Add 58 µL Nuclease-free Water to each aRNA sample (from step F.3.c on page 19) to bring the final volume to 100 µL.

b. Mix thoroughly by repeated pipetting or gentle vortexing.

2. Add 350 µL of aRNA Binding Buffer

Add 350 µL of aRNA Binding Buffer to each aRNA sample, and mix thoroughly by repeated pipetting or gentle vortexing.

3. Add 250 µL of 100% ethanol

Add 250 µL of ACS grade 100% ethanol to each aRNA sample, and mix thoroughly by repeated pipetting or gentle vortexing.

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. Pass sample through an aRNA Filter Cartridge(s)

a. Place an aRNA Filter Cartridge in an aRNA Collection Tube, and pipet each sample mixture from step 3 onto the center of the filter in the aRNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 X g, or until the mixture has passed through the filter.

c. Discard the flow-through and replace the aRNA Filter Cartridge in the aRNA Collection Tube.

5. Wash with 650 µL aRNA Wash Buffer

Make sure that the ethanol has been added to the bottle of aRNA Wash Buffer before using it.

a. Apply 650 µL aRNA Wash Buffer to each aRNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 X g, or until all the wash solution is through the filter.

c. Discard the flow-through and spin the aRNA Filter Cartridge for an additional ~1 min to remove trace amounts of ethanol.

d. Transfer Filter Cartridge(s) to a fresh aRNA Collection Tube.

6. Elute aRNA with 2 x 50 µL 50°C Nuclease-free Water

NOTE

(Optional) A single elution with 100 µL preheated Nuclease-free Water can replace the 2 x 50 µL elutions described in steps a–c.

a. To the center of the filter, add 50 µL Nuclease-free Water that is preheated to 50°C.
Replace the Nuclease-free Water back in the 50°C incubator.

b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 X g, or until the solution is through the filter.

c. Repeat the elution with a second 50 µL of Nuclease-free Water. The aRNA will now be in the aRNA Collection Tube in 100 µL of the solution used for elution.

d. Discard the aRNA Filter Cartridge.

7. Store purified aRNA at –20°C or continue the procedure

STOPPING POINT

The purified aRNA can be stored at –20°C at this point if desired, or determine the aRNA concentration using one of the methods described in section III.A. aRNA Quantitation on page 21. The aRNA concentration must be known in order to continue to the next step: the dye coupling reaction.

III. Assessing aRNA Yield and Quality

A. aRNA Quantitation

1. Assessing aRNA yield by UV absorbance

The concentration of aRNA can be determined by diluting an aliquot of the preparation (usually a 1:50 to 1:100 dilution) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and reading the absorbance in a spectrophotometer at 260 nm. Be sure to zero the spectrophotometer using the TE that was used for sample dilution.

An A_{260} of 1 is equivalent to 40 µg RNA/mL

The concentration (µg/mL) of RNA is therefore calculated by multiplying the A_{260} X dilution factor X 40 µg/mL.

Following is a typical example:

aRNA is in 100 µL
2 µL of the prep is diluted 1:50 into 98 µL of TE
A_{260} = 0.15
RNA conc = 0.15 x 50 x40 µg/mL = 300 µg/mL or 0.3 µg/µL
There are 98 µL of the aRNA left: 98 µL x 0.3 µg/µL = 29.4 µg

2. Assessing aRNA yield with RiboGreen®

If you have a fluorometer, or a fluorescence microplate reader, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen.

B. Analysis of aRNA on Agarose Gels

aRNA can form extensive secondary structure via intramolecular base pairing. Because of this, it is best to use a denaturing gel system to size-fractionate aRNA. Be sure to include a positive control on the gel so that any unusual results can be attributed to a problem with the gel or a problem with the RNA under analysis. RNA molecular weight markers, an RNA sample known to be intact, or both, can be used for this purpose. Ambion® NorthernMax® reagents for Northern Blotting include everything needed for denaturing agarose gel electrophoresis. These products are optimized for ease of use, safety, and low background, and they include detailed instructions for use. An alternative to using the NorthernMax reagents is to use the procedure described below for electrophoresis in a formaldehyde denaturing agarose gel. This procedure is modified from *Current Protocols in Molecular Biology*, Section 4.9 (Ausubel).

1. Prepare the gel

a. For 100 mL of gel, dissolve 1 g agarose in 72 mL water and cool to 60°C.

b. Add 10 mL 10X MOPS running buffer, and 18 mL of 37% formaldehyde (12.3 M).

CAUTION

Formaldehyde is toxic through skin contact and inhalation of vapors. Manipulations involving formaldehyde should be done in a chemical fume hood.

10X MOPS running buffer	
Concentration	Component
0.4 M	MOPS, pH 7.0
0.1 M	sodium acetate
0.01 M	EDTA

c. Pour the gel and allow it to set. The wells should be large enough to accommodate at least 60 µL. Remove the comb, and place the gel in the gel tank. Cover with a few millimeters of 1X MOPS running buffer.

2. Prepare the RNA samples

a. Determine the concentration of the aRNA (see section III.A on page 21).

b. Transfer 1–3 µg of each aRNA sample to a microfuge tube. Add Nuclease-free Water to bring the volume to 11 µL.

c. Add the following to each RNA sample, and heat to 55°C for 15 min.

Amount	Component
5 µL	10X MOPS running buffer
9 µL	12.3 M formaldehyde
25 µL	formamide

d. Add 10 µL formaldehyde loading dye

Formaldehyde loading dye	
1 mM	EDTA
0.25%	bromophenol blue
0.25%	xylene cyanol
50%	glycerol
60 µg/mL	(optional) ethidium bromide

3. Electrophoresis

a. Load the samples, and electrophorese at 5 V/cm until the bromophenol blue (the faster-migrating dye) has migrated one-half to two-thirds the length of the gel.

b. Visualize the gel on a UV transilluminator. (If ethidium bromide was not added to the formaldehyde loading dye, stain the gel for ~20 min in 1X MOPS running buffer with 0.5 µg/mL ethidium bromide, and destain with two 10 min incubations in water.)

4. Expected Results

Amplified aRNA should appear as a smear from 250 to 5000 nt. The average size of the aRNA should be approximately 1500 nt.

C. aRNA Analysis Using the Agilent 2100 Bioanalyzer

The Agilent 2100 Bioanalyzer with Caliper's LabChip® technology (RNA 6000 Assay) is a good alternative to conventional gel analysis for characterizing aRNA. The RNA 6000 assay can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis (section III.A on page 21). You will need an RNA 6000 LabChip Kit (Agilent Technologies Cat #5064-8229) and RNA 6000 Ladder (Ambion P/N AM7152). Follow the manufacturer's instructions for running the assay.

To rapidly analyze aRNA size distribution, run 1 µL of purified aRNA (from step II.G.6 on page 20) per LabChip well. Bioanalyzer quantitation should be confirmed with UV absorbance or RiboGreen quantitation.

The expected aRNA profile for unmodified aRNA is a distribution of sizes from 250–5500 nt with a peak centered at 1000–1500 nt (see Figure 5 on page 32). To compare bioanalyzer profiles of different aRNA samples, be sure to load equal mass amounts to get an accurate comparison. When comparing unmodified aRNA with amino allyl modified aRNA you will notice that amino allyl aRNA migrates at a slightly higher molecular weight than unmodified aRNA.

IV. Amino Allyl aRNA:Dye Coupling and Clean-up

This kit was developed with Cy3 and Cy5 dyes from Amersham Pharmacia Biotech, but mono-reactive NHS esters of any label moiety should be capable of coupling to the amino allyl modified aRNA generated with this kit. For a partial list of commercially available NHS ester dyes see section *I.C. Materials Not Provided with the Kit* on page 7. The choice of dye will depend on your preference and type of microarray scanning equipment.

A. Concentrate the Amino Allyl aRNA

1. Determine the aRNA concentration

(See section III.A on page 21 for instructions on determining the aRNA concentration.)

2. Vacuum dry 5–20 µg aRNA

- Place 5–20 µg of amino allyl aRNA in a nuclease-free microfuge tube, and vacuum dry to completion on the medium or low heat setting.
- Check the progress of drying every 5–10 min, and remove the sample from the concentrator as soon as it is dry; do not overdry.

B. NHS Ester Dye Preparation

Prepare dye immediately before starting the dye coupling procedure; the table below shows our recommendations for solubilizing several commercially available NHS ester dyes. The preparation and storage of solubilized dye is important for the efficient labeling of amino allyl modified aRNA. It is imperative that the dye compounds remain dry both before and after dissolving in DMSO because any water that is introduced will cause hydrolysis of the NHS esters, lowering the efficiency of coupling.

IMPORTANT

Store any unused solubilized dye in the dark at –80°C. Note that most dye manufacturers do not recommend storing solubilized dyes for more than ~1 month.

Table 1. NHS Ester Dye Preparation

Dye Type	Preparation Instructions
Amersham Biosciences CyDye Post Labelling Reactive Dyes	These dyes are supplied in single-use quantities. Resuspend one vial with 11 µL of DMSO and keep in the dark at room temp for up to 1 hr until you are ready to use it.
Amersham Biosciences FluoroLink Cy5 and Cy3 monofunctional dye 5-pack ***Molecular Probes*** Alexa Fluor Succinimidyl Esters	These dyes are supplied in relatively large aliquots; resuspend one dye vial in 88 µL of DMSO and keep in the dark at room temp for up to 1 hr until you are ready to use it.
Denovo Biolabels GmbH Oyster® Dyes	Resuspend one dye vial in 55 µL of DMSO and keep in the dark at room temp for up to 1 hr until you are ready to use it.

C. aRNA:Dye Coupling Reaction

1. Add 9 µL Coupling Buffer to the aRNA

To a tube containing the dried amino allyl aRNA (5–20 µg), add 9 µL Coupling Buffer and mix well.

2. Add 11 µL prepared dye to the aRNA and mix well

Add 11 µL of NHS ester dye in DMSO to the aRNA:Coupling Buffer mixture. Mix well.

3. Incubate 30 min at room temp in the dark

This 30 min incubation at room temp allows the dye coupling reaction to occur. To keep the samples in the dark, typically it is sufficient to simply put the tubes in a closed drawer.

4. Add 4.5 µL 4M Hydroxylamine, mix, and incubate 15 min in the dark

To quench the reaction, add 4.5 µL 4M Hydroxylamine and mix well. Incubate the reaction at room temperature in the dark for 15 minutes.

During this incubation, the large molar excess of hydroxylamine will quench the amine-reactive groups on the unreacted dye molecules.

D. Dye Labeled aRNA Purification

Repeat the aRNA purification procedure described in section II.G starting on page 19 using a fresh aRNA Filter Cartridge and aRNA Collection Tube.

The filter in the aRNA Filter Cartridge may acquire the color of the fluorescent dye during the purification. This is from the labeled aRNA binding to the filter. Most of the color should disappear when the purified aRNA is eluted.

If the sample will not be used the same day, store it at –20°C in the dark.

E. Spectrophotometric Analysis of Dye Incorporation

1. Determine the appropriate dilution for spectrophometer readings

Dilute 5 µL labeled aRNA in 95 µL 10 mM Tris-HCl, 1 mM EDTA (TE) (a 1:20 dilution). Mix well, place in a quartz cuvette and measure the A_{260} in a UV-Vis spectrophotometer.

If the A_{260} is below 0.1 reduce the dilution factor and check the A_{260} again. To obtain an accurate reading at both 260 nm and at the maximum absorbance wavelength for the dye, the aRNA dilution should result in an A_{260} between 0.1 and 1.0.

2. Measure the aRNA absorbance at 260 nm and at the absorbance max for the dye

Blank the instrument with the TE used for making dilutions. Measure the absorbance of each sample at 260 nm (A_{260}) and also at the maximum absorbance wavelength for the dye used in the coupling reaction (A_{dye}).

Absorbance maximum	Dye type	Extinction coefficient*
491 nm	fluorescein	66,000
491 nm	rhodamine	60,000
550 nm	Cy3	150,000
650 nm	Cy5	250,000
546 nm	Alexa Fluor 546	104,000
555 nm	Alexa Fluor 555	150,000
663 nm	Alexa Fluor 660	132,000
650 nm	Alexa Fluor 647	239,000
556 nm	Oyster 556	155,000
645 nm	Oyster 645	250,000
656 nm	Oyster 656	220,000

* Extinction coefficient (ε) at λ_{max} in $cm^{-1}M^{-1}$

3. Calculate the number of dye molecules incorporated per 1000 nt

- With these absorbance values (A_{dye} and A_{260}) in hand, use the convenient dye incorporation calculator on our web site. Find it at: http://www.ambion.com/techlib/misc/aama_dye_calc.html
- Alternatively use this formula to estimate the number of dye molecules incorporated per 1000 nt of labeled aRNA.

$$\frac{\text{\# dye molecules}}{\text{1000 nt}} = \frac{A_{dye}}{A_{260}} \times \frac{9010\ M^{-1}\ cm^{-1}}{\text{dye extinction coefficient}} \times 1000$$

Successful reactions should incorporate 20–50 dye molecules per 1000 nt.

4. (Optional) Calculate the RNA concentration

Use the A_{260} to calculate the RNA concentration if desired (see section *III.A.1. Assessing aRNA yield by UV absorbance* on page 21).

F. Preparing the Labeled aRNA for Hybridization

1. Vacuum dry the labeled RNA that will be used for hybridization

Typically microarrays are hybridized with 25–100 µL Hybridization Solution. Since the labeled aRNA is eluted from the aRNA Filter Cartridge in about 100 µL, it will need to be concentrated in order to use it for microarray hybridization.

We recommend vacuum drying the labeled aRNA in the dark until the volume is reduced to 1–10 µL (cover the lid of the vacuum drier with aluminum foil if necessary).

Bring the volume of the aRNA to 10 µL with either hybridization buffer or Nuclease-free Water and calculate the aRNA concentration based on the amount of RNA coupled to dye or the A_{260} reading after purification.

2. Fragment labeled RNA for hybridization to oligonucleotide micoroarrays

For microarrays printed with oligonucleotides, the labeled aRNA must be fragmented for proper hybridization. Any RNA hydrolysis method that produces RNA fragments in the 60–200 nucleotide size range can be used. We recommend Ambion RNA Fragmentation Reagents (P/N AM8740) for this procedure. Follow the procedure associated with the fragmentation method used.

3. Dilute the labeled aRNA into the hybridization solution

aRNA amount

The amount of aRNA to use for hybridization will depend on your microarray type and will have to be optimized for maximum sensitivity and minimal background.

We recommend hybridizing with 1–5 µg of labeled aRNA per microarray.

Choice of hybridization solution

The choice of hybridization buffer should be based on the type of microarray being hybridized.

V. (Optional) Second Round Amplification

If one cycle of amplification does not yield the amount of aRNA necessary for your experiments, a second round of amplification can be done to generate additional aRNA. In order to do two rounds of amplification, the first round of amplification must contain ***only*** unmodified UTP, ***amino allyl modified aRNA cannot undergo a second round of amplification***.

The procedure is similar to the first round of amplification, and the reaction products are equivalent, but different primers are used for first and second strand synthesis, and the reaction set-up is slightly different.

A. Synthesis of First Strand cDNA (Second Round)

Incubators needed:

- 70°C (thermal cycler recommended)
- 42°C (hybridization oven or air incubator recommended)
- 37°C (hybridization oven or air incubator recommended)

1. Mix RNA and Second Round Primers, and bring the volume to 12 µL

Incubate 10 min at 70°C

a. Place up to 2 µg of purified aRNA from the first round of amplification into a sterile RNase-free microfuge tube.

IMPORTANT

The volume of the aRNA must be ≤10 µL. If necessary, concentrate the aRNA in a vacuum centrifuge until the volume is reduced to ≤10 µL. Do not dry the aRNA completely, as this might impede reverse transcription.

b. Add 2 µL of Second Round Primers.

c. Add Nuclease-free Water to bring the volume to 12 µL.

d. Incubate 10 minutes at 70°C (see *Incubator type* on page 12).

e. Remove the RNA samples from the 70°C incubator and centrifuge briefly (~5 sec) to collect the sample at the bottom of the tube. Place the reaction on ice briefly before starting step 2.

2. At room temp, add the remaining reverse transcription reagents to each aRNA/Primer mixture:

Amount	Component
2 µL	10X First Strand Buffer
1 µL	Ribonuclease Inhibitor
4 µL	dNTP Mix
1 µL	Reverse Transcriptase

Mix well by gently pipetting up and down or flicking the tube a few times, then centrifuge briefly (~5 sec) to collect sample at bottom of tube.

3. Incubate 2 hr at 42°C

After incubation, centrifuge briefly (~5 sec) to collect the sample at the bottom of tube. Proceed immediately to the next step.

4. Add 1 µL RNase H and incubate for 30 minutes at 37°C

RNase H specifically degrades the aRNA leaving only the cDNA as template for second strand synthesis. This helps assure that the second strand synthesis reaction will be primed exclusively by the T7 Oligo(dT) Primer.

After this RNase H treatment is complete, proceed directly to Second Strand Synthesis (below).

B. Synthesis of Second Strand cDNA (Second Round)

Incubator needed:

- 70°C (thermal cycler recommended)
- 16°C (thermal cycler recommended)

1. Add 5 µL T7 Oligo(dT) Primer

a. Add 5 µL T7 Oligo(dT) Primer to the first strand reaction tube from step 4 above.

b. Mix well by gently pipetting up and down or flicking the tube a few times, then centrifuge briefly (~5 sec) to collect the sample at the bottom of the tube.

2. Incubate for 10 min at 70°C

After the 10 min incubation at 70°C, place the reaction on ice briefly before adding the remaining second strand cDNA synthesis reagents.

3. Add the second strand cDNA synthesis reagents in the order listed:

Amount	Component
(26 µL)	first strand cDNA + T7 Oligo(dT) Primer (from previous step)
58 µL	Nuclease-Free Water
10 µL	10X Second Strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase
100 µL	Total Volume

Mix well by gently pipetting up and down or flicking the tube a few times, then centrifuge briefly (~5 sec) to collect sample at bottom of tube.

4. Incubate for 2 hr at 16°C

Incubate 2 hr in a 16°C thermal cycler. If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the reactions with the heated lid turned off, or if the lid cannot be turned off—do not cover the reactions. (Do not use a water bath or a heat block in a 4°C refrigerator for this incubation because the temperature will fluctuate too much.)

5. Continue with the procedure at section II.E starting on page 16

Complete the rest of the second round amplification according to the instructions for the first round of amplification. Start at section *II.E. cDNA Purification* on page 16, and continue through the remainder of section II.

VI. Troubleshooting

A. Positive Control Reaction

Amplification of the Control RNA

To establish if the kit is working properly, a tube of Control RNA consisting of 1 mg/mL HeLa cell total RNA is provided. Use 2 µL of the Control RNA in an Amino Allyl MessageAmp reaction; follow the procedure starting at section II.C step 1 on page 14. Continue with procedure for making amino allyl aRNA through section II.G on page 19.

Analysis of the positive control amplification

After completing the aRNA purification, measure the A_{260} of the reaction product as described in section III.A.1 on page 21.

The positive control reaction should produce ≥70 µg of aRNA.

Be aware that often the positive control reaction cannot be compared to experimental reactions, because many experimental amplification experiments will use much less than the 2 µg of input RNA used in the positive control reaction, and the aRNA yield will be proportionately lower. Also the Control RNA is of exceptional quality and purity compared to typical experimental RNA, ensuring that it will amplify with extremely high efficiency.

Also run a 2 µg aliquot of the reaction products on a denaturing agarose gel (instructions in section III.B starting on page 21) or 100–200 ng on a bioanalyzer; ***the average size of the aRNA should be at ≥1 kb***.

Labeling the amino allyl modified aRNA

Use 10 µg of aRNA in a dye coupling reaction following the instructions in section IV.A through IV.E starting on page 24.

Evaluate the positive control reaction by determining the number of dye molecules incorporated per 1000 nt as described in section *IV.E. Spectrophotometric Analysis of Dye Incorporation* on page 26.

Successful reactions should incorporate 20–50 dye molecules per 1000 nt.

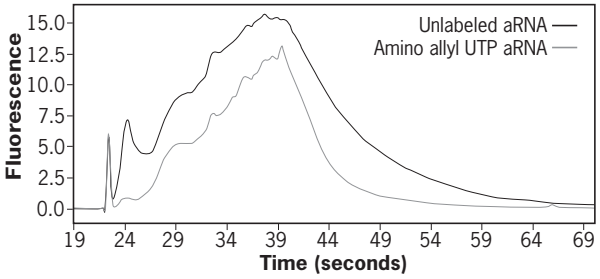


Figure 5. Bioanalyzer Electropherogram of aa-UTP Modified aRNA and Unmodified aRNA.

The same double-stranded cDNA was used as a template for in vitro transcription reactions containing either 50% aaUTP or 100% UTP. Each reaction was run on an Agilent bioanalyzer using the RNA 6000 Marker and Nano LabChip Kit. The average size and yield of aRNA is very similar regardless of whether 50% aaUTP was included in the amplification reaction.

B. Yield of aRNA is Low

1. Low aRNA yield from the Control RNA and from your RNA

a. Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective amplification of RNA.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.
- If a thermal cycler is used for incubation, be sure to use the lid with the heat turned off. If the heat cannot be turned off in the thermal cycler lid, do not cover the reaction vessel(s) with it.

b. Condensation formed in the tube during the reaction incubation(s)

Condensation occurs when the cap of the reaction vessel is cooler (e.g. room temperature) than the bottom of the tube. As little as 1–2 µL of condensate in a transcription reaction tube throws off the concentration of the nucleotides and the magnesium which are crucial for good yield.

If you see condensation occurring, spin the tube briefly and mix the reaction gently. Move the tube(s) to an incubator where condensation doesn't occur, or is minimized.

c. Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, etc. that are contaminated with nucleases can cleave the RNA or DNA being generated at each step in the procedure. This will reduce the size of the aRNA products and decrease aRNA yield. Both RNases and DNases can be removed from surfaces using Ambion® RNase*Zap*® RNase Decontamination Solution.

d. Absorbance readings are inaccurate

Confirm that your spectrophotometer is accurate by measuring the absorbance of an RNA or DNA sample of known concentration. Alternatively, assess the aRNA concentration by fractionating on an agarose gel adjacent to an RNA sample whose concentration is known. Comparing the ethidium bromide staining of the aRNA and control samples can approximate the concentration of the aRNA.

2. Low aRNA yield from your RNA, but the Control RNA works well

a. Impure RNA samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA or use the Ambion MEGAclear™ Kit (P/N AM1908) to further purify it before reverse transcription.

b. Lower than suspected total or poly(A) RNA concentration

Take another A_{260} reading of your RNA sample or try amplifying more RNA using the aRNA amplification procedure.

c. RNA sample integrity is poor

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See section *II.A. RNA integrity* on page 10 for more information).

d. The mRNA content of your total RNA sample is lower than expected

Different RNA samples contain different amounts of mRNA. mRNA constitutes from 1–3% of total cellular RNA. The actual amount of mRNA depends on the cell type and the physiological state of the sample. When calculating the amount of amplification, the starting mass of mRNA in a total RNA prep should always be considered a range from 10–30 ng per µg of total RNA (assuming good RNA quality). Most total RNA samples can be amplified up to 1000 fold producing 10–30 µg of aRNA from 1 µg of total RNA.

C. Average aRNA Size is <500 nt

1. The average size of aRNA from both your sample RNA and the Control RNA is <500 nt

a. Incubation temperature(s) were incorrect

The incubation temperatures are critical for the effective amplification of your RNA sample.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.
- If a thermal cycler is used for incubation, be sure to use the lid with the heat turned off. If the heat cannot be turned off in the thermal cycler lid, do not cover the reaction vessel(s) with it.

b. Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, etc. that are contaminated with nucleases can cleave the RNA or DNA being generated at each step in the procedure. This will reduce the size of the aRNA products and decrease aRNA yield. Both RNases and DNases can be removed from surfaces using Ambion RNase*Zap*® Solution.

c. There was a problem with the gel electrophoresis

Agarose gels that are not completely denaturing or that are not the proper percent agarose can provide inaccurate estimates of aRNA size. Fractionate a known RNA sample in an adjacent lane to effectively estimate the size of the aRNA.

2. Your sample RNA yields aRNA with an average size less than 500 nt, but the Control RNA works well

a. Impure RNA samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA or use the Ambion MEGAclear™ Kit (P/N AM1908) to further purify it before reverse transcription.

b. Integrity of RNA sample is poor

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See section *II.A. RNA integrity* on page 10 for more information).

D. aRNA is Not Efficiently Reverse Transcribed

1. The cDNA procedure relies on oligo(dT) priming

The aRNA has a poly(U) tract near the 5' end but lacks a poly(A) tract at its 3' end. Thus any reverse transcription procedures that rely on oligo(dT) primers will not effectively convert aRNA to cDNA. Try using gene specific or random primers.

2. The filter in the aRNA Filter Cartridge was not completely dried after the wash steps

If the aRNA contains ethanol carried over from the aRNA Wash Buffer, it can inhibit reverse transcription. Make sure that the filter is completely dry at step 5.c on page 20 just before eluting the aRNA.

3. Absorbance readings are inaccurate

Confirm that your spectrophotometer is accurate by measuring the absorbance of an RNA or DNA sample of known concentration. Alternatively, assess the quantity of aRNA by a different method such as fractionating on an agarose gel adjacent to an RNA sample whose concentration is known and comparing the ethidium bromide staining or using a sensitive RNA dye like Ribogreen.

E. Troubleshooting Dye Coupling

NHS ester dye coupling is a simple and robust chemical reaction, but unreacted NHS ester dye solutions are susceptible to hydrolysis and photobleaching. Dye esters that have been improperly handled or stored may lose much of their reactivity. Refer to information provided by the NHS ester dye supplier for more information.

VII. Appendix

A. (Optional) cDNA Purification using Phenol

This phenol cDNA purification is an alternative to the glass fiber filter purification described in section II.E.

1. Add 100 µL phenol/chloroform/isoamyl alcohol (25:24:1) to the cDNA preparation from step II.D.2 on page 15 and vortex well to mix.
2. Centrifuge at 14,000 rpm for 15 min at room temp. Transfer aqueous (upper) phase to a fresh tube.
3. Add the following ice-cold reagents to the tube

Amount	Component
2 µL	5 µg/µL glycogen
50 µL	5 M NH_4OAc (0.5 volumes)
375 µL	absolute ethanol (2.5 volumes)

4. Incubate 20 min at –20°C.
5. Centrifuge at 14,000 rpm for 20 min at 4°C. Carefully remove and discard supernatant.
6. Wash the pellet with 500 µL 70% cold ethanol, centrifuge at 14,000 rpm for 10 min at 4°C. Carefully remove and discard the ethanol.
7. To remove the last traces of ethanol, quickly re-spin the tube, and aspirate any residual fluid with a very fine tipped pipette, or with a syringe needle.
8. Air-dry the cDNA pellet.
9. Resuspend pellet in 8 µL Nuclease-free Water, and continue with the procedure.

B. References

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C. Quality Control

Functional Testing

The Control RNA (2 µg) is used in an aRNA amplification experiment following the instructions in section VI.A on page 31. The yield of amino allyl aRNA is >90 µg. Cy5 is used to label the 10 µg of the amino allyl aRNA following the instructions in section IV starting on page 24; 50–60 dye molecules per 1000 nt are incorporated.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

D. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.