PROTOCOL

ambion® | RNA
by life technologies™

Amino Allyl MessageAmp™ II aRNA Amplification Kit

life technologies™

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Amino Allyl MessageAmp™ II aRNA Amplification Kit

(Part Number AM1753, AM1795, AM1796, AM1797)

Protocol

I. Introduction

IMPORTANT

Before using this product, read and understand the "Safety Information" in the appendix in this document.

A. Product Description and Background

The Amino Allyl MessageAmp™ II aRNA Amplification Kit is based on the RNA amplification protocol developed in the Eberwine laboratory (Van Gelder et al. 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™ reverse transcriptase (RT), engineered to produce higher yields of first-strand cDNA than wild-type enzymes. ArrayScript RT catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second-strand synthesis and cleanup to become a template for in vitro transcription (IVT) with T7 RNA polymerase. To maximize aRNA yield, Ambion MEGAscript® IVT technology is used to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. (In this protocol the antisense amplified RNA is referred to as aRNA; it is also commonly called cRNA.) The IVT 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 N-hydroxysuccinimidyl ester-derivatized reactive dyes (NHS ester dyes), such as Cy™3 and Cy5, in a simple, efficient reaction (see Figure 1).

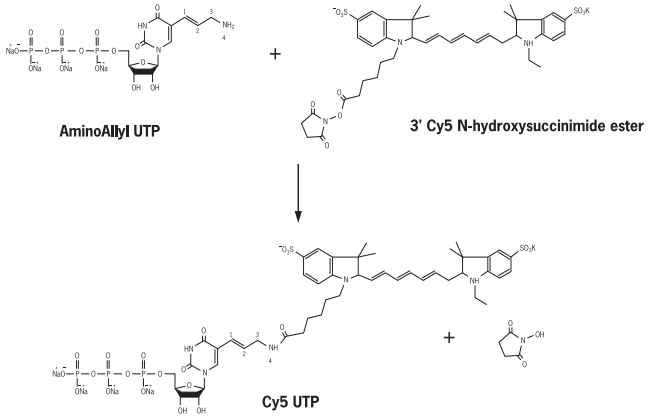
Once purified, the labeled aRNA is suitable for use on most commercially available microarray gene expression systems. RNA samples of limited amounts (0.1–100 ng) can be put through two rounds of amplification if desired. This strategy makes the production of microarray samples from picogram amounts of total RNA entirely possible (Luo et al. 1999).

Benefits of RNA amplification

RNA amplification was originally developed as a method to expand very small RNA samples to produce enough material for array hybridization (Yue et al. 2001). Several groups have conducted studies to determine whether amplification of RNA introduces bias and they report that any bias is minimal (Li et al. 2004, Feldman et al. 2002 and Polacek et al. 2003). Additionally, among the benefits of amplification is a more reproducible expression profile from a wide range of RNA inputs. Some researchers conclude that amplification actually improves the reliability of array results regardless of whether it is needed for sample expansion

(Feldman et al. 2002 and Polacek et al. 2003). As a result, RNA amplification has become the standard method for preparing RNA samples for array analysis (Kacharmina et al. 1999, Pabon et al. 2001).

Figure 1. Amino Allyl Labeling Reaction

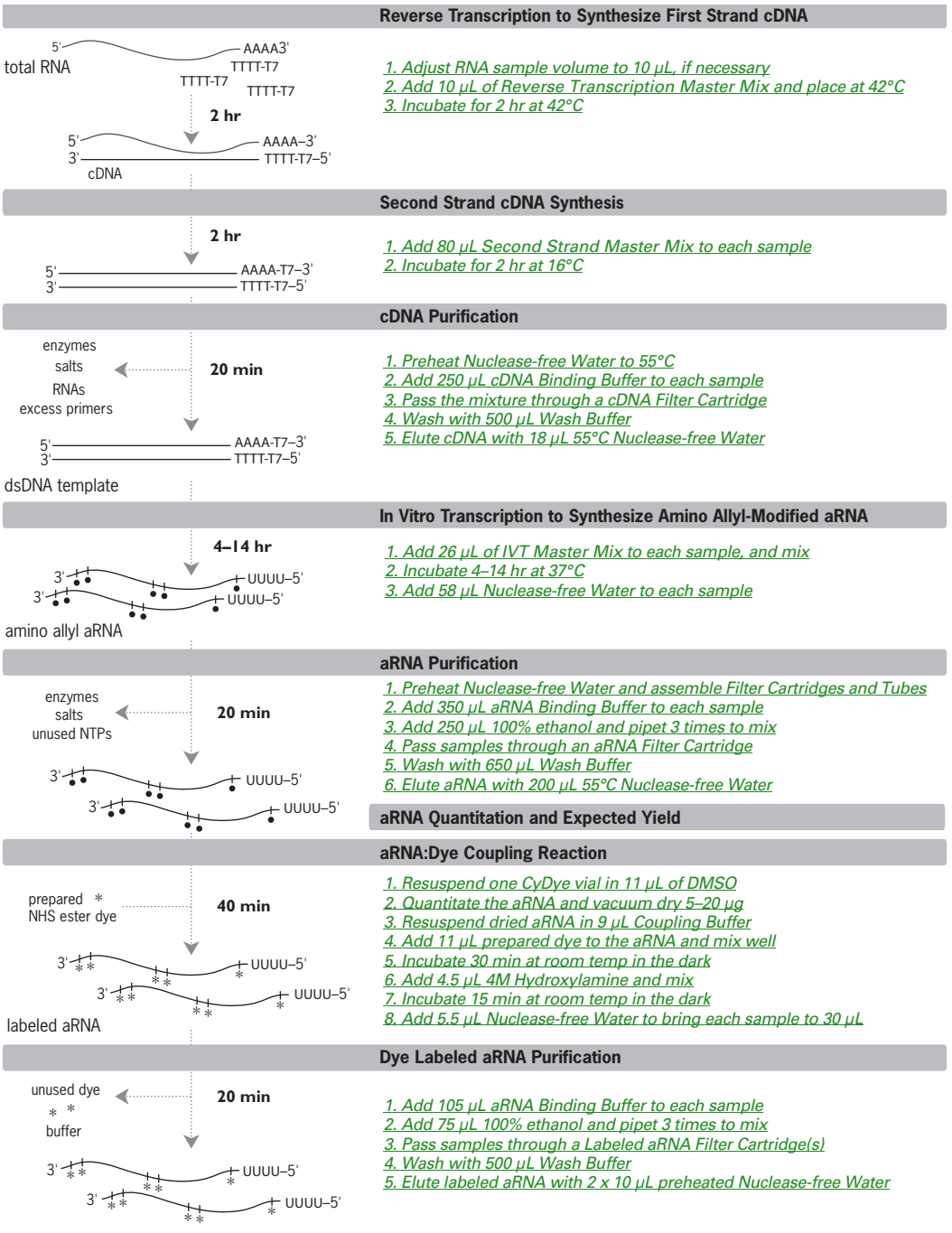


B. Procedure Overview

The Amino Allyl MessageAmp II aRNA amplification procedure is depicted in Figure 2.

- ***Reverse Transcription to Synthesize First Strand cDNA*** is primed with the T7 Oligo(dT) Primer to synthesize cDNA containing a T7 promoter sequence.
- ***Second Strand cDNA Synthesis*** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA Polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.
- ***cDNA Purification*** removes RNA, primers, enzymes, and salts that would inhibit in vitro transcription.
- ***In Vitro Transcription to Synthesize Amino Allyl-Modified aRNA*** with aaUTP generates multiple copies of amino allyl-modified aRNA from the double-stranded cDNA templates; this is the amplification step.
- ***aRNA Purification*** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the aRNA and to facilitate NHS ester coupling or subsequent enzymatic reactions.

Figure 2. Amino Allyl MessageAmp II aRNA Amplification Procedure

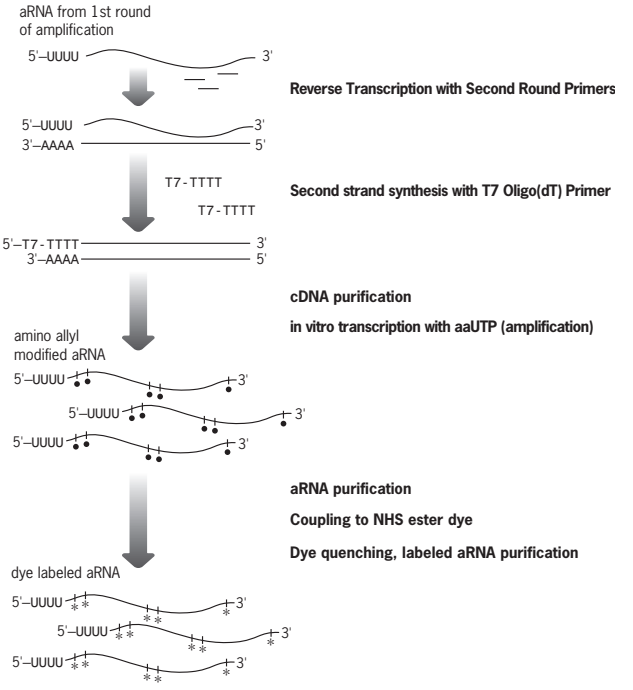


- ***aRNA:Dye Coupling Reaction*** takes place between the amino allyl-modified UTP residues on the aRNA and amine reactive Cy3 or Cy5 dyes.
- ***Dye Labeled aRNA Purification*** removes free dye and exchanges the buffer with Nuclease-free Water.

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 reagents and methodology used in the first and second rounds of amplification are only slightly different (see section V starting on page 28.) When doing 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) cannot be amplified.

Figure 3. Second Round Amplification



The Amino Allyl MessageAmp II advantage

Each step in the Amino Allyl MessageAmp II aRNA Amplification Kit amplification procedure has been streamlined and optimized. The first-strand cDNA synthesis reaction employs ArrayScript reverse transcriptase 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 also efficiently removes RNA from the cDNA sample. This eliminates the heating or enzymatic digestion step commonly used in other procedures to degrade RNA (especially ribosomal RNA). The IVT reaction features MEGAscript technology to maximize transcriptional amplification and yield of aRNA. It is optimized to ensure efficient transcription of limited amounts of input DNA and synthesis of long transcripts.

The NTPs are provided in three separate vials. 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 IVT reaction has only a minor effect on the reaction efficiency and yield (Hoen et al. 2003). Additionally, since the incorporation of aaUTP by IVT 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 by in vitro transcription. Simple, rapid aRNA purification procedures are used to prepare the aRNA for dye coupling and to remove free dye molecules after the coupling reaction in preparation for array hybridization.

C. Materials Provided with the Kit and Storage Conditions

Amino Allyl MessageAmp II aRNA Amplification Kits

The Amino Allyl MessageAmp II aRNA Amplification Kits are available with Cy™3 and Cy5 reactive dyes.

Store Cy3 and Cy5 reactive dyes at –20°C in a non-frost-free freezer.

P/N	Product
AM1753	Amino Allyl MessageAmp II aRNA Amplification Kit
AM1795	Amino Allyl MessageAmp II with Cy3 Dye • P/N AM1753 Amino Allyl MessageAmp II aRNA Amplification Kit • 20 single-use tubes of Cy3 reactive dye (in 4 packs of 5 tubes)
AM1796	Amino Allyl MessageAmp II with Cy5 Dye • P/N AM1753 Amino Allyl MessageAmp II aRNA Amplification Kit • 20 single-use tubes of Cy5 reactive dye (in 4 packs of 5 tubes)
AM1797	Amino Allyl MessageAmp II with Cy3/Cy5 Dye • P/N AM1753 Amino Allyl MessageAmp II aRNA Amplification Kit • 5 single-use tubes of Cy3 reactive dye (1 pack of 5 tubes) • 15 single-use tubes of Cy5 reactive dye (3 packs of 5 tubes)

P/N AM1753 Amino Allyl MessageAmp II Kit components

The Amino Allyl MessageAmp II aRNA Amplification Kit includes reagents for single-round amplification of 20 samples or two-round amplification of 10 samples.

cDNA synthesis and IVT reagents

Do ***not*** store reagents in a frost-free freezer.

Amount	Component	Storage
60 µL	T7 Oligo(dT) Primer	–20°C
22 µL	ArrayScript™ Reverse Transcriptase	–20°C
22 µL	RNase Inhibitor	–20°C
42 µL	10X First Strand Buffer	–20°C
170 µL	dNTP Mix	–20°C
210 µL	10X Second Strand Buffer	–20°C
42 µL	DNA Polymerase	–20°C
22 µL	RNase H	–20°C
84 µL	T7 Enzyme Mix	–20°C
84 µL	T7 10X Reaction Buffer	–20°C
95 µL	UTP Solution (50 mM)	–20°C
64 µL	aaUTP [(50 mM) 5-(3-amino allyl)-UTP]	–20°C
255 µL	ATP, CTP, GTP Mix	–20°C
40 µL	Second Round Primers	–20°C
10 µL	Control RNA (1 mg/mL HeLa total RNA)	–20°C
1.75 mL	Nuclease-free Water	any temp*

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

Some reagents may form a precipitate when stored at –20°C. If a precipitate is visible, redissolve it by warming the solution to room temperature with gentle mixing.

cDNA and aRNA purification components, and dye labeling reagents

Do ***not*** store reagents in a frost-free freezer.

Amount	Component	Storage
400 µL	Coupling Buffer	–20°C
440 µL	DMSO	–20°C
180 µL	4M Hydroxylamine	–20°C
40 mL	Wash Buffer (Add 32 mL 100% ethanol before use)	4°C or room temp
7 mL	cDNA Binding Buffer	room temp*
20 mL	aRNA Binding Buffer	room temp
20	aRNA Filter Cartridges	room temp

Amount	Component	Storage
40	aRNA Collection Tubes	room temp
20	cDNA Filter Cartridges + Tubes	room temp
20	cDNA Elution Tubes	room temp
10 mL	Nuclease-free Water	any temp†
20	Labeled aRNA Filter Cartridges + Tubes	room temp
20	Labeled aRNA Elution Tubes	room temp

* The cDNA Binding Buffer may form a precipitate if stored colder than room temperature. 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 temperature before use.

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

D. Materials Not Provided with the Kit

Lab equipment and supplies

- 100% Ethanol (to prepare the Wash Buffer)
- Additional 100% DMSO may be required, depending on how the fluorescent dye is supplied
- Thermal cycler with adjustable-temperature heated-lid (recommended), hybridization oven, or constant temperature incubators set at 70°C, 42°C, 37°C, and 16°C (See *Thermal cycler recommended* on page 12 for more information.)
- Heat block set at 55°C, for preheating the water for cDNA and aRNA purification
- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- Non-stick RNase-free 0.5 mL microcentrifuge tubes (P/N AM12350)
- RNase-free pipettors and tips, positive-displacement type recommended to increase the accuracy and precision of reaction inputs
- (Optional) RNA controls for microarrays analysis, such as Array Control™ RNA Spikes (P/N AM1780) or the GeneChip® Eukaryotic Poly-A RNA Control Kit from Affymetrix® (Cat #900433)
- (Optional) Non-stick RNase-free tubes for storage of cDNA (e.g., AM 12450)

Optional materials and equipment for RNA analysis

- Spectrophotometer—such as the NanoDrop ND-1000 or ND-8000 UV-Vis Spectrophotometer. Follow the manufacturer's instructions.
- (Optional) Agilent bioanalyzer and RNA LabChip Kits
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen (R11490) for use with a fluorescence microplate reader, standard spectrofluorometer, or filter fluorometer

E. Related Products

MessageAmp™ aRNA Amplification Kits See web or print catalog for P/Ns	A full line of Ambion® MessageAmp Kits is available, 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, the MessageAmp Premier and MessageAmp III RNA Amplification Kits are available. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits, which are available with and without Cy™3 and Cy5 dyes. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. The MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits are offered for high-throughput applications.
FirstChoice® Total RNA See web or print catalog for P/Ns	High-quality total 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 RNAs are ready for use in any application that requires highly purified, intact RNA. See the catalog or website (www.invitrogen.com/ambion) for a complete listing of available FirstChoice RNAs.
RNA Isolation Kits See web or print catalog for P/Ns	Family of kits for isolation of total 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. See the catalog or website (www.invitrogen.com/ambion).
GLOBINclear™ Whole Blood Globin Reduction Kits P/N AM1980, AM1981	The GLOBINclear Whole Blood Globin Reduction Kits employ a novel, non-enzymatic technology to remove >95% of the globin mRNA from whole blood total RNA samples. The resulting mRNA is a superior template for RNA amplification and synthesis of labeled cDNA for array analysis. Kits are available for treatment of human or mouse/rat whole blood total RNA.
ArrayControl™ RNA Spikes P/N AM1780	The ArrayControl RNA Spikes are a set of eight control RNA transcripts designed for the normalization and validation of glass microarray experiments. The RNA 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.
Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452	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.
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® RNA Fragmentation Reagents include a 10X Fragmentation Reagent and a Stop Solution.

II. aRNA Amplification Procedure

A. Important Parameters for Successful Amplification

Input RNA quantity and IVT reaction incubation time

Consider both the amount of sample RNA you have and the amount of aRNA needed for your analysis when planning Amino Allyl MessageAmp II kit experiments. These factors will influence how much input RNA to use, whether one or two rounds of amplification should be done, and how long to incubate the IVT reaction.

Accurate quantitation

For experiments where the aRNA yield from different samples will be compared, it is ***essential*** to accurately quantify the input RNA used in the Amino Allyl MessageAmp II kit procedure. The NanoDrop 1000A Spectrophotometer is recommended for rapid, accurate quantitation of nucleic acids; however, any reliable RNA quantitation method, such as traditional spectrophotometry or RiboGreen, can be used.

Recommended minimum and maximum amounts of input RNA

Table 1 shows the mass of total RNA that can be used in the Amino Allyl MessageAmp II aRNA Amplification procedure. The RNA volume must be ≤10 µL.

Table 1. Total RNA Input for Amino Allyl MessageAmp II Procedure

Amplification	Recommended	Minimum	Maximum
single round	1000 ng	100 ng	5000 ng
two rounds	100 ng	0.1 ng	100 ng

Determining input RNA amount and IVT reaction incubation time

The procedure can accommodate a wide range of input RNA amounts, but for reproducible and comparable results, use a fixed amount of input RNA for all experiments. Tailor both the amount of input RNA and the amplification procedure to produce the amount of aRNA needed for your microarray hybridizations. Figure 4 shows aRNA yields from different amounts of Control RNA amplified with increasing IVT incubation times. Typically 0.2–5 µg of cyanine dye labeled aRNA is used for hybridization, but the exact amount will depend on the array platform used.

Typically 100 ng of total RNA input is the lower limit for synthesizing ~10 µg of aRNA using a 4 hr IVT reaction. If your total RNA input will be ~100 ng or less, we advise conducting a preliminary amplification from a representative sample to determine how much aRNA you can expect from your experimental samples. If the preliminary experiment

does not produce enough aRNA for two array hybridizations, repeat the experiment using an IVT incubation as long as 14 hr and/or consider using two rounds of amplification.

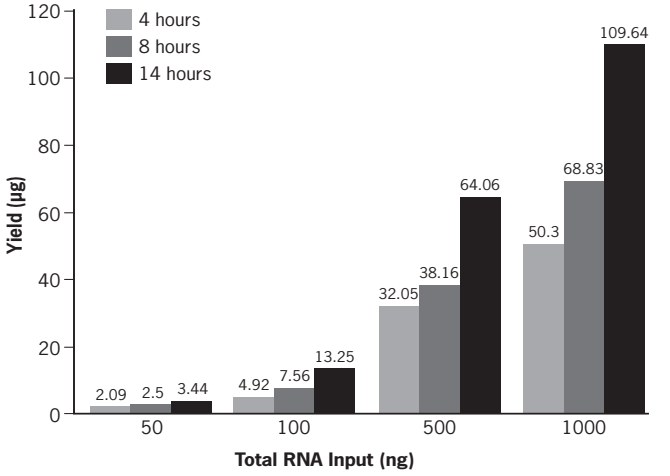


Figure 4. aRNA Yield vs. IVT Incubation Time and Total RNA Input.

The indicated amounts of Control RNA (HeLa total RNA) were amplified for the IVT incubation times shown. Although longer IVT incubation times produced more aRNA, the amount of aRNA needed for most microarrays was obtained using a 4-hour incubation time with ≥500 ng input RNA. Note that this is empirical data obtained using the Control RNA provided with the kit; aRNA yield from experimental samples may be considerably different.

RNA purity

The quality of the RNA is the single most important factor affecting how efficiently an RNA sample will be amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit. 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 must be suspended in high quality water or TE (10 mM Tris-HCl, 1 mM EDTA) or THE RNA Storage Solution (P/N AM7000, AM7001).

RNA integrity

The integrity of the RNA sample, or the proportion that is full-length, is another important component of RNA quality. Reverse transcription of partially degraded mRNAs will typically generate relatively short cDNAs that potentially lack portions of the coding region. RNA integrity can be evaluated by microfluidic analysis using the Agilent

2100 bioanalyzer and RNA LabChip® Kits. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity.

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e., no significant smearing below each band), with the 28S rRNA band appearing 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 gel electrophoresis is that it requires microgram amounts of RNA.

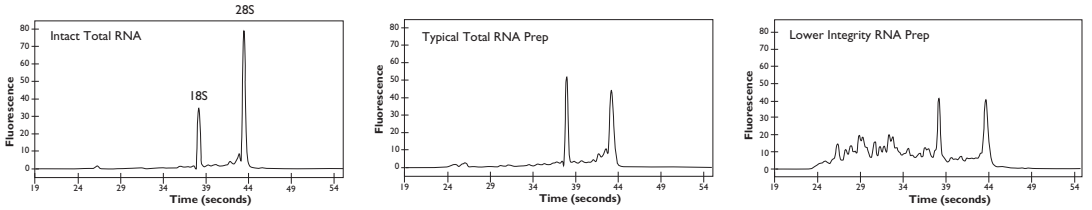


Figure 5. Bioanalyzer Images of Total RNA Preparations

These electropherograms (from the Agilent 2100 bioanalyzer) show RNA samples with decreasing integrity that are all of sufficient quality to use as input for the Amino Allyl MessageAmp II aRNA Amplification Kit. The trace labeled "Intact Total RNA" represents the ideal for a bioanalyzer trace of total RNA. Notice how the ribosomal RNA peaks are at a ratio of about 2 (28S:18S) in this sample; this represents ultrahigh quality RNA in terms of integrity. Most RNA samples will more closely resemble the center trace where there are nearly equal amounts of 28S and 18S rRNA. The trace on the right shows a fairly typical human RNA prep with rRNA peaks that are lower than in the other two traces and where lower molecular weight degradation products become apparent. RNA samples with suboptimal integrity can yield meaningful array analysis results if the data are subjected to rigorous statistical analysis (Schoor et al. 2003).

Reaction incubation times should be precise and consistent

The incubation times for most of the enzymatic reactions in the procedure were optimized in conjunction with the kit reagents to ensure the maximum yield of nucleic acid product in each step—adhere to them closely. An exception is the IVT reaction, where a range of 4–14 hr incubation time is acceptable (step II.F.2 on page 19). Refer to Figure 4 on page 10 to help determine what incubation time to use. Although differences in IVT incubation time among samples has had very little, if any, effect on array results in our hands, we recommend using uniform IVT incubation times if aRNA yield from different samples will be compared or if you want to have equal amplification of different samples—this will provide the most reproducible amplification and array analysis.

Master mixes

We strongly recommend preparing master mixes for the Amino Allyl MessageAmp II aRNA Amplification procedure. This approach reduces the effects of pipetting error, saves time, and improves reproducibility. Using master mixes is especially important when aRNA yield from different samples will be compared.

Thorough mixing is very important for reproducibility

Below are specific instructions for mixing kit reagents, master mixes, and individual reactions. For maximum reproducibility and aRNA yield, follow these instructions closely.

Mix each kit component after thawing.
Mix enzyme solutions by ***gently*** flicking the tube a few times before adding them to reactions. Thaw frozen reagents completely ***at room temperature*** (i.e., primers, nucleotides, and 10X buffers), then mix thoroughly by vortexing, and keep on ice before use.

Mix master mixes by gentle vortexing.
After assembling master mixes, ***gently*** vortex to make a homogenous mixture without inactivating the enzyme(s).

Mix individual reactions by pipetting and flicking the tube.
After adding master mixes or other reagents to individual reactions, pipet up and down 2–3 times to rinse reagents from the pipet tip. Then flick the tube with your finger 3–4 times to mix thoroughly, and finish by centrifuging briefly to collect the reaction at the bottom of the tube.

Thermal cycler recommended

The Amino Allyl MessageAmp II aRNA Amplification procedure is very sensitive to temperature; variable or inaccurate incubation temperatures can limit aRNA synthesis. 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, which can greatly reduce yield.

- ***A thermal cycler with a temperature adjustable heated lid is recommended.***
  A calibrated thermal cycler, with a temperature-adjustable heated lid, is recommended, for the greatest temperature control and stability during Amino Allyl MessageAmp II aRNA Amplification reaction incubations. Allow the thermal cycler to equilibrate to the required temperature before placing the tubes in the block for incubation. Follow the recommended settings for the lid temperatures. Too high a lid setting may inhibit the reaction; too low a setting may cause condensation.

NOTE

Even if you use a hybridization oven or incubator for most of the Amino Allyl MessageAmp II aRNA Amplification reactions, a thermal cycler is strongly recommended for the 16°C second-strand synthesis reaction incubation (step II.D.2 on page 16). Turn off the heated lid if it cannot be adjusted to match the 16°C block temperature.

If your thermal cycler does not have a temperature-adjustable lid, or a thermal cycler is unavailable, calibrated hybridization ovens or incubators (at constant temperature) may also be used. Preheat incubators so that the correct temperature has stabilized before reactions are placed in the incubator. To avoid any potential influence on the reaction temperature from the tube holder, let tube holders equilibrate in the incubator for sufficient time, or use a tube holder that doesn't touch the sides and bottoms of the tubes—for example a floating tube support.

- ***Heat blocks or water baths are not recommended for Amino Allyl MessageAmp II aRNA Amplification reaction incubations.***

Maintaining consistency

Procedural consistency is very important for amplification experiments. Consider implementing a detailed procedural plan that will be used by everyone in the lab to maintain consistency. This type of plan will minimize variation due to subtle procedural differences that can influence RNA amplification and may complicate gene expression studies. The plan should include basic information such as the method of RNA isolation, the amount of RNA to use in the procedure, and how long to incubate the IVT reaction. It should also address specifics that are not often included in protocols such as which tubes, tube racks, and incubators to use for each step in the process. Finally, develop a consistent work flow. For example standardize stopping points in the method. The idea is to standardize all of the variables discussed in this section of the Protocol and carefully follow all the steps in order to maximize amplification consistency among samples.

Tubes: use 0.5 mL RNase-free nonstick tubes

If a 60-well thermal cycler with temperature-adjustable lid is available, it is most convenient to conduct the Amino Allyl MessageAmp II aRNA Amplification procedure in 0.5 mL nonstick tubes (for example, P/N AM12350). These can be thin-wall (PCR) tubes or ordinary-weight nonstick tubes. 0.5 mL tubes are large enough to accommodate the cDNA Binding Buffer without having to transfer reactions to a larger tube. Their small size and nonstick properties also keep the reaction components at the bottom of the tube.

If your thermal cycler is equipped with a standard 96-well block, 0.2 mL non-stick tubes can be used.

B. Prepare the Wash Buffer

Add 32 mL 100% ethanol (ACS grade or better) to the bottle labeled Wash Buffer. Mix well and mark the label to indicate that the ethanol was added.

C. Reverse Transcription to Synthesize First Strand cDNA

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
42°C (50°C lid)	2 hr	1
4°C	hold	

It is important to follow the lid temperature recommendation; higher lid temperatures may inhibit reverse transcription, while lower temperatures may cause condensation, resulting in changes to the reaction composition.

1. Adjust RNA sample volume to 10 µL, if necessary

a. Place a maximum volume of 10 µL of total RNA (1000 ng recommended) into a nonstick, sterile, RNase-free, 0.5 mL tube. RNA must be in high quality water or TE.

NOTE

If your experiment will include RNA spikes (e.g., Ambion ArrayControl RNA Spikes, P/N AM1780, or Affymetrix GeneChip Poly-A Control Kit, Cat #900433), add them to samples at this step.

b. If necessary, add Nuclease-free Water to a final volume of 10 µL, vortex briefly to mix, then centrifuge to collect the mixture at the bottom of the tube.

2. Add 10 µL of *Reverse Transcription Master Mix* and place at 42°C

a. At room temperature, prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

Reverse Transcription Master Mix (for a single 20 µL reaction)	
Amount	Component
1 µL	Nuclease-free Water
1 µL	T7 Oligo(dT) Primer
2 µL	10X First Strand Buffer
4 µL	dNTP Mix
1 µL	RNase Inhibitor
1 µL	ArrayScript

b. ***Gently*** vortex the tube to make a homogenous mixture without inactivating the enzyme, then centrifuge briefly (~5 sec) to collect the Reverse Transcription Master Mix at the bottom of the tube and place on ice.

c. Transfer 10 µL of Reverse Transcription Master Mix to each RNA 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 samples in the thermal cycler, and start the run.

3. Incubate for 2 hr at 42°C

Incubate the reactions for 2 hr at 42°C, then centrifuge the tubes briefly (~5 sec) to collect the contents at the bottom of the tubes.

Place the tubes on ice and immediately proceed to second strand cDNA synthesis (below).

IMPORTANT

Proceed immediately to the next step.

D. Second Strand cDNA Synthesis

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
16°C (heat-disabled lid, or no lid)	2 hr	1
4°C	hold	

Disable the heated lid of the thermocycler if the lid temperature cannot be set in the range 16°C to room temperature. If the lid is too hot, inhibition of the reaction may occur.

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. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Second Strand Master Mix at the bottom of the tube and place on ice.

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 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 tubes with it. (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.)

3. Place reactions on ice briefly or freeze immediately

After the 2 hr incubation at 16°C, place the reactions on ice and proceed to section *E. cDNA Purification* (below), or immediately freeze reactions at –20°C. Do not leave the reactions on ice for more than 1 hr.

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.

E. cDNA Purification

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temperature.
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 55°C

Before beginning the cDNA purification, preheat at least 18 µL per sample of Nuclease-free Water to 55°C.

IMPORTANT

Preheat the Nuclease-free Water to a maximum of 55°C; temperatures above 58°C can partially denature the cDNA, compromising final aRNA yield.

2. Add 250 µL cDNA Binding Buffer to each sample

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 temperature before use.

Add 250 µL of cDNA Binding Buffer to each sample, and mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times. Follow up with a quick spin to collect the reaction in the bottom of the tube. Proceed quickly to the next step.

3. Pass the mixture through a cDNA Filter Cartridge

Check that the cDNA Filter Cartridge is firmly seated in its wash tube (supplied).

a. Pipet the cDNA sample/cDNA Binding Buffer (from step 2) onto the center of the cDNA Filter Cartridge.

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

c. Discard the flow-through and replace the cDNA Filter Cartridge in the wash tube.

IMPORTANT

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

4. Wash with 500 µL Wash Buffer

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

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

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

5. Elute cDNA with 18 µL 55°C Nuclease-free Water

It is important to use Nuclease-free Water that is at 50–55°C for the cDNA elution. Colder water will be less efficient at eluting the cDNA, and hotter water (≥58°C) may result in reduced aRNA yield.

a. Apply 18 µL of preheated Nuclease-free Water (55°C) to the center of the filter in the cDNA Filter Cartridge.

b. Leave at room temperature for 2 min and then centrifuge for 1 min at 10,000 x g, or until all the Nuclease-free Water is through the filter. The double-stranded cDNA will now be in the eluate (~16 µL).

STOPPING POINT

The purified cDNA can be stored overnight at –20°C at this point if desired. Transfer the cDNA to a lidded, non-stick, nuclease-free tube for storage.

F. In Vitro Transcription to Synthesize Amino Allyl-Modified aRNA

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
37°C (default lid; 100–105°C)	4–14 hr; see step 2	1
4°C	hold	

It is important to use a thermal cycler with a heated lid (100–105°C), to maintain uniform temperature, and because it is extremely important that condensation does not form inside the tubes; this would change the reagent concentrations and reduce yield.

1. Add 26 µL of *IVT Master Mix* to each sample, and mix

Because the amino allyl UTP nucleotide (aaUTP) does not contain a "bulky" modification, it is possible to replace all of the UTP with aaUTP in the IVT reaction. However, we recommend using a 1:1 ratio of UTP to aaUTP (i.e. 50%) for most samples. Using a higher ratio of aaUTP will produce aRNA that can be coupled with more dye molecules, which could lead to brighter signal on microarrays, but which may cause a decrease in the signal to noise ratio. The kit contains 50 mM aaUTP for 20 IVT reactions at the 1:1 ratio recommended. If more aaUTP is needed, it is available separately (P/N AM8437).

IMPORTANT

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

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. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

IVT Master Mix (for a single reaction, ~42 µL)		
Amino allyl	Unmodified	Component
3 µL	– –	aaUTP (50 mM)
12 µL	12 µL	ATP, CTP, GTP Mix
3 µL	6 µL	UTP Solution (50 mM)
4 µL	4 µL	T7 10X Reaction Buffer
4 µL	4 µL	T7 Enzyme Mix

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the IVT Master Mix at the bottom of the tube and place on ice.

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.

d. Once assembled, place the tubes in the thermal cycler and start the run.

2. Incubate 4–14 hr at 37°C

The minimum recommended incubation time is 4 hr; the maximum is 14 hr. (The reactions can be held post-IVT at 4°C for up to 48 hr, for convenience.)

There are data and a detailed discussion of the length of the IVT incubation in section II.A *Input RNA quantity and IVT reaction incubation time* starting on page 9.

3. Add 58 µL Nuclease-free Water to each sample

Stop the reaction by adding 58 µL Nuclease-free Water to each aRNA sample to bring the final volume to 100 µL. Mix thoroughly by gentle vortexing.

Proceed to the 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

Incubator needed: heat block set at 55°C.

This purification removes unincorporated aaUTP and Tris from IVT reactions that would otherwise compete with the aRNA for dye coupling; it also removes enzymes, salts, and other unincorporated nucleotides.

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temperature.
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.

1. Preheat Nuclease-free Water and assemble Filter Cartridges and Tubes

- Preheat a minimum of 200 µL per sample of Nuclease-free Water to 55°C.
- For each sample, place an aRNA Filter Cartridge into an aRNA Collection Tube and set aside for use in step 4.

2. Add 350 µL aRNA Binding Buffer to each sample

a. Check to make sure that each IVT reaction was brought to 100 µL with Nuclease-free Water.

b. Add 350 µL of aRNA Binding Buffer to each aRNA sample. Proceed to the next step immediately.

3. Add 250 µL 100% ethanol and pipet 3 times to mix

Add 250 µL of ACS grade 100% ethanol to each aRNA sample, and mix by pipetting the mixture up and down 3 times. ***Do NOT vortex to mix and do NOT centrifuge***.

IMPORTANT

It is crucial to follow these mixing instructions exactly, and to proceed quickly to the next step.

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 samples through an aRNA Filter Cartridge

a. Pipet each sample mixture from step 3 onto the center of the filter in the aRNA Filter Cartridge/Collection Tube assembly.

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

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

5. Wash with 650 µL Wash Buffer

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

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

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

6. Elute aRNA with 200 µL 55°C Nuclease-free Water

a. To the center of the filter, add 200 µL Nuclease-free Water (preheated to 55°C).

b. Incubate the samples in the 55°C heat block for 10 min (recommended).
Alternatively, incubate at room temperature for 2 min. This results in ~80% recovery of the aRNA.

c. Centrifuge for ~1.5 min at 10,000 x g, or until the Nuclease-free Water is through the filter.

d. The aRNA will now be in the aRNA Collection Tube in ~200 µL of Nuclease-free Water.

e. Store purified aRNA at –20°C or determine its concentration using one of the methods described in section *III.A. aRNA Quantitation and Expected Yield* on page 21. The aRNA concentration must be known in order to continue to the dye coupling reaction.

STOPPING POINT

The purified aRNA can be stored at –20°C overnight, or at –80°C for longer times if desired.

III. Assessing aRNA Yield and Quality

A. aRNA Quantitation and Expected Yield

1. Assessing aRNA yield by UV absorbance

The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm using a spectrophotometer.

- Use a NanoDrop spectrophotometer and measure 1.5 µL of the RNA sample directly.
- With a traditional spectrophotometer, dilute an aliquot of the RNA 1:50 to 1:100 in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and read the absorbance. (Be sure to zero the spectrophotometer with the TE used for sample dilution.) The buffer used for dilution need not be RNase-free, since slight degradation of the RNA will not significantly affect its absorbance.

Find the concentration in µg/mL by multiplying the A_{260} by the dilution factor and the extinction coefficient. (1 A_{260} = 40 µg RNA/mL):

A_{260} X dilution factor X 40 = µg RNA/mL

2. Assessing aRNA yield with the RiboGreen® assay

If a fluorometer or a fluorescence microplate reader is available, Invitrogen's 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.

NOTE

Cy5-labeled aRNA cannot be accurately quantitated using RiboGreen.

3. Expected yield

The aRNA yield will depend on the amount and quality of poly(A) RNA in the input total RNA. Since the proportion of poly(A) RNA in total RNA is affected by influences such as health of the organism and the organ from which it is isolated, aRNA yield from equal amounts of total RNA may vary considerably.

B. Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with LabChip technology, or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA (from step II.G.6 on page 20). Instructions for denaturing agarose gel electrophoresis are available at:

www.invitrogen.com/ambion/techlib/append/supp/rna_gel.html

Expected aRNA size

Agilent bioanalyzer analysis

The expected aRNA profile is a distribution of sizes 250–5500 nt with most of the aRNA 1000–1500 nt (Figure 6 on page 33). 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.

Denaturing agarose gel analysis

Amplified aRNA should appear as a smear from 250 to 5000 nt. The average size of amino allyl aRNA should be approximately 1400 nt; the average size of unmodified aRNA should be ~1150 nt.

IV. Dye Coupling and Labeled aRNA Cleanup

Life Technologies offers the Amino Allyl MessageAmp II Cy3 and Cy5 aRNA Amplification Kits (P/N AM1795, AM1796, AM1797) that are supplied with single use tubes of mono-reactive NHS esters of Cy3 dye, Cy5 dye, or both. We recommend these kits for convenience and reliability. However, 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, and instructions for preparing them for use with this kit, see section *VII.A. NHS Ester Dyes from Other Suppliers* on page 36. The choice of dye will depend on your preference and on the microarray scanning equipment available.

A. aRNA:Dye Coupling Reaction

1. Resuspend one CyDye vial in 11 µL of DMSO

Prepare dye immediately before starting the dye coupling procedure; add 11 µL of DMSO to one tube of Cy3 or Cy5 reactive dye, and vortex to mix thoroughly.

Keep the resuspended dye in the dark at room temperature for up to 1 hr until you are ready to use it.

Our recommendations for solubilizing several other commercially available NHS ester dyes are shown in section VII.A on page 36.

IMPORTANT

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.

2. Quantitate the aRNA and vacuum dry 5–20 µg

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

- Place 5–20 µg of amino allyl-modified aRNA in a nuclease-free microfuge tube and vacuum dry on medium or low heat until no liquid remains.
- 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.

3. Resuspend dried aRNA in 9 µL Coupling Buffer

To a tube containing the dried amino allyl aRNA (5–20 µg), add 9 µL Coupling Buffer and resuspend thoroughly by vortexing gently.

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

Add 11 µL of prepared dye (from step 1) to the aRNA:Coupling Buffer mixture. Mix well by vortexing gently.

5. 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, simply put the tubes in a closed drawer.

6. Add 4.5 µL 4M Hydroxylamine and mix

To quench the reaction, add 4.5 µL 4M Hydroxylamine and mix well by vortexing gently.

7. Incubate 15 min at room temp in the dark

Incubate the reaction in the dark at room temp for 15 min.

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

8. Add 5.5 µL Nuclease-free Water to bring each sample to 30 µL

Add 5.5 µL Nuclease-free Water to each sample to bring the volume to 30 µL.

B. Dye Labeled aRNA Purification

This purification removes excess dye from the labeled 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

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

1. Add 105 µL aRNA Binding Buffer to each sample

Add 105 µL of aRNA Binding Buffer to each aRNA sample. Proceed to the next step immediately.

2. Add 75 µL 100% ethanol and pipet 3 times to mix

IMPORTANT

It is crucial to follow these mixing instructions exactly, and to proceed quickly to the next step.

Add 75 µL of ACS grade 100% ethanol to each labeled aRNA sample, and mix by pipetting the mixture up and down 3 times. ***Do NOT vortex and do NOT centrifuge.***

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.

3. Pass samples through a Labeled aRNA Filter Cartridge(s)

a. Pipet each sample mixture onto the center of the filter in the Labeled aRNA Filter Cartridge.

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

c. Discard the flow-through and replace the Labeled aRNA Filter Cartridge back into the Labeled aRNA Collection Tube.

NOTE

The filter in the Labeled 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 in step 5.

4. Wash with 500 µL Wash Buffer

a. Apply 500 µL Wash Buffer to each Labeled aRNA Filter Cartridge.

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

c. Discard the flow-through and centrifuge for an additional ~1 min to remove trace amounts of Wash Buffer.

d. Transfer the Labeled aRNA Filter Cartridge to a Labeled aRNA Elution Tube.

5. Elute labeled aRNA with 2 x 10 µL preheated Nuclease-free Water

a. To the center of the filter, add 10 µL Nuclease-free Water that is preheated to 50–60°C.

b. 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.

c. Repeat steps 5.a–b with a second 10 µL of preheated Nuclease-free Water.

d. The aRNA will now be in the Labeled aRNA Elution Tube in ~20 µL of Nuclease-free Water.

6. Store purified labeled aRNA at –20°C in the dark or continue the procedure

Transfer the labeled aRNA to a lidded, non-stick, nuclease-free tube. If the sample will not be used the same day, store it at –20°C in the dark. We do not recommend storing labeled aRNA for long periods of time; prepare labeled aRNA as it is needed for your experiments.

To continue, determine the aRNA concentration using one of the methods described in section *III.A. aRNA Quantitation and Expected Yield* on page 21. The aRNA concentration must be known in order to continue to the next step: fragmentation and hybridization.

STOPPING POINT

The purified labeled aRNA can be stored at –20°C overnight, or at –80°C for longer times if desired.

C. Spectrophotometric Analysis of Dye Incorporation

1. Determine the appropriate dilution for spectrophotometer readings

Dilute labeled aRNA 1:10 in TE (10 mM Tris-HCl, 1 mM EDTA). Mix well and measure the A_{260} in a UV-Vis spectrophotometer.

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. If the A_{260} is below 0.1 reduce the dilution factor and check the A_{260} again.

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}). (Specifications for other fluorescent dyes are provided in section *VII.A. Dye specifications for calculation of incorporation* on page 37.)

Dye	Absorbance maximum	Extinction coefficient*
Cy3	550 nm	150,000
Cy5	650 nm	250,000

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

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

Use this formula to estimate the number of dye molecules incorporated per 1000 nt of labeled aRNA.

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

The expected incorporation rate is 30–60 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).

D. Preparing Labeled aRNA for Hybridization

1. If necessary, vacuum dry the labeled RNA

Typically microarrays are hybridized with 25–100 µL Hybridization Solution. Since the labeled aRNA is eluted in only 20 µL, it may or may not need to be concentrated in order to use it for microarray hybridization.

If it is necessary to concentrate the labeled aRNA, we recommend vacuum drying in the dark until the volume is reduced to 1–10 µL (cover the lid of the vacuum drier with aluminum foil if necessary to keep out ambient light).

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.

2. Fragment labeled RNA for hybridization to oligonucleotide microarrays

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.

Typically 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 conducted to generate additional aRNA (see Figure 3 on page 4 for an overview of second round amplification). In order to conduct 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 setup is slightly different.

Second round amplification products are typically shorter than first round amplification products, but we have not seen that this has adverse effects on array hybridization results if all samples are prepared using two rounds of amplification.

A. Synthesis of First Strand cDNA (Second Round)

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
37°C (default lid; 100–105°C)	30 min	1
4°C	hold	

It is important to use a thermal cycler with a heated lid (100–105°C), to maintain uniform temperature, and because it is extremely important that condensation does not form inside the tubes; this would change the reagent concentrations and reduce yield.

1. Mix ≤2 µg aRNA with 2 µL Second Round Primers, and adjust the volume to 12 µL

a. Place up to 2 µg of purified aRNA from the first round of amplification into a sterile RNase-free microcentrifuge tube.
With very small RNA samples, we have dried the entire first round amplification reaction, and used it as starting material for the second round amplification.

IMPORTANT

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

b. Add 2 µL of Second Round Primers.

c. Add Nuclease-free Water to bring the volume to 12 µL, vortex briefly to mix, and centrifuge briefly to collect the reaction in the bottom of the tube.

2. Incubate 10 min at 70°C

a. Program a thermal cycler for the annealing step:

Temp	Time	Cycles
70°C (default lid; 100–105°C)	10 min	1
4°C	hold	

b. Place the samples in the equilibrated thermal cycler, start the run, and incubate for 10 minutes at 70°C.
It is important to follow the lid temperature recommendation; higher lid temperatures may inhibit reverse transcription, while lower temperatures may cause condensation, resulting in changes to the reaction composition.

c. Remove the RNA samples from the 70°C incubation and centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube. Place the reaction on ice briefly before starting step 3.

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

a. While the samples are incubating at 70°C, prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube at room temperature. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Reverse Transcription Master Mix at the bottom of the tube and place on ice.

c. Transfer 8 µL of Reverse Transcription 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. Program the thermal cycler for the reverse transcription reaction:

Temp	Time	Cycles
42°C (50°C lid)	2 hr	1
4°C	hold	

e. Place the tubes in the thermal cycler which has equilibrated to 42°C and start the run.

4. Incubate 2 hr at 42°C

Incubate reactions for 2 hr at 42°C, then centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube.

5. Add 1 µL RNase H and incubate 30 min 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.

a. Add 1 µL RNase H to each reaction. 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.

b. Incubate for 30 min at 37°C in a thermal cycler. After the incubation, proceed directly to Second Strand Synthesis (below).

B. Synthesis of Second Strand cDNA (Second Round)

Incubator needed:
Thermal cycler with a temperature-adjustable lid

1. Add 5 µL T7 Oligo(dT) Primer to each sample

a. Add 5 µL T7 Oligo(dT) Primer to cDNA sample.

b. Mix well by gently vortexing, then centrifuge briefly (~5 sec) to collect the sample at the bottom of the tube.

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

a. Program a thermal cycler for the annealing step:

Temp	Time	Cycles
70°C (default lid; 100–105°C)	10 min	1
4°C	hold	

b. Place the samples in the equilibrated thermal cycler, start the run, and incubate 10 min at 70°C in a thermal cycler.

c. Place the reaction on ice briefly before adding the remaining second strand cDNA synthesis reagents.

3. Add 74 µL *Second Strand Master Mix* to each sample

a. On ice, Assemble *Second Strand Master Mix* for all the samples in the experiment. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

Second Strand Master Mix (for a single 100 µL reaction)	
Amount	Component
58 µL	Nuclease-Free Water
10 µL	10X Second Strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Second Strand Master Mix at the bottom of the tube and place on ice.

c. Transfer 74 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. Program a thermal cycler for the reverse transcription reaction:

Temp	Time	Cycles
16°C (heat-disabled lid, or no lid)	2 hr	1
4°C	hold	

Disable the heated lid of the thermocycler if the lid temperature cannot be set in the range 16°C to room temperature. If the lid is too hot, inhibition of the reaction may occur.

e. Place the tubes in the pre-cooled 16°C thermal cycler. It is important to cool the thermal cycler block to 16°C before placing the reaction tubes; subjecting the reactions to temperatures >16°C could compromise aRNA yield.

4. Incubate 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.)

STOPPING POINT

This is a potential overnight stopping point, but it is better to complete the cDNA purification (section II.E on page 16) before stopping.

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

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

Complete the rest of the second round 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

Control RNA amplification instructions

To establish if the kit is working properly, Control RNA consisting of 1 mg/mL HeLa cell total RNA is provided.

1. Use 1 µL of the Control RNA in a single-round Amino Allyl MessageAmp II kit reaction; follow the procedure starting at step II.C.1 on page 14.
2. At step II.F.2 on page 19, use a ***14 hr*** incubation for the IVT reaction.
3. Continue with the procedure for making amino allyl aRNA through section II.G.

Analysis of the positive control amplification reaction

- 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 less than the 1 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, ensuring that it will amplify with extremely high efficiency.
- Also run a 2 µg aliquot of the reaction products on a denaturing agarose gel or analyze 100–200 ng on a bioanalyzer; ***the average size of the aRNA should be ≥1 kb.*** Figure 6 on page 33 shows bioanalyzer data of both amino allyl-modified aRNA and unmodified aRNA produced from the Control RNA.

Labeling the aRNA produced in the positive control amplification reaction

Use 10 µg of aRNA in a dye coupling reaction following the instructions in section IV.A through IV.C starting on page 23.

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

The expected incorporation rate is 30–60 dye molecules per 1000 nt.

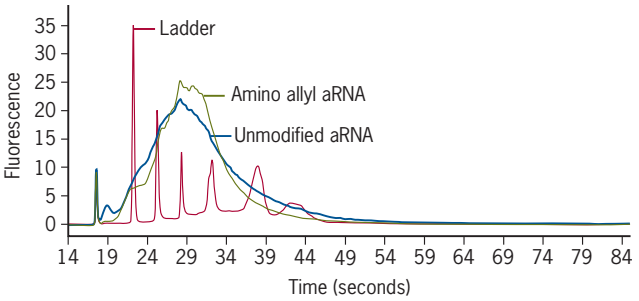


Figure 6. Bioanalyzer Electropherogram of aa-UTP Modified aRNA and Unmodified aRNA.

The same double-stranded cDNA was used as a template for in vitro transcription reactions containing either 50% aaUTP (amino allyl aRNA) or 100% UTP (unmodified aRNA). 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. Factors that Affect Both the Positive Control and Experimental Samples

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions. These suggestions also apply to problems with amplification of experimental RNA.

Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective RNA amplification.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.
- If a thermal cycler is used for incubation, check the accuracy of the adjustable temperature lid. If the lid temperature cannot be adjusted to match the desired reaction temperature, use the lid with the heat turned off, or do not use the lid to cover the reaction vessel(s).

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 an IVT reaction tube throws off the concentrations of the nucleotides and magnesium, which are crucial for good yield.

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

Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, or equipment 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 (P/N AM9780, AM9786).

Absorbance readings were 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.

C. Troubleshooting Low Yield and Small Average aRNA Size

Consider the following troubleshooting suggestions if the positive control reaction produced the expected results, but amplification of your experimental samples results in less or smaller (average <500 nt) aRNA than expected.

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 your RNA before reverse transcription.

Lower than expected input RNA concentration

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

RNA integrity is compromised

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 *RNA integrity* on page 10 for more information).

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

Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 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 within a range of 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.

D. aRNA is Not Efficiently Reverse Transcribed

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.

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

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

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 such as 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 photo bleaching. Dye esters that have been exposed to water, or stored improperly may lose much of their reactivity.

VII. Appendix

A. NHS Ester Dyes from Other Suppliers

Amine reactive fluorescent dye suppliers

Mono-reactive NHS esters of any label moiety should be capable of coupling to the amino allyl modified aRNA generated with this kit. Common dyes are listed below.

- 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
- Amersham Biosciences
  CyDye™ Post-Labeling Reactive Dye Pack Cat #RPN 5661
- Pierce Biotechnology
  NHS-Fluorescein, Product #46100
- 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

Preparation of dye for coupling

Prepare dye immediately before starting the dye coupling procedure; the table below shows our recommendations for solubilizing several commercially available NHS ester dyes.

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 2.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.
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.
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.

Dye specifications for calculation of incorporation

The specifications listed below are provided for calculation of dye incorporation into labeled aRNA described in section IV.C on page 26,

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

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

B. References

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Pabon C, Modrusan Z, Ruvolo MV, Coleman IM, Daniel S, Yue H, Arnold LJ Jr. (2001) Optimized T7 amplification system for microarray analysis. *Biotechniques* **31**(**4**):874–879.

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Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M, Johnston R (2001) An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. *Nucleic Acids Res* **29**(**8**):E41–1.

C. Quality Control

Functional testing

The Control RNA is used in a Amino Allyl MessageAmp II kit reaction following the instructions in section VI.A on page 32. The aRNA yield is assessed by measuring the A_{260} on the NanoDrop ND1000A spectrophotometer. The median size of the aRNA is assessed using the mRNA smear assay on the Agilent 2100 bioanalyzer. CyDye is used to label 10 µg of the amino allyl aRNA following the instructions in section IV.A through IV.C starting on page 23.

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.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

D. Safety Information

WARNING

GENERAL SAFETY. *Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.*

- *Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.*
- *Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.*

1. Chemical safety

WARNING

GENERAL CHEMICAL HANDLING. *To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:*

- *Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.*
- *Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).*
- *Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).*
- *Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.*
- *Handle chemical wastes in a fume hood.*
- *Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)*
- *After emptying a waste container, seal it with the cap provided.*
- *Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.*
- *Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.*
- ***IMPORTANT!*** *Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.*

2. Biological hazard safety

WARNING

Potential Biohazard. *Depending on the samples used on the instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.*

WARNING

BIOHAZARD. *Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable*

regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- *U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety*
- *Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html*
- *Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.*
- *Additional information about biohazard guidelines is available at: www.cdc.gov*

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

VIII. Documentation and Support

A. Obtaining SDSs

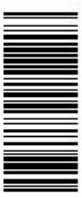
Safety Data Sheets (SDSs) are available from:
www.invitrogen.com/sds

B. Obtaining support

For the latest services and support information for all locations, go to:
www.invitrogen.com/ambion

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches



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