PROTOCOL

ambion® | RNA
by life technologies™

Amino Allyl MessageAmp™ II-96 Kit

life technologies™

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TRADEMARKS

Part Number 1821M Rev. H
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Amino Allyl MessageAmp™ II-96 Kit

(Part Number AM1821)

Protocol

I. Introduction

IMPORTANT

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

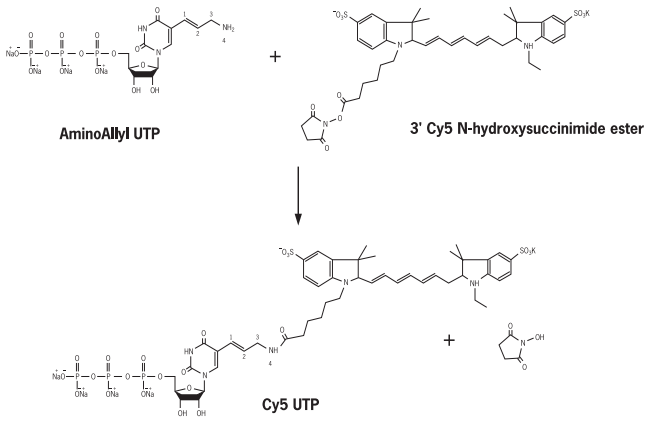
A. Product Description and Background

The Amino Allyl MessageAmp™ II-96 Kit is designed for amplification of RNA in a 96-well format. The procedure is based on the RNA amplification protocol developed in the laboratory of James Eberwine (Figure 2 on page 4) (Van Gelder et al. 1990). consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™, a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild type enzymes. ArrayScript 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 clean-up to become a template for in vitro transcription with T7 RNA Polymerase. To maximize aRNA yield, Life Technologies proprietary MEGAscript® in vitro transcription (IVT) technology is used in the kit 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, in scientific literature 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) 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.

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). 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



The Amino Allyl MessageAmp™ II-96 advantage

Optimized protocols

All reagents and protocols have been optimized to produce high quality amino allyl-modified aRNA with maximum yield and reproducibility. The simple, rapid aRNA purification procedure prepares the aRNA for post-synthesis labeling reactions.

The reagents used for enzymatic reactions in the Amino Allyl MessageAmp™ II-96 Kit are the same as those used in the single prep version of the kit (P/N AM1751). However, magnetic beads are used for cDNA, aRNA, and labeled aRNA cleanup steps in the high throughput kits to streamline the amplification process.

Built-in flexibility

The NTPs are provided in three separate tubes: a mixture of ATP, CTP and GTP, a UTP Solution, and an aaUTP Solution. Separating the UTP and the aaUTP provides the flexibility to make unlabeled aRNA or to incorporate aaUTP during the amplification. Unlike most modified nucleotides, the inclusion of aaUTP in an IVT reaction has only a minor effect on the reaction efficiency and yield. 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.

Ready for automation

This protocol was designed with both manual and robotic liquid handling systems in mind. It can easily be adapted for use on a robotic platform. For more information, go to www.invitrogen.com/site/us/en/home/support/technical-support.html

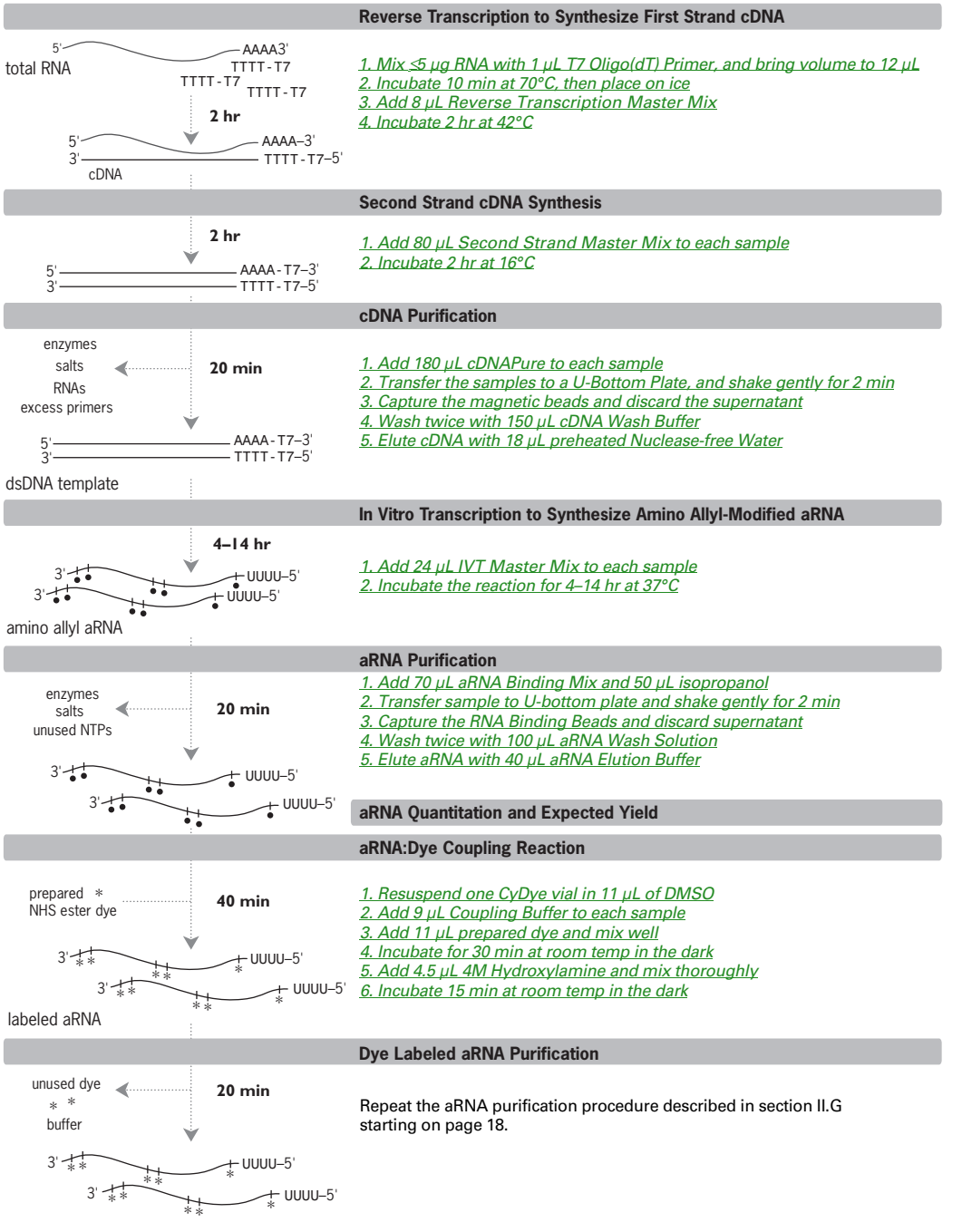
Compatible with second round amplification

This kit is designed for a single round of aRNA amplification, which provides sufficient aRNA for most uses. If more aRNA is needed, RNA can be subjected to two sequential rounds of amplification. If your experiments require two rounds of amplification, you will need to purchase the Ambion MessageAmp II aRNA Amplification Kit (P/N AM1751). For the first round amplification, use the MessageAmp II Kit to make unmodified aRNA.

For the second round of amplification, use the Amino Allyl MessageAmp™ II-96 Kit components plus the Second Round Primers from the MessageAmp II Kit, and follow instructions from both protocols as detailed below:

- For second round reverse transcription and second strand cDNA synthesis, follow the instructions in the MessageAmp II protocol.
- For the second round amplification IVT reaction, follow the instructions in the Amino Allyl MessageAmp™ II-96 Kit protocol.

Figure 2. Procedure Overview



Repeat the aRNA purification procedure described in section II.G starting on page 18.

B. Materials Provided with the Kit and Storage

The Amino Allyl MessageAmp™ II-96 Kit includes reagents for amplification and labeling of 96 samples using a single round of amplification in a 96-well plate. Reagents for 4 control reactions are also included.

cDNA synthesis and in vitro transcription reagents

Store at –20°C in a non-frost-free freezer.

Amount	Component
110 µL	T7 Oligo(dT) Primer
110 µL	ArrayScript™
110 µL	RNase Inhibitor
220 µL	10X First Strand Buffer
880 µL	dNTP Mix
220 µL	DNA Polymerase
110 µL	RNase H
10 µL	Control RNA (1 µg/µL HeLa total RNA)
1.1 mL	10X Second Strand Buffer
440 µL	T7 Enzyme Mix
440 µL	T7 10X Reaction Buffer
220 µL	T7 UTP Solution (75 mM)
220 µL	aaUTP Solution (75 mM) [5-(3-amino allyl)-UTP]
1.32 mL	ATP, CTP, GTP Mix (25 mM each nucleotide)

cDNA and aRNA purification components

Amount	Component	Storage
50 mL	Nuclease-free Water	any temp*
20 mL	cDNAPure†	4°C ‡
2.2 mL	RNA Binding Beads	4°C‡
40 mL	cDNA Wash Buffer Concentrate Add 32 mL 100% ethanol before use	room temp
50 mL	aRNA Wash Solution Concentrate Add 40 mL 100% ethanol before use	room temp
900 µL	Bead Resuspension Solution	room temp
22 mL	aRNA Elution Buffer	room temp
11 mL	aRNA Binding Buffer Concentrate	room temp
3	U-Bottom Plate	room temp
5	PCR Plate	room temp
1	Reservoir	room temp

* Store Nuclease-free Water at room temp, 4°C, or –20°C.
† cDNAPure is manufactured by Agencourt Bioscience Corporation.
‡ Do not freeze.

Dye labeling reagents

Store at –20°C in a non-frost-free freezer.

Amount	Component
1 mL	4M Hydroxylamine
2.6 mL	DMSO
2 mL	Coupling Buffer

C. Materials Not Provided with the Kit

Lab equipment and supplies

- ACS grade (or higher quality) alcohol:
  – 100% ethanol
  – 100% isopropanol
- Additional 100% DMSO may be needed depending on how the dye is supplied.
- Thermal cycler (recommended) or fixed temperature air incubators set at 70°C, 42°C, 37°C, and 16°C. (See Procedural notes on page 10 for more information.)
- Vortex mixer
- Orbital shaker for 96-well plates such as the Barnstead/Lab-Line Titer Plate Shaker (available from Fisher or VWR)
- Magnetic stand for 96-well plates (we recommend the Ambion 96-well Magnetic-Ring Stand, P/N AM10050, for its high strength magnets and quality design)
- Microcentrifuge
- Aluminum sealing foil or tape for sealing reactions in 96-well plates during incubations

(optional) Materials and equipment for RNA analysis

- Spectrophotometer—such as the NanoDrop® ND-8000 UV-Vis Spectrophotometer
- Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen (R11490) for use with a fluorescence microplate reader, standard spectrofluorometer, or filter fluorometer
- Bioanalyzer (Agilent) and RNA LabChip® Kits

NHS esters of fluorescent dyes

This kit was developed using CyDye™ fluorescent dyes from GE Healthcare (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. Some suppliers of compatible dyes are listed below:

- Amersham Biosciences CyDye fluors
- Molecular Probes Alexa Fluor® Succinimidyl Esters
- Pierce Biotechnology NHS-Fluorescein and -Rhodamine
- Denovo Biolabels GmbH Oyster® Dyes

D. Related Products

96-well Magnetic-Ring Stand P/N AM10050	The Ambion 96-well Magnetic-Ring Stand features 96 powerful ring-shaped magnets arranged to cradle each well of a 96-well plate for quick, thorough bead capture. Captured magnetic beads form evenly distributed donut-shaped pellets with a large hole in the center. This capture pattern facilitates both supernatant removal and subsequent bead resuspension. The stand is suitable for high throughput applications conducted with multichannel pipettors or with robotic liquid handlers. However, because the pellets will be evenly distributed around the edge of the wells, it may require practice for efficient manual removal of supernatants.
Magnetic Stand-96 P/N AM10027	The Ambion Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96-well Magnetic-Ring Stand (P/N AM10050).
MagMAX™-96 Total RNA Isolation Kit P/N AM1830	The MagMAX™-96 Total RNA Isolation Kit is a magnetic bead based total RNA purification system designed for rapid high throughput processing of cells in 96-well plates. High yield and high quality total RNA can be obtained from 100 to 500,000 cultured eukaryotic cells. The kit can also be used for total RNA isolation from small tissue samples.
MagMAX™-96 for Microarrays Total RNA Isolation Kit P/N AM1839	The MagMAX-96 for Microarrays Total RNA Isolation Kit is designed for rapid, high throughput isolation of total RNA from mammalian cells and tissues in 96-well plates. The procedure employs the robust and reliable lysis/denaturant, TRI Reagent® and Ambion MagMAX magnetic bead-based RNA purification technology to provide high yields of pure, intact RNA that can be used directly for quantitative reverse transcriptase PCR (qRT-PCR) and microarray analysis. The kit can also be used to process fewer than 96 samples at one time.
RNAqueous®-96 Automated Kit P/N AM1812	The RNAqueous-96 Automated Kit employs a simple and rapid procedure to purify total RNA from source material without using organic solvents (such as phenol). It is formatted specifically for high throughput RNA isolation on robotic platforms.
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 RNA Fragmentation Reagents include a 10X Fragmentation Reagent and a Stop Solution.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette See web or print catalog for P/Ns	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. Life Technologies also offers the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.

II. aRNA Amplification Protocol

A. Planning the Experiment

We understand that the needs of our high throughput customers are very diverse; if you have questions that are not covered in this Protocol, please contact our high throughput experts by emailing:
automation@ambion.com

RNA quantity and IVT incubation time

The Amino Allyl MessageAmp™ II-96 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. Typically ***100 ng–5 µg of total RNA*** should be used in an Amino Allyl MessageAmp™ II-96 amplification reaction. ***The recommended amount of RNA per reaction is 1 µg of total RNA.***

The amount of input RNA needed will depend on how much aRNA is required for your experiments, and how long the IVT amplification is incubated.
Using 1 µg or more total RNA and a 4 hr IVT incubation typically produces enough aRNA for several microarray hybridizations. When amplifying small RNA samples (e.g. ~250 ng or less), however, incubating the IVT reaction for 14 hr will maximize the amount of aRNA produced.


NOTE

Note that aRNA yield may vary considerably depending on the purity and integrity of the RNA used in the reaction and on the other parameters discussed in these instructions.

Other input RNA requirements

RNA purity

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 of full-length mRNA, can significantly impact the amplification reaction. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that lack the sequence upstream of the break in the RNA molecule.

The most common method for evaluating the relative integrity of mRNA is to measure the ratio of the 28S and 18S ribosomal RNAs (rRNA) using an RNA LabChip Kit with an Agilent 2100 bioanalyzer. The RNA LabChip fractionates RNA molecules according to size, and the relative amounts of 18S and 28S rRNA can be determined. The ratio of the 28S to 18S rRNA peaks will approach 2:1 in RNA samples comprising primarily full-length RNA (Figure 3).

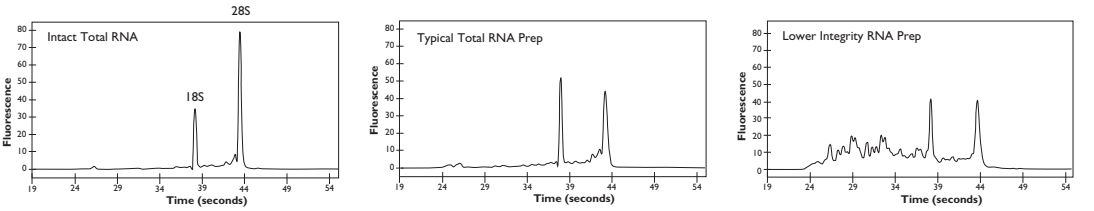


Figure 3. Bioanalyzer Images of Increasingly Degraded Total RNA

These electropherograms from the Agilent 2100 bioanalyzer show progressive RNA degradation. Notice that the ribosomal RNA (rRNA) peaks are at a ratio of about 2:1 (28S:18S) in an intact total RNA sample (left). Total RNA that is somewhat degraded shows nearly equal amounts of 28S and 18S rRNA (middle). Further degradation decreases the rRNA peaks, and degradation products become apparent in the lower molecular weight range (right).

Procedural notes

We recommend a thermal cycler for all enzymatic reaction incubations

Ideally, reactions should be incubated in a calibrated thermal cycler with a lid temperature that adjusts to the block temperature (typically 5°C above the block temperature). If the lid temperature is static (~100°C), turn off the lid heat or do not close the heated lid onto your samples. Otherwise, heat from the lid will raise the temperature of the solution in the tube, compromising the reaction.

If a thermal cycler is not available, you may use a calibrated hybridization oven or other constant temperature air incubator for second strand cDNA synthesis and IVT reactions (see next section). We do ***not*** recommend using heat blocks or water baths for any Amino Allyl MessageAmp™ II-96 reaction incubations.

Use calibrated, preheated incubators for enzymatic reaction incubations (Alternative to thermal cycler)

The Amino Allyl MessageAmp™ II-96 procedure is very sensitive to temperature; therefore use incubators that have been professionally calibrated according the manufacturer's recommended schedule. Variable or inaccurate incubation temperatures can limit aRNA synthesis.

Preheat incubators if necessary so that the correct temperature has stabilized before reactions are placed in the incubator. It is also very important that condensation does not form in the plate wells during any of the incubations. Condensation changes the composition of reaction mixtures and can greatly reduce yield.

Orbital shaker settings

We recommend using an orbital shaker with a 2–3 mm orbit. Using too large an orbit will cause the liquid to spill out of the wells, while too small of an orbit may not mix the sample effectively.

Determine the maximum speed that can be used in this procedure by filling the wells of a 96-well plate with 290 µL of water. Turn the shaker on and increase the speed slowly while observing the movement of the water in the wells. Note the maximum speed at which the plate can be shaken without causing the water to splash or spill out of the wells. When shaking experimental samples, always increase the orbital shaker speed slowly to avoid splashing. Always choose the lowest speed that is strong enough for complete mixing.

Master mixes

We strongly recommend preparing master mixes, instead of pipetting components individually, to improve pipetting consistency. When using a multichannel pipettor to dispense a master mix that consists of <2 mL, we recommend that you minimize the void volume by aliquoting the master mix into a PCR plate instead of using a reservoir.

B. Reagent Preparation

1. Add 32 mL 100% ethanol to the cDNA Wash Buffer Concentrate

Add 32 mL 100% ethanol (ACS grade or better) to the bottle labeled cDNA Wash Buffer Concentrate. Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as ***cDNA Wash Buffer*** in these instructions.

Store at room temperature.

2. Add 40 mL 100% ethanol to the aRNA Wash Solution Concentrate

Add 40 mL 100% ethanol (ACS grade or better) to the bottle labeled aRNA Wash Solution Concentrate. Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as ***aRNA Wash Solution*** in these instructions.

Store at room temperature.

3. Prepare *aRNA Binding Mix* for the experiment

IMPORTANT

Prepared aRNA Binding Mix can be stored at room temp for one week; prepare only the amount needed for all samples in the experiment plus ~5% overage to cover pipetting error.

a. First, mix RNA Binding Beads with Bead Resuspension Solution and isopropanol

Mixing beads with the Bead Resuspension Solution will facilitate bead dispersion in Binding Mix. At room temp, combine Bead Resuspension Solution with RNA Binding Beads in a nuclease-free tube and mix briefly. Then add 100% isopropanol and mix thoroughly by vortexing.

a. Combine the following (for a single reaction):	
RNA Binding Beads*	10 µL
Bead Resuspension Solution	4 µL
b. Mix briefly, then add:	
100% isopropanol	6 µL
c. Mix well by vortexing.	

* Mix the RNA Binding Beads by vortexing before dispensing.

b. Next, add aRNA Binding Buffer Concentrate

At room temp, add aRNA Binding Buffer Concentrate to the bead mixture from the previous step in a nuclease-free container. Mix by vortexing:

aRNA Binding Mix (for a single reaction)	
Component	Amount
aRNA Binding Buffer Concentrate	50 µL
RNA Binding Bead Mixture (from step 3.a)	20 µL

This mixture is referred to as aRNA Binding Mix in these instructions.

C. Reverse Transcription to Synthesize First Strand cDNA

Incubators needed:

- 70°C: thermal cycler recommended
- 42°C: thermal cycler recommended

1. Mix ≤5 µg RNA with 1 µL T7 Oligo(dT) Primer, and bring volume to 12 µL

a. Place up to 5 µg of total RNA (typically 100 ng–5 µg) into the wells of a PCR Plate.

b. Add 1 µL T7 Oligo(dT) Primer.

c. Add Nuclease-free Water to a final volume of 12 µL and cover with aluminum sealing foil.


NOTE

If all samples use the same RNA volume, premix the Nuclease-free Water and T7 Oligo(dT) Primer to simplify the procedure.

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

a. Incubate 10 min at 70°C.

b. Place the samples on ice. If there is condensation on the foil, centrifuge briefly (~5 sec) to collect samples at the bottom of the wells.

3. Add 8 µL *Reverse Transcription Master Mix*

a. Prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube at room temp. 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 ~5 sec to collect the master mix at the bottom of the tube and place it on ice.

c. Transfer 8 µL of the Reverse Transcription Master Mix to each RNA sample, mix by gently pipetting up and down 3–4 times. Cover the reactions with aluminum sealing foil.
To facilitate the transfer process, you can distribute the master mix into 8 wells of a PCR plate and use an 8-channel pipette to transfer the master mix.

4. Incubate 2 hr at 42°C

Incubate the reaction for 2 hr at 42°C. If condensation is apparent after the incubation, centrifuge the plate briefly (~5 sec) to collect the samples at the bottom of the wells.

Place the plate on ice, or change the thermal cycler temperature to 4°C, and immediately proceed to the second strand cDNA synthesis.

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*** by adding the following reagents to a reservoir 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.

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 gently by rocking the reservoir back and forth.

c. Transfer 80 µL of Second Strand Master Mix to each sample in the PCR Plate. Mix by gently pipetting up and down 3–4 times. Cover the reactions with aluminum sealing foil.

2. Incubate 2 hr at 16°C

Incubate in a 16°C thermal cycler (do not incubate in a heat block in a 4°C refrigerator because the temperature may fluctuate). It is important to cool the thermal cycler to 16°C before placing the PCR Plate inside; subjecting the reactions to temperatures >16°C could compromise aRNA yield.

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

Before beginning the cDNA purification:

- Remove the cDNAPure from the refrigerator and allow it to equilibrate to room temp for 30 min before use.
- Preheat the bottle of Nuclease-free Water to 50–60°C for at least 10 min.
- Shake the container of cDNAPure to fully resuspend the magnetic beads before use. cDNAPure is a slurry of DNA binding magnetic beads in binding buffer. The magnetic beads will settle over time.

1. Add 180 µL cDNAPure to each sample

Add 180 µL of resuspended cDNAPure to each second strand cDNA synthesis reaction.

2. Transfer the samples to a U-Bottom Plate, and shake gently for 2 min

a. Transfer the samples from the PCR Plate to a U-Bottom Plate.

NOTE

This transfer step facilitates good mixing of the cDNAPure with the cDNA.

b. Gently shake for at least 2 min to thoroughly mix the sample with the cDNAPure. The cDNA in the samples will bind to the magnetic beads in the cDNAPure during this incubation.

3. Capture the magnetic beads and discard the supernatant

a. Move the U-Bottom Plate to a magnetic stand to capture the magnetic beads. Leave the plate on the magnetic stand until the mixture becomes transparent, indicating that capture is complete. This may take 5–8 min with the Ambion 96-well Magnetic-Ring Stand (P/N AM10050).

b. Carefully aspirate the supernatant without disturbing the RNA Binding Beads. Discard the supernatant.

c. Remove the plate from the magnetic stand.
For effective washing, it is critical to remove the plate from the magnetic stand before adding cDNA Wash Buffer (next step).

4. Wash twice with 150 µL cDNA Wash Buffer

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

a. Add 150 µL cDNA Wash Buffer to each sample and shake at moderate speed for 1 min.

NOTE

The beads will not resuspend at this point because of the low surface tension of the cDNA Wash Buffer.

b. Move the plate to a magnetic stand, and capture the magnetic beads until the mixture becomes transparent. This takes 1–2 min with an Ambion magnetic stand.

c. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, and remove the plate from the magnetic stand.

d. Repeat steps a–c to wash a second time with 150 µL of cDNA Wash Buffer.

e. Move the plate to a shaker and shake the plate vigorously for 2 min to evaporate residual ethanol from the plate.
Do not overdry the beads as this may make them difficult to resuspend in the next step.

5. Elute cDNA with 18 µL preheated Nuclease-free Water

a. Elute the cDNA from the magnetic beads by adding 18 µL preheated (50–60°C) Nuclease-free Water to each sample.

b. Vigorously shake the plate for 3 min, then check to make sure the magnetic beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

c. Move the plate to the magnetic stand, and capture the magnetic beads until the mixture becomes transparent.

d. Carefully transfer 16 µL of the eluted cDNA into a new PCR Plate.

NOTE

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

F. In Vitro Transcription to Synthesize aRNA

Because the amino allyl UTP (aaUTP) does not have a bulky modification group, it is possible to replace 100% of the UTP with aaUTP in the IVT reaction. We recommend, however, using a 1:1 ratio of UTP to aaUTP (i.e. 50%) for most samples. If desired, it is possible to adjust the level of aaUTP incorporation by adjusting the UTP:aaUTP ratio. Increasing the amount of aaUTP in the ratio will produce aRNA with more dye-coupling sites, which in some cases will lead to brighter signal on microarrays. This kit contains enough 75 mM aaUTP to set up 100 IVT reactions at the 1:1 ratio recommended. If more aaUTP is needed, it can be purchased from Life Technologies (P/N AM8437).

1. Add 24 µL *IVT Master Mix* to each sample

a. Assemble the ***IVT Master Mix*** at room temperature 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.

IVT Master Mix (for a single 40 µL reaction)	
Amount	Component
2 µL	aaUTP Solution (75 mM)
12 µL	ATP, CTP, GTP Mix
2 µL	T7 UTP Solution (75 mM)
4 µL	T7 10X Reaction Buffer
4 µL	T7 Enzyme Mix

b. Mix well by gently vortexing. Centrifuge ~5 sec to collect the master mix at the bottom of the tube and place it on ice.

c. Transfer 24 µL of IVT Master Mix to each sample; mix by pipetting up and down twice. Cover the reactions with aluminum sealing foil.

2. Incubate the reaction for 4–14 hr at 37°C

The minimum recommended incubation time is 4 hr. The maximum incubation time is 14 hr. It is important to maintain a constant 37°C incubation temperature. We recommend incubating in a thermal cycler with a heated lid (lid temp set at 42°C). An alternative is to use a mechanical convection oven, such as a hybridization oven, with the PCR Plate in an aluminum heat block (preferred), or on the oven shelf (rather than on the oven floor). It is extremely important that condensation does not form inside the tubes; this would change the concentration of the reaction components and reduce yield.

G. aRNA Purification

Before beginning the aRNA purification:

- Preheat the aRNA Elution Buffer to 50–60°C for at least 10 min.

1. Add 70 µL aRNA Binding Mix and 50 µL isopropanol

a. Add 70 µL aRNA Binding Mix (prepared as described in section II.B.3 on page 11) to each sample.

b. Add 50 µL of 100% isopropanol to each sample.

2. Transfer sample to U-bottom plate and shake gently for 2 min

Transfer sample into a U-Bottom Plate. Gently shake for ≥2 min to thoroughly mix. The aRNA in the samples will bind to the RNA Binding Beads during this incubation.

3. Capture the RNA Binding Beads and discard supernatant

a. Move the plate to a magnetic stand and capture the magnetic beads, until the mixture becomes transparent.

b. Carefully aspirate and discard the supernatant without disturbing the magnetic beads; then remove the plate from the magnetic stand.

4. Wash twice with 100 µL aRNA Wash Solution

a. Add 100 µL aRNA Wash Solution (prepared as described in step II.B.2 on page 11) to each sample, and shake at moderate speed for 1 min.

NOTE

The RNA Binding Beads may not fully disperse during this step; this is expected and will not affect RNA purity or yield.

b. Move the plate to a magnetic stand and capture the RNA Binding Beads until the mixture becomes transparent.

c. Carefully aspirate and discard the supernatant without disturbing the RNA Binding Beads and remove the plate from the magnetic stand.

d. Repeat steps a–c to wash a second time with 100 µL of aRNA Wash Solution.

e. Move the plate to a shaker and shake the plate vigorously for 1 min to evaporate residual ethanol from the beads.

5. Elute aRNA with 40 µL aRNA Elution Buffer

a. Elute the purified aRNA from the RNA Binding Beads by adding 40 µL preheated (50–60°C) aRNA Elution Buffer to each sample.

b. Vigorously shake the plate for 3 min, then check to make sure the RNA Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

c. Move the plate to a magnetic stand, and capture the RNA Binding Beads until the mixture becomes transparent.

d. Transfer the supernatant, which contains the eluted aRNA, to a nuclease-free PCR plate.

6. Store the aRNA at –20°C or determine the aRNA concentration and continue

STOPPING POINT

The purified aRNA can be stored at –20°C at this point.

The purified aRNA can be stored at –20°C if desired. Alternatively, determine its concentration using one of the methods described in section *III.A. aRNA Quantitation* on page 20. The aRNA concentration must be known in order to continue to the dye coupling reaction.

III. Assessing aRNA Yield and Size

A. aRNA Quantitation

1. Assessing aRNA yield by UV absorbance

The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm. We recommend using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just measure 2 µL of the aRNA sample directly.

Alternatively, the aRNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. Find the concentration in µg/mL using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

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

2. Assessing aRNA yield with RiboGreen

If a fluorometer or a fluorescence microplate reader is available, the RiboGreen fluorescence-based assay for RNA quantitation (Life Technologies) is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen.

B. Analysis of aRNA Size with a Bioanalyzer

The Agilent 2100 bioanalyzer with RNA LabChip Kits provides better qualitative data than conventional gel analysis for characterizing aRNA. When used with the Ambion RNA 6000 Ladder (P/N AM7152), this system can provide a fast and accurate size distribution profile of aRNA samples. Follow the manufacturer's instructions for performing the assay.

Expected results

Using 1 µg of high quality input total RNA, 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 4 on page 26). Reaction products may be smaller when less input RNA or lower quality input RNA is used.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. Dye Coupling and Cleanup

This kit was developed using Cy™3 and Cy™5 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. For a partial list of suppliers of NHS ester dyes, see section *I.C. Materials Not Provided with the Kit* on page 6. The choice of dye will depend on your preference and on the type of microarray scanning equipment that will be used.

A. Concentrate the Amino Allyl aRNA

1. Determine the aRNA concentration

(See section III.A on page 20 for instructions on determining the aRNA concentration.)

2. Vacuum dry 5–20 µg aRNA to completion

- Place 5–20 µg of amino allyl aRNA into separate wells of a PCR Plate and vacuum dry using the low heat setting 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.

B. 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 Amersham Biosciences Cy3 or Cy5 Post Labelling 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.

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. Add 9 µL Coupling Buffer to each sample

To each well containing dried amino allyl aRNA, add 9 µL Coupling Buffer. Resuspend samples by shaking vigorously for 1 min.

3. Add 11 µL prepared dye and mix well

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

4. Incubate for 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, cover the plate with aluminum foil or incubate it in a closed drawer.

5. Add 4.5 µL 4M Hydroxylamine and mix thoroughly

To quench the reaction, add 4.5 µL 4M Hydroxylamine to each sample and mix thoroughly.

6. Incubate 15 min at room temp in the dark

Incubate the reaction at room temp 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.

C. Dye Labeled aRNA Purification

Repeat the aRNA purification procedure described in section II.G starting on page 18.

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

D. Spectrophotometric Analysis of Dye Incorporation

The Nanodrop ND-1000A UV-Vis Spectrophotometer is recommended for UV-Vis spectrophotometry measurements because there is no need to dilute samples. Measure 1.5 µL of undiluted sample.

1. Determine the appropriate dilution for spectrophometer readings

To use a conventional spectrophotometer, dilute labeled aRNA 1:20 by mixing 5 µL labeled aRNA in 95 µL 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 absorbance readings (A_{260} and A_{dye})between 0.1 and 1.0. If absorbance readings are below 0.1, reduce the dilution factor and check the A_{260} and A_{dye} 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}).

Dye type	Absorbance maximum	Extinction coefficient*
fluorescein	491 nm	66,000
Cy3	550 nm	150,000
Cy5	650 nm	250,000
Alexa Fluor 546	546 nm	104,000

Dye type	Absorbance maximum	Extinction coefficient*
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 $cm^{-1}M^{-1}$

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.

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

The expected incorporation rate is 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 20).

E. Preparing Labeled aRNA for Hybridization

1. Concentrate the labeled aRNA if necessary

Typically, microarrays are hybridized with 25–100 µL hybridization solution. Since the labeled aRNA is eluted in about 40 µL, it may need to be concentrated 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 (to keep out ambient light 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.

2. Fragment labeled RNA for hybridization to oligonucleotide microarrays

For microarrays printed with oligonucleotides, the labeled aRNA must typically 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 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.

Most arrays require 1–10 µg of labeled aRNA.

Choice of hybridization solution

The choice of hybridization solution should be based on the type of microarray being hybridized. For glass arrays, we recommend Ambion SlideHyb™ Glass Array Hybridization Buffers (P/N AM8861–AM8863).

V. Troubleshooting

A. Positive Control Reaction

Control RNA amplification instructions

To establish if the reaction conditions are optimal, a tube of Control RNA is provided. ***Use 1 µL of the Control RNA*** in an Amino Allyl MessageAmp™ II-96 reaction; follow the protocol starting at section II.C step 1 on page 12. At step II.F.2 on page 17, ***use a 14 hr incubation for the IVT reaction.*** Continue with the procedure for making amino allyl aRNA through section II.G on page 18.

Analysis of the positive control amplification reaction

Quantitative analysis

After completing the aRNA purification, measure the A_{260} of the reaction product as described in section III.A.1 on page 20; ***the positive control reaction should produce≥ 80 µg of aRNA.***

Qualitative analysis

Also, run 100–200 ng of the reaction products on a bioanalyzer as described in section III.B on page 20; ***the expected aRNA profile*** for unmodified aRNA ***is a distribution from 250–5500 nt with a peak centered at 1000–1500 nt*** (Figure 4).

Alternatively, if a bioanalyzer is not available, run 1 µg of aRNA on a denaturing agarose gel.

Labeling the aRNA produced in the positive control amplification reaction

Use 15 µg of aRNA (made from Control RNA) in a dye coupling reaction following the instructions in section IV.A through IV.D starting on page 21.

Evaluate the positive control reaction by determining the number of dye molecules incorporated per 1000 nt as described in section IV.D on page 22.

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

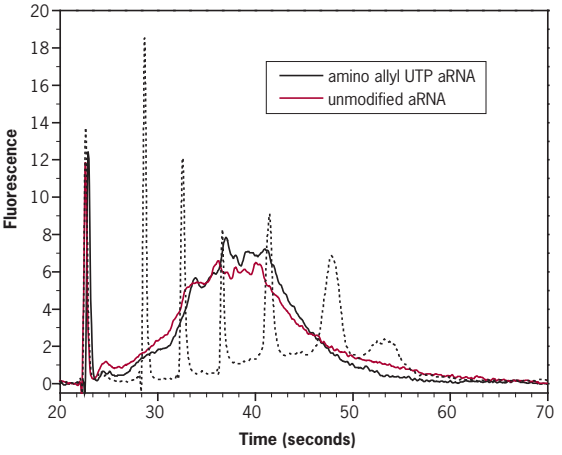


Figure 4. Bioanalyzer Electropherogram of aa-UTP Modified aRNA and Unmodified aRNA.

Double stranded cDNA produced with the Amino Allyl MessageAmp™ II-96 Kit was used as template for in vitro transcription reactions containing either 50% aaUTP or 100% UTP. Approximately 500 ng of aRNA was analyzed on an Agilent bioanalyzer with the RNA 6000 Ladder. The Ladder consists of six RNA transcripts with lengths of 0.2, 0.5, 1, 2, 4, and 6 kb. aRNA of similar size and yield are produced in reactions containing either 50% aaUTP or 100% UTP.

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 it 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 changes the concentrations of the nucleotides and magnesium which can reduce yields.

The best way to avoid condensation is to use a thermal cycler for all incubations and to set the lid temp 5°C above the reaction temp.

If you see condensation, spin the plate briefly and mix the reaction gently. Move the plate 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).

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.

Incomplete resuspension or dispersion of magnetic beads

In general, the cDNAPure and the RNA Binding Beads will disperse more easily when the temperature of the mixture is warmer than 20°C.

a. Be sure to resuspend RNA Binding Beads just before each step in preparing the aRNA Binding Mix (section II.B.3 on page 11).

b. Make sure that magnetic bead mixtures are fully resuspended before adding them to cDNA or aRNA samples for purification procedures (steps II.E.1 on page 15, and II.G.2 on page 18).

c. For efficient elution of purified reaction products, make sure that magnetic beads are fully resuspended in the elution solution (steps II.E.5 on page 16 and II.G.5 on page 18).

d. Avoid overdrying magnetic beads before eluting the RNA (in steps II.E.4.e on page 16 and II.G.4.e on page 18) because this may make the beads more difficult to resuspend. If the beads are inadvertently overdried, increase the mixing time (to 10 min) during the elution step to allow the beads to rehydrate.

Loss of the cDNAPure and/or the RNA Binding Beads

Since the reaction products are purified by immobilization on the cDNAPure and the RNA Binding Beads, any magnetic bead loss during the cDNA or aRNA purifications will result in lower final aRNA yield. Magnetic beads can be lost by incomplete magnetic bead capture and/or by inadvertently aspirating beads when removing supernatant from captured beads.

To determine whether cDNAPure or RNA Binding Beads have been aspirated along with supernatant, it may be helpful to collect all supernatants (except the final cDNA and aRNA-containing supernatant) in a single container. Observe the color of the collected supernatant, if magnetic beads are present, the solution will be light brown.

To prevent aspiration of magnetic beads in subsequent experiments, reduce the aspiration speed and make sure that the tip openings are not touching the bottom of the well when removing supernatant from captured cDNAPure and RNA Binding Beads.

To avoid incomplete magnetic bead capture, make sure that the solution becomes transparent before removing supernatant. This is especially important during the cDNA Purification in section II.E, because the reaction mixture may be somewhat viscous.

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 aRNA than expected or average aRNA size below approximately 500 nt.

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 the samples 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 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 9 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 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.

D. Bead Carryover in Eluted Sample

If cDNAPure or RNA Binding Beads are carried over into the eluate containing the RNA, they will cause the solution to be light brown in color.

To avoid bead carryover, allow sufficient time for bead capture and aspirate slowly and carefully when transferring eluent from the beads. Also avoid touching the bottom of the well with the pipette tip when removing solutions from captured beads.

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 details.

VI. Appendix

A. References

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B. Quality Control

Functional testing

The Control RNA (2 µg) is used in an aRNA amplification experiment following the instructions in section V.A on page 25. The amino allyl aRNA yield is assessed by measuring the A_{260} on the NanoDrop ND1000A spectrophotometer. Cy5 is used to label the 10 µg of the amino allyl aRNA following the instructions in section IV starting on page 21; 20–50 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.

Protease testing

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

C. 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/

VII. Documentation and Support

A. Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

www.invitrogen.com/sds

or

www.appliedbiosystems.com/sds

B. Obtaining support

For the latest services and support information for all locations, go to:

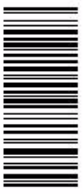
www.invitrogen.com

or

www.appliedbiosystems.com

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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