MessageAmp™ III RNA Amplification Kit

RNA Amplification and Biotin Labeling Kit for Microarray Analysis with GFF aRNA Clean-Up

Part Numbers 4383451, AM1793

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(Part Number 4383451, AM1793)

Protocol

P/N 4393639 Revision D **Revision Date: September 6, 2010**

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I. Introduction

A. Product Description and Background

The Ambion MessageAmp™ III RNA Amplification Kit is the latest innovation for the preparation of RNA samples for microarray analysis. The kit builds upon the linear RNA amplification method and reagents developed for Ambion MessageAmp II Kits with enhancements that create a simplified workflow, improved performance, and more flexible RNA input requirements. All Ambion MessageAmp kits employ the proven methodology for RNA sample preparation and labeling based on the original T7 in vitro transcription (IVT) amplification technology, known as the Eberwine or reverse transcription-IVT (RT-IVT) method (Van Gelder et al. 1990). The RT-IVT method is considered the gold standard for sample preparation in microarray-based expression profiling. It is well documented in the current scientific literature and was experimentally validated using TaqMan® RT-PCR (MAQC Consortium et al., 2006).

Based on this proven enzymatic amplification and labeling strategy, the MessageAmp III procedure starts with a simple cDNA synthesis reaction using ArrayScript™ reverse transcriptase. No cleanup step is required after the RT; cDNA is used directly in a high yield IVT reaction using Ambion MEGAscript® technology. The IVT is configured to incorporate the modified nucleotide, biotin-UTP, into the aRNA synthesized. (In this Protocol the amplified RNA is referred to as aRNA; in the literature, it is also commonly called cRNA.) Once purified, the aRNA is suitable for use on microarray gene expression analysis platforms designed for biotin-modified antisense RNA samples.

MessageAmp III Kit features

- Lowest RNA input requirements for a single round of amplification: 35 ng total RNA yields enough biotin-modified aRNA for most microarray formats, with results equivalent to those from samples prepared from significantly more starting material using other RT-IVT kits.
- Very flexible RNA input requirements: 35–500 ng total RNA, depending on the tissue or cell type
- Streamlined workflow: depending on the RNA input amounts, only a single day of processing time is required to complete a microarray experiment.
- Simplified protocol and streamlined reagents
- Complete kit: everything needed to prepare biotin-modified aRNA is included.

- Rigorous quality control that includes GeneChip analysis
- T7 MEGAscript® IVT delivers up to 50,000-fold amplification.
- Single-tube format

B. Procedure Overview

The MessageAmp™ aRNA amplification procedure is depicted in Figure 1.

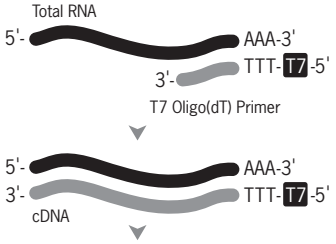
- ***Reverse Transcription to Synthesize First-Strand cDNA*** is primed with T7 oligo(dT) primer to synthesize cDNA containing a T7 promoter sequence.
- ***Second-Strand cDNA Synthesis*** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.
- ***In Vitro Transcription to Synthesize Biotin-Modified aRNA*** with T7 Biotin IVT Mix generates multiple copies of biotin-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 biotin-modified aRNA.

Control RNA

Use the included Control RNA to familiarize yourself with the MessageAmp™ III RNA Amplification procedure. Instructions for the positive control reaction are provided in section IV.A on page 17.

Figure 1. MessageAmp™ III RNA Amplification Procedure

Reverse Transcription to Synthesize First Strand cDNA



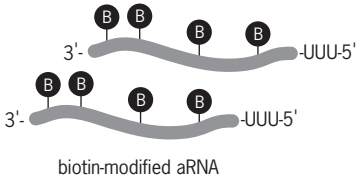
1. Assemble First Strand Master Mix and dispense 5 µL into a reaction tube on ice
2. Add 5 µL RNA and mix thoroughly
3. Incubate at 42°C for 2 hr

Second Strand cDNA Synthesis

1. Assemble Second Strand Master Mix and add 20 µL to each sample
2. Incubate for 1 hr at 16°C, then 10 min at 65°C

Potential stopping point

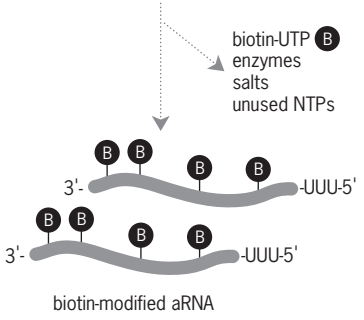
In Vitro Transcription to Synthesize Biotin-Modified aRNA



1. Assemble a T7 IVT Master Mix and add 30 µL to each sample
2. Incubate for 2–14 hr at 40°C
3. Place the aRNA on ice briefly or freeze immediately

Potential stopping point

aRNA Purification



1. Add 40 µL Nuclease-free Water to each sample
2. Add 350 µL aRNA Binding Buffer to each sample
3. Add 250 µL 100% ethanol, pipet 3 times to mix, then spin samples through an aRNA Filter Cartridge
4. Wash with 650 µL Wash Buffer
5. Elute aRNA with 100 µL preheated Nuclease-free Water
6. Store aRNA at –20°C or below

C. Materials Provided with the Kit and Storage Conditions

P/N 4383451 10 rxn kit	P/N AM1793 30 rxn kit	Component	Storage
11 µL	33 µL	First Strand Enzyme Mix	–20°C
44 µL	132 µL	First Strand Buffer Mix	–20°C
22 µL	66 µL	Second Strand Enzyme Mix	–20°C
55 µL	165 µL	Second Strand Buffer Mix	–20°C
66 µL	198 µL	T7 Enzyme Mix	–20°C
220 µL	660 µL	T7 Biotin IVT Mix	–20°C
10 µL	10 µL	Control RNA (1 mg/mL HeLa total RNA)	–20°C
1.75 mL	1.75 mL	Nuclease-free Water	any temp*
10 mL	30 mL	Wash Buffer Add 100% ethanol before use	4°C or room temp
4 mL	12 mL	aRNA Binding Buffer	room temp†
10 ea.	30 ea.	aRNA Filter Cartridges	room temp
20 ea.	60 ea.	aRNA Collection Tubes	room temp
10 ea.	10 ea.	8-Strip PCR Tubes & Caps (0.2 mL)	room temp
1 mL	1 mL	5X Array Fragmentation Buffer	room temp
10 mL	10 mL	Nuclease-free Water	any temp*

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

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

D. Materials Not Provided with the Kit

Lab equipment and supplies

- 100% Ethanol (ACS reagent grade or equivalent)
- Thermal cycler capable of holding 0.2 mL tubes for reaction incubations Recommended: Applied Biosystems Veriti™ 96-Well Thermal Cycler, or GeneAmp® PCR System 9700
- Vortex mixer
- Microcentrifuge with an adapter for the PCR strip-tubes supplied with the kit
- (Optional) RNA controls for microarray analysis, such as Array Control™ RNA Spikes from Ambion (P/N AM1780) or the GeneChip® Poly-A RNA Control Kit from Affymetrix® (P/N 900433)
- (Optional) Vacuum centrifuge concentrator

Optional materials and equipment for RNA analysis

- Spectrophotometer—such as the NanoDrop® ND-8000 UV-Vis Spectrophotometer.
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) Quant-iT™ RiboGreen® RNA Reagent (Invitrogen)

E. Related Products Available from Applied Biosystems

FirstChoice® Total and Poly(A) RNA See web or print catalog for P/Ns	Ambion provides high quality total and poly(A) RNA from a variety of human, mouse and rat tissues and from human cell lines. DNA is removed with a stringent DNase treatment. These RNAs are shown to be intact by denaturing agarose gel electrophoresis, Northern analysis, reverse transcription, and capillary electrophoresis using the Agilent 2100 bioanalyzer, and they are precisely quantitated. Please see our catalog or our website (www.ambion.com) for a complete listing.
RNA Isolation Kits See web or print catalog for P/Ns	Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits.
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.

II. aRNA Amplification Procedure

A. Important Parameters for Successful Amplification

Input RNA quantity and IVT reaction incubation time

Consider both the type and amount of sample RNA available and the amount of aRNA needed for your analysis when planning MessageAmp experiments using the MessageAmp III Kit. Because mRNA content varies significantly with tissue type, the optimal amount of total RNA input should be determined empirically for each experimental system. The recommended input RNA amounts listed in Table 1 are based on using total RNA from HeLa cells; use these recommendations as a starting point. Table 2 shows the corresponding recommended IVT incubation times.

NOTE

The RNA volume must be ≤5 µL.

Table 1. Input RNA Limits

Recommendations	Amount
Recommended	100 ng
Minimum	35 ng
Maximum	500 ng

Table 2. Recommended IVT Incubation Times

RNA Amount	IVT Incubation Time
35–50 ng	14 hr
50–100 ng	8 hr
100–200 ng	4–8 hr
200–500 ng	2–8 hr

RNA purity

RNA quality is the single most important factor affecting how efficiently an RNA sample will be amplified using the MessageAmp III 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, 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 transcribing partially degraded mRNAs will generate cDNAs that may lack portions of the coding region. RNA integrity can be evaluated by microfluidic analysis using the Agilent 2100 bioanalyzer with an RNA LabChip® Kit. 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. The RIN, a metric developed by Agilent, includes information from both the rRNA bands and outside the rRNA peaks (potential degradation products) to provide a picture of RNA degradation states. Search for "RIN" at the following web address for more information:

http://www.chem.agilent.com

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.

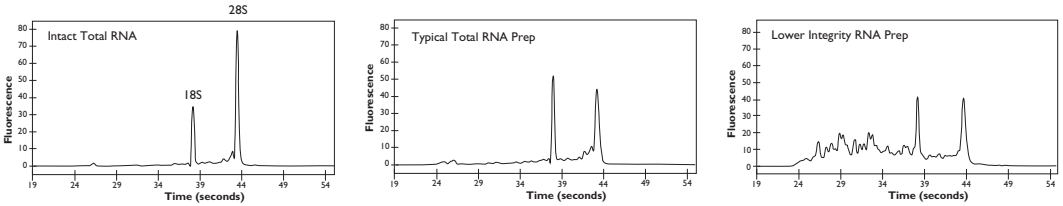


Figure 2. Bioanalyzer Images of Total RNA Preparations

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 MessageAmp III 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 typical example of a 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).

Other important parameters

Keep reaction incubation times precise and consistent

The incubation times for the enzymatic reactions in the protocol were optimized in conjunction with the kit reagents for maximum yield in each step—adhere to them closely. An exception is the IVT reaction, where a range of 2–14 hr incubation time is acceptable (step II.E.2 on page 12). Refer to Table 2 on page 6 to help determine what incubation time to use.

Use master mixes

We strongly recommend preparing master mixes for each step of the MessageAmp III procedure. This reduces the effects of pipetting error, saves time, and improves reproducibility. A web-based master mix calculator is available at: www.ambion.com/tools/ma3

Mix each kit component before use

- Mix enzyme solutions by ***gently*** flicking the tube a few times before adding them to master mixes.
- Thaw frozen reagents completely at room temperature, then mix thoroughly by vortexing, and place on ice.

Incubate MessageAmp reactions in a calibrated thermal cycler

- We do ***not*** recommend using ordinary laboratory heat blocks, water baths, or hybridization ovens for any of the reaction incubations.
- The MessageAmp procedure is very sensitive to temperature; therefore use a thermal cycler that has been calibrated according to the manufacturer's recommended schedule. Variable or inaccurate incubation temperatures can negatively impact aRNA synthesis.
- ***Heated lids:*** It is important that condensation does not form in the tubes during any of the incubations, because it would change the reaction composition and can greatly reduce yield. If possible, set the lid temperature to match the block temperature. Otherwise, incubate all reactions with the heated lid on (~100°C).

Maintain procedural 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 and thermal cycler to use for each step in the process. Finally, develop a consistent workflow. 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 procedure steps in order to maximize amplification consistency among samples.

B. Equipment and Reagent Preparation

Prepare Wash Buffer

- Add 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled Wash Buffer, as indicated on the label.
- Mix well and mark the label to indicate that the ethanol was added. Store at room temperature.

Program the thermal cycler

Incubate all MessageAmp III reactions in a thermal cycler. We find it convenient to set up the thermal cycler programs for each incubation before starting the procedure. The specifications for each incubation are shown in Table 3.

Table 3. Thermal Cycler Programs for MessageAmp™ III RNA Amplification

Program (or Method)			
First-Strand cDNA Synthesis	42°C for 2 hr	4°C indefinite hold	
Second-Strand cDNA Synthesis	16°C for 1 hr	65°C for 10 min	4°C indefinite hold
IVT	40°C for 2–14 hr	4°C indefinite hold	

C. Reverse Transcription to Synthesize First Strand cDNA

1. Assemble *First Strand Master Mix* and dispense 5 µL into a reaction tube on ice

a. Thaw first strand synthesis reagents and place on ice.

b. On ice, assemble ***First Strand Master Mix*** in a nuclease-free tube in the order listed in Table 4. Include ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts: www.ambion.com/tools/ma3

Table 4. First-Strand Master Mix (for a single reaction)

Component	Amount
First Strand Buffer Mix	4 µL
First Strand Enzyme Mix	1 µL
Total volume	5 µL

c. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the mix at the bottom of the tube.

d. Place the supplied PCR Tubes or Plate on ice and transfer 5 µL First Strand Master Mix to individual tubes.

2. Add 5 µL RNA and mix thoroughly

a. Add 5 µL RNA sample (plus Nuclease-free Water if necessary) to each aliquot of First Strand Master Mix for a final volume of 10 µL.

NOTE

To include RNA Spikes (e.g., the Ambion ArrayControl™ RNA Spikes, P/N AM1780 or Affymetrix GeneChip Poly-A Control Kit, P/N 900433), add them to samples at this step.

b. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube and place on ice.

3. Incubate at 42°C for 2 hr

a. Incubate for 2 hr at 42°C in a thermal cycler using the program for "First-Strand cDNA Synthesis" (page 9).

b. After the incubation, centrifuge briefly (~5 sec) to collect the first strand cDNA at the bottom of the tube. Place the sample on ice and immediately proceed to second strand cDNA synthesis (below).

D. Second Strand cDNA Synthesis

1. Assemble *Second Strand Master Mix* and add 20 µL to each sample

a. On ice, prepare a *Second Strand Master Mix* in a nuclease-free tube in the order listed in Table 5. Prepare master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts: www.ambion.com/tools/ma3

Table 5. Second-Strand Master Mix *(*for a single reaction)

Component	Amount
Nuclease-free Water	13 µL
Second Strand Buffer Mix	5 µL
Second Strand Enzyme Mix	2 µL
Total volume	20 µL

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

c. Transfer 20 µL Second Strand Master Mix to each (10 µL) cDNA sample. Mix thoroughly by gently vortexing or flicking the tube 3–4 times. Centrifuge briefly to collect the reaction at the bottom of the tube and place on ice.

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

2. Incubate for 1 hr at 16°C, then 10 min at 65°C

a. Incubate for 1 hr at 16°C followed by 10 min at 65°C in a thermal cycler using the program for "Second-Strand cDNA Synthesis" (page 9).

NOTE

Cover reactions with the heated lid of the thermal cycler even if its temperature cannot be adjusted to match the block temperature.

b. After the incubation, centrifuge briefly (~5 sec) to collect the double-stranded cDNA at the bottom of the tube.

c. Place on ice and ***immediately*** proceed to the IVT (below) or freeze at –20° C.

STOPPING POINT

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

E. In Vitro Transcription to Synthesize Biotin-Modified aRNA

1. Assemble a *T7 IVT Master Mix* and add 30 µL to each sample

a. At room temp, prepare a *T7 IVT Master Mix* in a nuclease-free tube in the order listed in Table 6. Prepare master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts: www.ambion.com/tools/ma3

Table 6. T7 IVT Master Mix (for a single reaction)

Component	Amount
Nuclease-free Water	4 µL
T7 Biotin IVT Mix	20 µL
T7 Enzyme Mix	6 µL
Total volume	30 µL

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

c. Transfer 30 µL of T7 IVT Master Mix to each (30 µL) double-stranded cDNA sample. Mix thoroughly by gently vortexing, and centrifuge briefly to collect the reaction at the bottom of the tube.

d. Once assembled, place the reaction in the thermal cycler block.

2. Incubate for 2–14 hr at 40°C

Incubate the IVT reaction for 2–14 hr at 40°C in a thermal cycler using the program for "IVT" (page 9). The recommended incubation time is based on the amount of input RNA and is shown in Table 7 below.

Table 7. Recommended IVT Incubation Times

RNA Amount	IVT Incubation Time
35–50 ng	14 hr
50–100 ng	8 hr
100–200 ng	4–8 hr
200–500 ng	2–8 hr

3. Place the aRNA on ice briefly or freeze immediately

Place the reaction on ice and proceed to the aRNA purification step (below) or immediately freeze at –20°C for overnight storage.

STOPPING POINT

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

F. aRNA Purification

After synthesis, the aRNA is purified to remove enzymes, salts, and unincorporated nucleotides.

IMPORTANT

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

Before beginning the aRNA purification:

- Preheat the bottle of Nuclease-free Water to 50–60°C for at least 10 min.
- For each sample, place an aRNA Filter Cartridge into an aRNA Collection Tube and set aside for use in step 3.

1. Add 40 µL Nuclease-free Water to each sample

a. Add 40 µL Nuclease-free Water to bring each sample to 100 µL.

b. Vortex to mix thoroughly, then centrifuge briefly (~5 sec) to collect the contents at the bottom of the tube.

c. Transfer sample to a nuclease-free 1.5 mL tube (not provided with the kit).

2. Add 350 µL aRNA Binding Buffer to each sample

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

3. Add 250 µL 100% ethanol, pipet 3 times to mix, then spin samples through an aRNA Filter Cartridge

IMPORTANT

It is crucial to follow these mixing instructions exactly and to work quickly.

a. Add 250 µL of ACS grade 100% ethanol to each aRNA sample.

b. Mix by pipetting the mixture up and down 3 times. ***Do not vortex to mix and do not centrifuge.***
Proceed ***immediately*** to the next step—any delay in proceeding could result in loss of aRNA because once the ethanol is added, the aRNA will be in a semiprecipitated state.

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

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

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

4. 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, replace the filter in the tube, and spin the aRNA Filter Cartridge for an additional ~1 min to remove trace amounts of Wash Buffer.

d. Transfer each Filter Cartridge to fresh aRNA Collection Tube.

5. Elute aRNA with 100 µL preheated Nuclease-free Water

a. To the center of the filter, add 100 µL Nuclease-free Water (preheated to 50–60°C) and close the tube.

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. The aRNA will now be in the aRNA Collection Tube in ~100 µL of Nuclease-free Water.

6. Store aRNA at –20°C or below

Store aRNA at –20°C or below for up to 1 year. As with any RNA preparation, the number of freeze-thaw cycles should be minimized to maintain aRNA integrity.

III. Evaluation and Fragmentation of aRNA

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. 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 (Invitrogen) is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for 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.

4. (Optional) Concentrate the purified aRNA

If necessary, concentrate the aRNA by vacuum centrifugation. If the heater on the vacuum centrifuge has different settings, use medium or low. Check the progress of drying every 5–10 min, and remove the sample from the concentrator when it reaches the desired volume. Avoid drying aRNA samples to completion.

B. Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with the Agilent RNA 6000 Nano Kit (part number 5067-1511), 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. Instructions for denaturing agarose gel electrophoresis are provided on our website at the following address:

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

Expected aRNA size

We recommend analyzing aRNA size distribution using an Agilent bioanalyzer and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 850–1500 nt.

C. Fragmentation of Biotinylated aRNA for GeneChip® Arrays

Most protocols for array hybridization begin with a sample fragmentation step prior to hybridization. The 5X Array Fragmentation Buffer supplied with the MessageAmp III Kit is compatible with the Affymetrix GeneChip® array platform. You can use the 5X Array Fragmentation Buffer following either the protocol in the Affymetrix GeneChip Expression Analysis Technical Manual, or the equivalent procedure included here. The composition of the 5X Array Fragmentation Buffer is shown below.

Table 8. 5X Array Fragmentation Buffer Composition

Concentration	Component
200 mM	Tris Acetate, pH 8.2
500 mM	Potassium Acetate
150 mM	Magnesium Acetate

1. Assemble the aRNA fragmentation mixture

The aRNA fragmentation reaction employs metal-induced hydrolysis to fragment input aRNA.

aRNA quantity and reaction volume

Refer to the Affymetrix GeneChip Expression Analysis Technical Manual for the following information:

- The amount of aRNA needed for hybridization with your GeneChip array format
- The recommended fragmentation reaction volume—this will be based on the volume of the hybridization mixture for your GeneChip array format.

Table 9. Example aRNA Fragmentation Reactions

40 µL rxn*	30 µL rxn*	Component
1–32 µL	1–24 µL	5–20 µg aRNA (depending on GeneChip array format)
8 µL	6 µL	5X Array Fragmentation Buffer [1X final]
to 40 µL	to 30 µL	Nuclease-free Water

* Use the reaction volume recommended for your GeneChip array platform.

2. Incubate at 94°C for 35 min, then place in ice

a. Incubate the fragmentation reaction at 94°C for 35 min.

b. Place the reaction on ice immediately after the incubation.

3. (Optional) Evaluate a sample of the reaction on a bioanalyzer

Analyze the size of the fragmentation reaction products by running a 300 ng sample of the reaction on an Agilent bioanalyzer using an Agilent RNA 6000 Nano Kit. Figure 3 shows a typical result of such analysis. (Follow the manufacturer's instructions for this analysis.)

The reaction should produce a distribution of 35–200 nt aRNA fragments with a peak at approximately 105 nt.

4. Use fragmented aRNA immediately or store frozen

Use the fragmented aRNA immediately in a GeneChip hybridization following the instructions in the Affymetrix GeneChip Expression Analysis Technical Manual, or store undiluted, fragmented aRNA at –20°C or below.

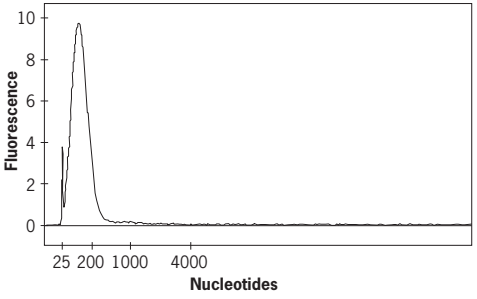


Figure 3. Fragmented aRNA.

Agilent bioananlyzer analysis of a 1 µL sample of a 30 µL fragmentation reaction containing 10 µg of aRNA.

IV. Troubleshooting

A. Positive Control Reaction

Control RNA amplification instructions

To verify that the process is working as expected, a Control RNA sample isolated from HeLa cells is provided with the kit.

a. Dilute 2 µL of the Control RNA into 18 µL of Nuclease-free Water.

b. Use 1 µL of the diluted Control RNA (100 ng) in a MessageAmp III reaction; follow the procedure starting at step II.C.1 on page 9.

c. At step II.E.2 on page 12, use an 8 hr incubation for the IVT reaction.

d. Continue with the procedure for making biotin-modified aRNA through section II.F.

Expected results

- The positive control reaction should produce ≥30 µg of aRNA.
- The average size of the aRNA should be ~1000 nucleotides.

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. Use only properly calibrated thermal cyclers for the MessageAmp procedure.

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, check to make sure that the heated lid feature of the thermal cycler is working properly.

Nuclease contamination

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).

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.

Lower than expected input RNA concentration

Take another A_{260} reading of your RNA sample or, if it is available, try using 100–200 ng of RNA in the amplification procedure.

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.

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 section *II.A. RNA integrity* on page 7 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 1–10% of total cellular RNA (Johnson 1974, Sambrook and Russell 2001). 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).

V. Appendix

A. References

Johnson LF, Abelson HT, Green H, and Penman S (1974) Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA and tRNA in Resting and Growing Cell. *Cell* **1(2):** 95–100.

Chapter 7 Extraction, purification, and analysis of mRNA from eukaryotic cells. In: Sambrook and Russell, editors (2001) *Molecular Cloning, a Laboratory Manual, third edition Vol I* Cold Spring Harbor, New York: Cold Spring Harbor Press.

MAQC Consortium et al. (2006) The MicroArray Quality Control (MAQC) project shows inter- and intra-platform reproducibility of gene expression measurements. *Nature Biotech.* **24(9):** 1151–1161.

Schoor O, Weinschenk T, Hennenlotter J, Corvin S, Stenzl A, Rammensee H-G, and Stevanovic S (2003) Moderate degradation does not preclude microarray analysis of small amounts of RNA. *Biotechniques* **35**:1192–1201.

Van Gelder RN, von Xastrow ME, Yool A, Dement DC, Barchas JD, Eberwine JH (1990) Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci USA*, **87**:1663–1667.

B. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

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

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

C. Quality Control

Functional testing

The Control RNA is used in a MessageAmp III reaction following the instructions in section IV.A on page 17. The aRNA yield is assessed by measuring the A_{260} on a spectrophotometer. The median size of the aRNA is assessed using the mRNA smear assay on the Agilent 2100 bioanalyzer. The aRNA is functionally evaluated by hybridization to an Affymetrix GeneChip microarray.

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.