MessageAmp™ II-Biotin *Enhanced* Kit

(Part Number AM1791)

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P/N 1791M Revision B **Revision Date: May 19, 2008**

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I. Introduction

A. Product Description and Background

The MessageAmp™ II-Biotin *Enhanced* Single Round aRNA Amplification Kit (patent pending) is based on the RNA amplification protocol developed in the Eberwine laboratory (Van Gelder et al. 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™ (patent pending), 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 (IVT) in a reaction containing biotin-modified UTP and T7 RNA polymerase. To maximize biotin-labeled aRNA yield, an optimized mixture of biotin-labeled and unlabeled NTPs are supplied with the kit, and Ambion's proprietary MEGAscript® IVT technology is used to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. (In this Protocol the antisense amplified RNA is referred to as aRNA; it is also commonly called cRNA.) Once purified, the biotin-labeled aRNA is suitable for use on microarray gene expression systems designed for biotin-labeled antisense RNA samples.

Systematically optimized for production of biotin-labeled aRNA for array analysis

In order to develop the MessageAmp II-Biotin *Enhanced* Kit, we systematically optimized the MEGAscript T7 IVT amplification reaction for appropriate incorporation of biotin-modified UTP. We found that both the length of the linker arm connecting the biotin to the uridine base and the concentration of the modified nucleotide in the IVT reaction were important for maximal aRNA synthesis. Previous widely accepted labeling protocols included two biotin-modified nucleotides (CTP and UTP). We and others (Dorris et al.), however, found that the contribution of signal from biotin-CTP is minimal, and that the majority of the signal on arrays results from incorporated biotin-UTP. To further optimize the reaction, Ambion evaluated several biotin-modified UTP compounds. In these studies, biotin-11-UTP proved to have good incorporation, minimal effect on aRNA recovery during purification, and high signal on most commercial microarrays.

Benefits of RNA amplification

RNA amplification was originally developed as a method to expand very small RNA samples to produce enough material for array hybridization (Yue et al. 2001). Several groups have conducted studies to determine whether amplification of RNA introduces bias and they report that any bias is minimal (Li et al. 2004, Feldman et al. 2002 and Polacek et al. 2003). Additionally, among the benefits of amplification is a more

reproducible expression profile from a wide range of RNA inputs. Some researchers conclude that amplification actually improves the reliability of array results regardless of whether it is needed for sample expansion (Feldman et al. 2002 and Polacek et al. 2003). As a result, RNA amplification has become the standard method for preparing RNA samples for array analysis (Kacharmina et al. 1999, Pabon et al. 2001).

B. Procedure Overview

The MessageAmp II-Biotin aRNA amplification procedure is depicted in Figure 1.

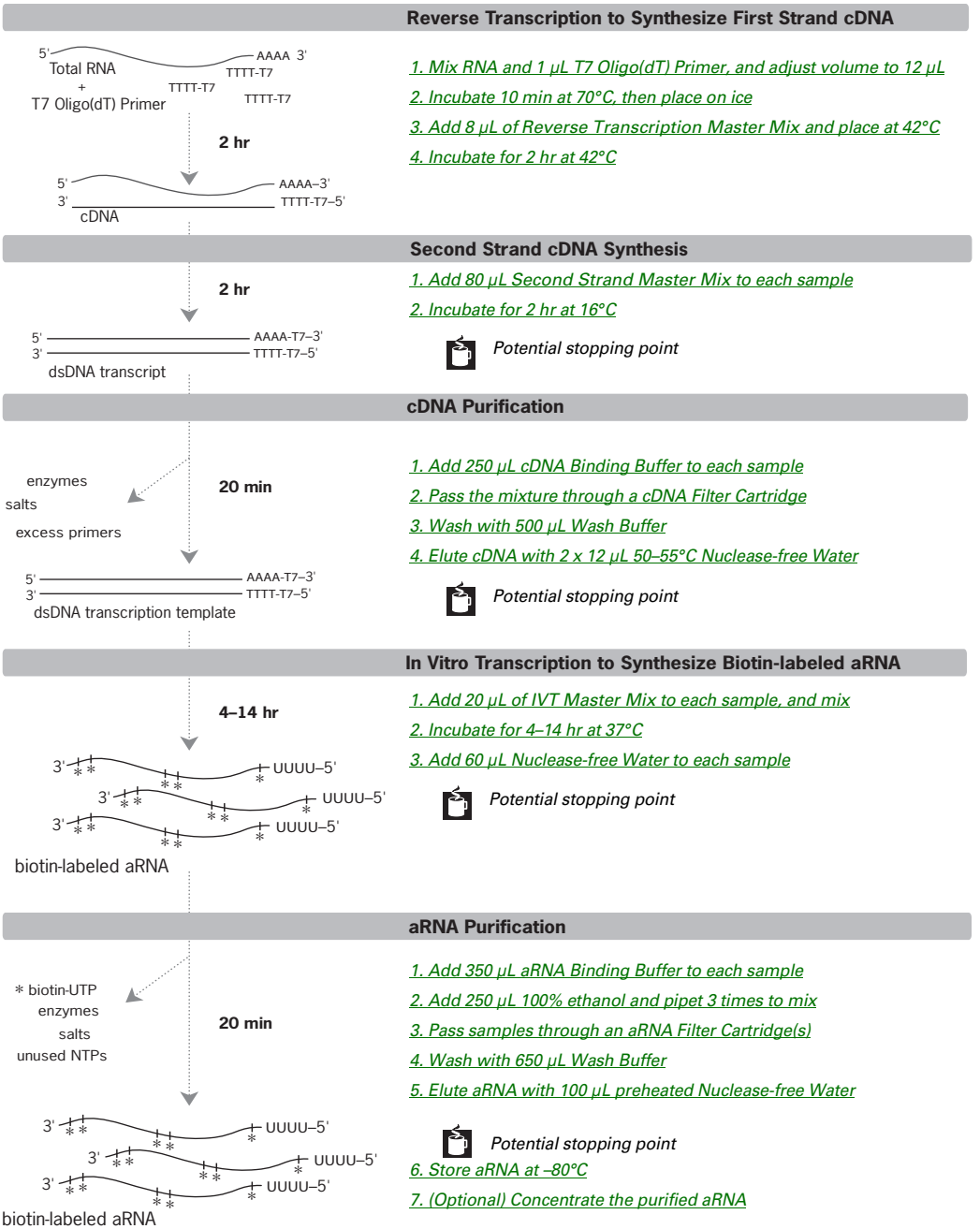
- ***Reverse Transcription to Synthesize First Strand cDNA*** is primed with the T7 Oligo(dT) Primer to synthesize cDNA containing a T7 promoter sequence.
- ***Second Strand cDNA Synthesis*** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA Polymerase and RNase H to simultaneously degrade the RNA and synthesize second strand cDNA.
- ***cDNA Purification*** removes RNA, primers, enzymes, and salts that would inhibit in vitro transcription.
- ***In Vitro Transcription to Synthesize aRNA*** with Biotin-NTP 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.

To include two rounds of amplification, purchase the MessageAmp II Kit

If your experiments require two rounds of amplification, you will need to purchase the Ambion® MessageAmp II aRNA Amplification Kit (P/N AM1751) in addition to the MessageAmp II-Biotin *Enhanced* Kit. For the first round amplification, use the MessageAmp II Kit to make unmodified aRNA.

For the second round of amplification, continue to use the MessageAmp II aRNA Amplification Kit until you have completed the second round cDNA synthesis step. At this point, follow the cDNA purification protocol for the MessageAmp II-Biotin *Enhanced* Kit; this will provide the correct cDNA elution volume for the IVT reaction. Continue to follow the MessageAmp II-Biotin *Enhanced* Kit protocol for the second round amplification IVT reaction to generate biotin-labeled aRNA.

Figure 1. MessageAmp II-Biotin *Enhanced* Kit Procedure



The MessageAmp II-Biotin advantage

Each step in the MessageAmp II-Biotin *Enhanced* Kit amplification procedure has been streamlined and optimized. The first strand cDNA synthesis reaction employs Ambion's proprietary, engineered reverse transcriptase, ArrayScript, to ensure that every cDNA bears a T7 promoter at its 5' end and that even very limited amounts of mRNA are fully converted to full-length cDNA. The second strand cDNA synthesis reaction is designed for the efficient synthesis of full-length, double-stranded cDNAs and the complete conversion of single-stranded cDNA into double-stranded transcription templates. The cDNA purification procedure not only removes enzymes, salts, and unincorporated dNTPs, but also efficiently removes RNA from the cDNA sample. This eliminates the heating or enzymatic digestion step commonly used in other procedures to degrade RNA (especially ribosomal RNA). The IVT reaction features Ambion's patented MEGAscript technology to maximize transcriptional amplification and yield of aRNA. It is optimized to ensure efficient transcription of limited amounts of input DNA and synthesis of long transcripts.

C. Materials Provided with the Kit and Storage Conditions

The MessageAmp II-Biotin *Enhanced* Kit includes reagents for single-round amplification and biotin-labeling of 20 samples.

The MessageAmp II-Biotin Kit is configured for single-round amplification. To perform two rounds of amplification, the Ambion MessageAmp II aRNA Amplification Kit (P/N AM1751) must be used in conjunction with the MessageAmp II-Biotin *Enhanced* Kit. See section I.B on page 2 for more information.

cDNA synthesis and IVT reagents

Amount	Component	Storage
20 µL	T7 Oligo(dT) Primer*	–20°C
22 µL	ArrayScript™	–20°C
22 µL	RNase Inhibitor	–20°C
42 µL	10X First Strand Buffer	–20°C
170 µL	dNTP Mix	–20°C
210 µL	10X Second Strand Buffer	–20°C
42 µL	DNA Polymerase	–20°C
22 µL	RNase H	–20°C
84 µL	T7 Enzyme Mix	–20°C
84 µL	T7 10X Reaction Buffer	–20°C
255 µL	Biotin-NTP Mix	–20°C
10 µL	Control RNA (1 mg/mL HeLa total RNA)	–20°C
1.75 mL	Nuclease-free Water	any temp†

* The T7 Oligo(dT) Primer is available separately from Ambion (P/N AM5710).
† Store the Nuclease-free Water at –20°C, 4°C, or room temp.

cDNA and aRNA purification, and fragmentation reagents

Amount	Component	Storage
30 mL	Wash Buffer (Add 24 mL 100% ethanol before use)	4°C or room temp
7 mL	cDNA Binding Buffer	room temp*
9 mL	aRNA Binding Buffer	room temp
1 mL	5X Array Fragmentation Buffer	room temp
20	aRNA Filter Cartridges	room temp
40	aRNA Collection Tubes	room temp
20	cDNA Filter Cartridges + Tubes	room temp
20	cDNA Elution Tubes	room temp
10 mL	Nuclease-free Water	any temp†

* The cDNA 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.

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

D. Materials Not Provided with the Kit

Lab equipment and supplies

- 100% Ethanol (to prepare the Wash Buffer)
- Thermal cycler with adjustable-temperature heated-lid, hybridization oven, or constant temperature incubators set at 70°C, 42°C, 37°C, and 16°C (See *Incubator recommendations* on page 10 for more information.)
- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- Non-stick RNase-free 0.5 mL microfuge tubes (Ambion P/N AM12350)
- (Optional) RNA controls for microarrays analysis, such as Array Control™ RNA Spikes from Ambion (P/N AM1780) or the GeneChip® Eukaryotic Poly-A RNA Control Kit from Affymetrix® (Cat #900433)

Optional materials and equipment for RNA analysis

- Spectrophotometer—such as the NanoDrop® ND-1000A UV-Vis Spectrophotometer.
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes Inc.)

E. Related Products Available from Applied Biosystems

MessageAmp™ aRNA Amplification Kits see our web or print catalog	Ambion offers a full line of MessageAmp Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion offers the MessageAmp Premier and MessageAmp III RNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.
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.
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.
Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452, AM8453	Ambion's biotinylated UTPs are ideal for use as substrates in vitro transcription reactions, and can be utilized by a variety of RNA polymerases, including T7, T3, and SP6 RNA polymerases. Biotinylated RNA can be used in place of radioactively labeled RNA in many applications with detection via one of a variety of streptavidin-based methods.
RNA Fragmentation Reagents P/N AM8740	Amplified RNA is commonly fragmented prior to hybridization on oligonucleotide microarrays to improve the hybridization kinetics and signal produced on oligonucleotide microarrays. Ambion's RNA Fragmentation Reagents include a 10X Fragmentation Reagent and a Stop Solution.
Amino Allyl cDNA Labeling Kit P/N AM1705	The Amino Allyl cDNA Labeling Kit generates cDNA for secondary fluorescent dye labeling to be used for glass array analysis. It includes all the reagents, except the amine-reactive labeling moiety (e.g. cyanine dyes) for 2-step labeling of cDNA. The reaction produces more labeled cDNA, more efficiently than direct dye incorporation.
RETROscript® Kit P/N AM1710	First strand cDNA synthesis kit. RETROscript® can be used to incorporate dye modified nucleotides into cDNA using aRNA prepared with the MessageAmp II Kit as a template.
5-(3-aminoallyl)-dUTP P/N AM8439	This 50 µM solution of amino allyl modified dUTP can be used with the RETROscript® Kit (P/N AM1710) to synthesize amine-reactive cDNA from aRNA. The amine-reactive cDNA can then be postlabeled with any amine-reactive label moiety.

II. aRNA Amplification Procedure

A. Important Parameters for Successful Amplification

Input RNA quantity and IVT reaction incubation time

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

Accurate quantitation

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

Recommended minimum and maximum amounts of input RNA

Table 1 shows the mass of total RNA that can be used in the MessageAmp II-Biotin *Enhanced* procedure. Alternatively, 10–100 ng of poly(A) selected RNA can be used in the procedure. The RNA volume must be ≤10 µL.

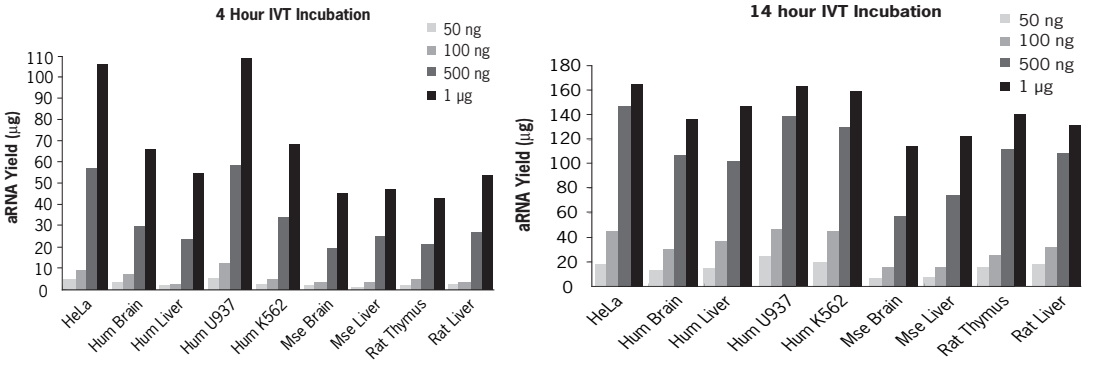
Table 1. Recommended Amount of Total RNA Input

Recommended	Minimum	Maximum
1000 ng	50 ng	5000 ng

Determining input RNA amount and IVT reaction incubation time

The procedure can accommodate a wide range of input RNA amounts, but for reproducible and comparable results, use a fixed amount of input RNA for all experiments. Tailor both the amount of input RNA and the amplification procedure to produce the amount of aRNA needed for your microarray hybridizations. For instance, Affymetrix GeneChips require 10–15 µg of aRNA for each hybridization, but other commercial and core facility arrays may require slightly more or less aRNA.

Figure 2 shows aRNA yield data from amplification of increasing amounts of input RNA from different sources. The experiment included either a 4 hr or a 14 hr IVT reaction incubation. With most samples, amplification of 50 ng of total RNA for 14 hr produced enough aRNA for a microarray experiment. When amplifying small RNA samples (e.g., ~250 ng or less), incubating the IVT reaction for 14 hr will maximize the amount of aRNA produced.



Input RNA	HeLa		Human Brain		Human Liver		U937 (Human)		K562 (Human)	
	4 h	14 h	4 h	14 h	4 h	14 h	4 h	14 h	4 h	14 h
50 ng	4.8	17.9	3.4	13.3	1.5	15.0	5	25.6	2.6	20.1
100 ng	9.4	45.1	7.4	31.0	2.6	37.3	12.5	47.1	4.8	45.4
500 ng	57.1	147.3	29.8	108.1	23.3	102.3	58.7	139.5	34.1	130.6
1 µg	105.8	165.6	66.0	136.5	54.7	147.3	109.1	163.2	68.8	158.9

Input RNA	Mouse Brain		Mouse Liver		Rat Thymus		Rat Liver		**Overall Average**	
	4 h	14 h	4 h	14 h	4 h	14 h	4 h	14 h	4 h	14 h
50 ng	1.8	7.7	1.2	8.3	1.8	16.5	2.1	18.4	2.7	15.9
100 ng	3.6	15.7	3.6	16.6	5.1	26.0	3.9	32.1	5.9	32.9
500 ng	19.1	57.7	24.6	75.0	21.1	112.2	26.9	108.6	32.7	109.0
1 µg	45.4	115.1	47.1	122.2	43.2	140.6	53.8	131.9	66.0	142.4

Figure 2. aRNA Yield from Nine Different Tissue/Cell Types

Four different amounts of total RNA from nine different sources were amplified using the MessageAmp™ II-Biotin *Enhanced* Kit. Reactions were performed in triplicate using either a 4 h or a 14 h IVT reaction time. The average aRNA yields from the triplicate reactions are shown as bar graphs and in tabular format. This data is useful for determining both the amount of total RNA needed to obtain enough labeled aRNA for an array hybridization (typically ~10 µg) and the length of the IVT incubation that should be used. Note that there is a ~3-fold difference in aRNA yield between some of the samples. With most RNA sources, 50–100 ng of input total RNA amplified with the MessageAmp II-Biotin *Enhanced* Kit using a 14 hour IVT incubation will yield enough labeled aRNA for a microarray hybridization.

RNA purity

The quality of the RNA is the single most important factor affecting how efficiently an RNA sample will be amplified using the MessageAmp II-Biotin *Enhanced* Kit. RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is

the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1. RNA must be suspended in high quality water or TE (10 mM Tris-HCl, 1 mM EDTA) or THE RNA Storage Solution (P/N AM7000, AM7001).

RNA integrity

The integrity of the RNA sample, or the proportion that is full-length, is another important component of RNA quality. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that potentially lack portions of the coding region. RNA integrity can be evaluated by microfluidic analysis using the Agilent 2100 bioanalyzer and Caliper RNA LabChip® Kits. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity.

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e., no significant smearing below each band), with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that it requires microgram amounts of RNA.

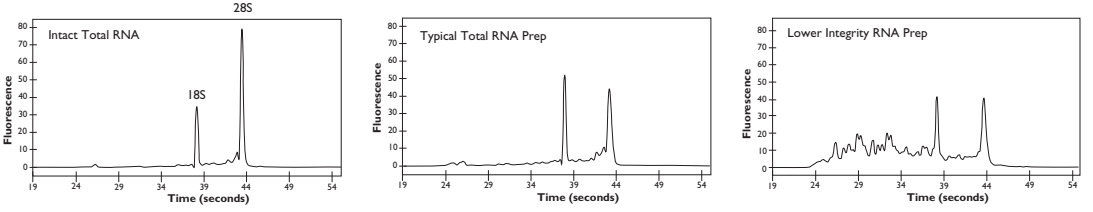


Figure 3. Bioanalyzer Images of Total RNA Preparations

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

Reaction incubation times should be precise and consistent

The incubation times for most of the enzymatic reactions in the procedure were optimized in conjunction with the kit reagents to ensure the maximum yield of nucleic acid product in each step—adhere to them closely. An exception is the IVT reaction, where a range of 4–14 hr incubation time is acceptable (step II.F.2 on page 16). Refer to Table 2 on page 17 to help determine what incubation time to use. Although

differences in IVT incubation time among samples has had very little, if any, effect on array results in our hands, we recommend using uniform IVT incubation times if aRNA yield from different samples will be compared or if you want to have equal amplification of different samples—this will provide the most reproducible amplification and array analysis.

Master mixes

We strongly recommend preparing master mixes for the MessageAmp II-Biotin *Enhanced* procedure. This approach reduces the effects of pipetting error, saves time, and improves reproducibility. Using master mixes is especially important when aRNA yield from different samples will be compared. We provide a web-based master mix calculator at the following address:

www.ambion.com/tools/ma2biotin

Thorough mixing is very important for reproducibility

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

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

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

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

Incubator recommendations

We recommend a calibrated hybridization oven, thermal cycler, or other constant temperature air incubator for most enzymatic reaction incubations.
We do ***not*** recommend using ordinary laboratory heat blocks or water baths for any MessageAmp II-Biotin reaction incubations.

To avoid any potential influence on the reaction temperature from the tube holder, let tube holders equilibrate in the incubator for sufficient time or use a tube holder that does not touch the sides and bottoms of the tubes—for example a floating tube support.

For the 16°C second strand synthesis reaction incubation (step II.D.2 on page 14), we recommend using a thermal cycler. Ideally these reactions should be incubated in a calibrated thermal cycler with a lid temperature that matches the block temperature. If your machine does not have this feature, and the lid temperature is static (~100°C), use it with the lid heat turned off or do not close the heated lid. Otherwise, heat from the lid will raise the temperature of the solution in the tube, compromising the reaction.

The 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 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 reaction tubes during any of the incubations. Condensation changes the composition of reaction mixtures and can greatly reduce yield.

Maintaining consistency

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

Tubes: use non-stick, RNase-free 0.5 mL tubes

It is most convenient to conduct the MessageAmp II-Biotin procedure in 0.5 mL nonstick tubes (e.g., Ambion P/N AM12350). These can be thin-wall (PCR) tubes or ordinary-weight nonstick tubes. 0.5 mL tubes are large enough to accommodate the cDNA Binding Buffer without having to transfer reactions to a larger tube. Their small size and nonstick properties also keep the reaction components at the bottom of the tube.

B. Prepare the Wash Buffer

Add 24 mL 100% ethanol (ACS grade or better) to the bottle labeled Wash Buffer. Mix well and mark the label to indicate that the ethanol was added.

C. Reverse Transcription to Synthesize First Strand cDNA

Incubators needed:

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

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

a. Place a maximum volume of 10 µL of total RNA (1000 ng recommended) or poly(A) selected RNA (typically 10–100 ng) into a nonstick, sterile, RNase-free, 0.5 mL tube. RNA must be in high quality water or TE. (See Table 2 on page 17 for minimum and maximum RNA input amounts.)

b. Add 1 µL of T7 Oligo(dT) Primer.

c. Add Nuclease-free Water to a final volume of 12 µL, vortex briefly to mix, then centrifuge to collect the mixture at the bottom of the tube.

NOTE

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

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

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

b. Centrifuge samples briefly (~5 sec) to collect them at the bottom of the tube. Place the mixtures on ice.

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

a. At room temp, prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube. Assemble enough to synthesize first strand cDNA from all the RNA 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/ma2biotin

At room temp, assemble the Reverse Transcription Master Mix in the order shown:

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

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

c. Transfer 8 µL of Reverse Transcription Master Mix to each RNA sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

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

4. Incubate for 2 hr at 42°C

Incubate reactions for 2 hr at 42°C (hybridization oven or air incubator is recommended). After the incubation, centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube.

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

D. Second Strand cDNA Synthesis

Incubator needed:

- 16°C: thermal cycler recommended

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

a. On ice, prepare a ***Second Strand Master Mix*** in a nuclease-free tube in the order listed below. Assemble enough to synthesize second strand cDNA from all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts:
www.ambion.com/tools/ma2biotin

Assemble the Second Strand Master Mix on ice in the order shown:

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

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

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

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

2. Incubate for 2 hr at 16°C

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

NOTE

You may want to preheat the Nuclease-free Water, for use in step II.E.4, during this incubation.

3. Place reactions on ice briefly or freeze immediately

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

STOPPING POINT

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

E. cDNA Purification

IMPORTANT

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

Preheat Nuclease-free Water to 50–55°C

Before beginning the cDNA purification, preheat the 10 mL bottle of Nuclease-free Water to 50–55°C for at least 10 min.

IMPORTANT

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

1. Add 250 µL cDNA Binding Buffer to each sample

IMPORTANT

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

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

2. Pass the mixture through a cDNA Filter Cartridge

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

a. Pipet the cDNA sample\cDNA Binding Buffer (from step 1) onto the center of the cDNA Filter Cartridge.

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

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

IMPORTANT

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

3. Wash with 500 µL Wash Buffer

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

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

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

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

4. Elute cDNA with 2 x 12 µL 50–55°C Nuclease-free Water

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

a. Apply 12 µL of Nuclease-free Water (preheated to 50–55°C) to the center of the filter in the cDNA Filter Cartridge.

b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until all the Nuclease-free Water is through the filter.

c. Elute with a second 12 µL of preheated Nuclease-free Water. The double-stranded cDNA will now be in the eluate (~20 µL).

d. Proceed directly to section F (next), or place the cDNA at –20°C.

STOPPING POINT

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

F. In Vitro Transcription to Synthesize Biotin-labeledaRNA

Incubator needed:

- 37°C: thermal cycler, hybridization oven, or air incubator recommended

1. Add 20 µL of *IVT Master Mix* to each sample, and mix

a. At room temp, prepare an ***IVT Master Mix*** by adding the following reagents to a nuclease-free microcentrifuge tube in the order listed below. Assemble enough for all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts:
www.ambion.com/tools/ma2biotin

IMPORTANT

*If two rounds of amplification will be done, the MessageAmp II Kit must be used for this first round transcription to make **unmodified** aRNA, (not biotin-labeled aRNA).*

Assemble the IVT Master Mix at room temp in the order shown:

Amount	Component
20 µL	double-stranded cDNA (from step E.4 on page 15)
IVT Master Mix for a single reaction	
12 µL	Biotin-NTP Mix
4 µL	T7 10X Reaction Buffer
4 µL	T7 Enzyme Mix

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

c. Transfer 20 µL of IVT Master Mix to each sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

d. Once assembled, place the tubes at 37°C.

2. Incubate for 4–14 hr at 37°C

The minimum recommended incubation time is 4 hr; the maximum is 14 hr. For overnight incubations, it is typically most convenient to incubate in a thermal cycler set to hold at 4°C after the 37°C incubation is complete.

Use the table below as a guide to determine how long to continue your IVT reaction. There are more data and a detailed discussion of the length of the IVT incubation in section II.A *Input RNA quantity and IVT reaction incubation time* starting on page 7.

Table 2. Recommended IVT Incubation TImes

aRNA Needed	Input Total RNA	IVT Incubation
10–100 µg	1–5 µg	4 hr
1–10 µg	50ng–1 µg	8 hr
0.1–1 µg	≤50 ng	14 hr

IMPORTANT

It is important to maintain a constant 37°C incubation temperature.

3. Add 60 µL Nuclease-free Water to each sample

Stop the reaction by adding 60 µL Nuclease-free Water to each aRNA sample to bring the final volume to 100 µL. Mix thoroughly by gentle vortexing.

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

STOPPING POINT

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

G. aRNA Purification

This purification removes enzymes, salts, and unincorporated nucleotides from the aRNA. At the end of the purification the aRNA is eluted from the filter with Nuclease-free Water.

IMPORTANT

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

aRNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Preheat Nuclease-free Water to 50–60°C

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

Assemble aRNA Filter Cartridges and tubes

For each sample, place an aRNA Filter Cartridge into an aRNA Collection Tube and set aside for use in step 3.

1. Add 350 µL aRNA Binding Buffer to each sample

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

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

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

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

IMPORTANT

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

Proceed ***immediately*** to the next step as soon as you have mixed the ethanol into each sample. Any delay in proceeding could result in loss of aRNA because once the ethanol is added, the aRNA will be in a semi-precipitated state.

3. Pass samples through an aRNA Filter Cartridge(s)

a. Pipet each sample mixture from step 2 onto the center of the filter in the aRNA Filter Cartridge.

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

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

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

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).

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 –80°C

Store aRNA at –80°C for up to 1 year, and minimize repeated freeze-thawing. Splitting samples into 5–20 µg aliquots for microarray labeling and hybridizations is a good way to prevent multiple freeze-thaw events.

7. (Optional) Concentrate the purified aRNA

If necessary, concentrate the aRNA by vacuum centrifugation or by precipitation with ammonium acetate (NH_4OAc)/ethanol.

(Optional) Concentrate 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.

(Optional) Precipitate with 5 M NH_4OAc and ethanol

a. Add 1/10th volume of 5 M NH_4OAc to the purified aRNA (10 µL if the aRNA was eluted in 100 µL Nuclease-free water).

b. Add 2.5 volumes of 100% ethanol (275 µL if the aRNA was eluted in 100 µL). Mix well and incubate at –20°C for 30 min.

c. Microcentrifuge at top speed for 15 min at 4°C or room temp. Carefully remove and discard the supernatant.

d. Wash the pellet with 500 µL 70% cold ethanol, centrifuge again, and remove the 70% ethanol.

e. To remove the last traces of ethanol, quickly respin the tube, and aspirate any residual fluid with a fine-tipped pipette or syringe needle.

f. Air dry the pellet.

g. Resuspend the aRNA pellet using the desired solution and volume.

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 using a spectrophotometer. With a traditional spectrophotometer, dilute an aliquot of the RNA 1:50–1:100 in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and read the absorbance. (Be sure to zero the spectrophotometer with the TE used for sample dilution.) The buffer used for dilution need not be RNase-free, since slight degradation of the RNA will not significantly affect its absorbance. NanoDrop spectrophotometers are more convenient—no dilutions or cuvettes are needed, just measure 1.5 µL of the RNA sample directly.

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

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

2. Assessing aRNA yield with RiboGreen

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

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 (see Figure 2 on page 8 for empirical aRNA yield data obtained using this kit).

B. Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with Caliper's LabChip technology, or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA (from step II.G.5 on page 18). 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

Agilent bioanalyzer analysis

The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 1000–1500 nt (Figure 4). To compare bioanalyzer profiles of different aRNA samples, be sure to load equal mass amounts to get an accurate comparison.

Denaturing agarose gel analysis

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

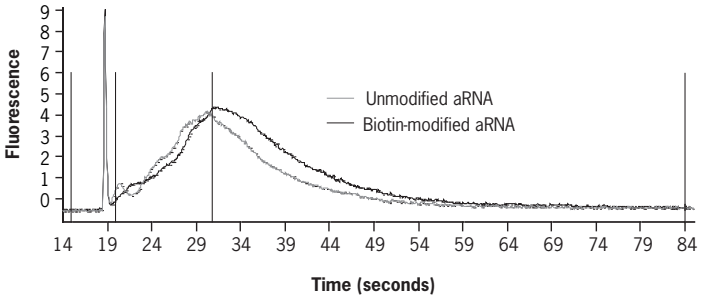


Figure 4. Biotin-labeled and Unlabeled aRNA Made with the MessageAmp II-Biotin *Enhanced* Kit and the MessageAmp II Kits.

HeLa RNA samples (1 µg) were amplified with either the MessageAmp II-Biotin *Enhanced* Kit to produce biotin-labeled aRNA, or the MessageAmp II Kit to produce unlabeled aRNA. The IVT reactions were carried out for 4 hr. The data were generated by running a sample of the reactions on an Agilent bioanalyzer using an RNA LabChip Kit.

C. Fragmentation of Biotinylated aRNA for GeneChip® Arrays

Most procedures for array hybridization begin with a sample fragmentation step prior to hybridization. The 5X Array Fragmentation Buffer supplied with the MessageAmp II-Biotin *Enhanced* Kit is designed for perfect compatibility with the Affymetrix GeneChip® array platform. You can use the 5X Array Fragmentation Buffer following either the procedure 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 3. 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 amount of aRNA needed for hybridization with your GeneChip array format, and
- the recommended fragmentation reaction volume. This will be based on the volume of the hybridization mixture for your GeneChip array format.

Table 4. 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 sample of the reaction on an Agilent bioanalyzer using an RNA LabChip Kit. Figure 5 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 for 1–3 days or at –80°C for long-term storage.

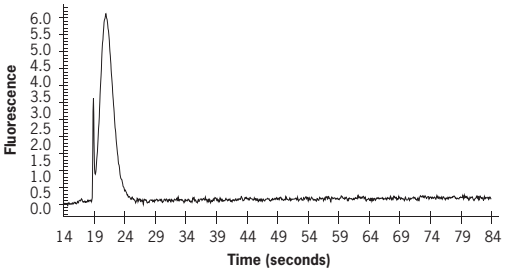


Figure 5. Fragmented aRNA.

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 establish if the kit is working properly, Control RNA consisting of 1 mg/mL HeLa cell total RNA is provided. ***Use 1 µL of the Control RNA*** in a MessageAmp II-Biotin *Enhanced* reaction; follow the procedure starting at step II.C.1 on page 12. At step II.F.2 on page 16, ***use a 14 hr incubation for the IVT reaction***. Continue with the procedure through section II.G on page 17.

Analysis of the positive control amplification reaction

- After completing the aRNA purification, measure the A_{260} of the reaction product as described in section III.A.1 on page 20.
  The positive control reaction should produce ≥80 µg of aRNA.
  Be aware that often the positive control reaction cannot be compared to experimental reactions, because many experimental amplification experiments will use less than the 1 µg of input RNA used in the positive control reaction, and the aRNA yield will be proportionately lower. Also the Control RNA is of exceptional quality and purity, ensuring that it will amplify with extremely high efficiency.
- Also run a 2 µg aliquot of the reaction products on a denaturing agarose gel or analyze 100–200 ng on a bioanalyzer; ***the average size of the aRNA should be ≥1 kb.***

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.

a. Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective RNA amplification.

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

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

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

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

c. Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, or equipment that are contaminated with nucleases can cleave the RNA or DNA being generated at each step in the procedure. This will reduce the size of the aRNA products and decrease aRNA yield. Both RNases and DNases can be removed from surfaces using Ambion RNase*Zap*® RNase Decontamination Solution (P/N AM9780, AM9786).

d. Absorbance readings were inaccurate

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

C. Troubleshooting Low Yield and Small Average aRNA Size

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

1. 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 Ambion MEGAclear™ Kit (P/N AM1908) to further purify your RNA before reverse transcription.

2. 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.

3. 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 9 for more information).

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

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

V. Appendix

A. References

Dorris DR, Ramakrishnan R, Trakas D, Dudzik F, Belval R, Zhao C, Nguyen A, Domanus M, Mazumder A (2002) A highly reproducible, linear, and automated sample preparation method for DNA microarrays. *Genome Res* **12(6):** 976–984.

Feldman AJ, Costouros NG, Wang E, Qian M, Marincola FM, Alexander HR, and Libutti SK (2002) Advantages of mRNA amplification for microarray analysis. *Biotechniques* **33(4)**:906–914.

Kacharmina JE, Crino PB, Eberwine J (1999) Preparation of cDNA from single cells and subcellular regions. *Methods Enzymol*, **303:**3–18.

Li Y, Li T, Liu S, Qiu M, Han Z, Jiang Z, Li R, Ying K, Xie Y, Mao Y (2004) Systematic comparison of the fidelity of aRNA, mRNA and T-RNA on gene expression profiling using cDNA microarray. *J Biotechnology* **107(1)**:19–28.

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

Polacek DC, Passerini AG, Shi C, Francesco NM, Manduchi E, Grant GR, Powell S, Bischof H, Winkler H, Stoeckert CJ Jr, Davies PF (2003) Fidelity of enhanced sensitivity of differential transcription profiles following linear amplification of nanogram amounts of endothelial mRNA. *Physiol Genomics* **13**:147–156.

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.

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

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 II-Biotin reaction following the instructions in section IV.A on page 24. The aRNA yield is assessed by measuring the A_{260} on the NanoDrop ND1000A spectrophotometer. The median size of the aRNA is assessed using the mRNA smear assay on the Agilent 2100 bioanalyzer.

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.