USER GUIDE

ambion®
by life technologies™

MessageAmp™ Premier RNA Amplification Kit

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life technologies™

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Information in this guide is subject to change without notice.

Contents

MessageAmp™ Premier RNA Amplification Kit

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Introduction

Product description and background

The Ambion® MessageAmp™ Premier 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).

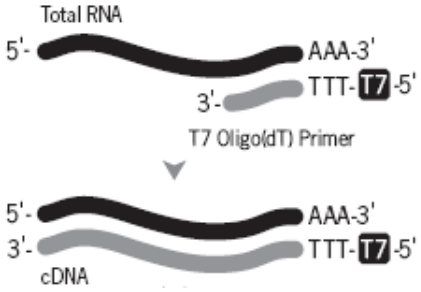
Based on this proven enzymatic amplification and labeling strategy, the MessageAmp™ Premier procedure starts with a simple cDNA synthesis reaction using ArrayScript™ reverse transcriptase. No cleanup step is required after the RT; cDNA is used directly in a high yield IVT reaction using Ambion® MEGAscript® 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™ Premier Kit features

- Lowest RNA input requirements for a single round of amplification: 20 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: 20–500 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
- Easy-to-use, high-recovery, magnetic-bead aRNA purification

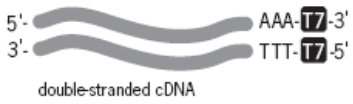
Figure 1 MessageAmp™ Premier RNA Amplification Procedure

Reverse Transcription to Synthesize First Strand cDNA



1. "Assemble First Strand Master Mix and dispense 5 µL into a reaction tube on ice" on page 11
2. "Add 5 µL RNA and mix thoroughly" on page 12
3. "Incubate at 42°C for 2 hr" on page 12

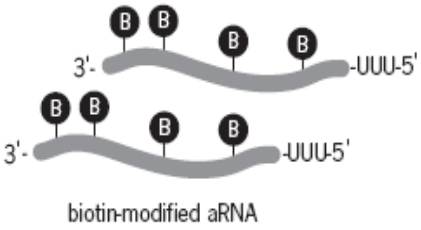
Second Strand cDNA Synthesis



1. "Assemble Second Strand Master Mix and add 20 µL to each sample" on page 12
2. "Incubate for 1 hr at 16°C, then 10 min at 65°C" on page 12

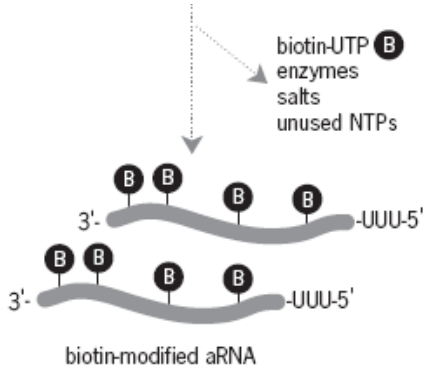
(*Potential Stopping Point)*

In Vitro Transcription to Synthesize Biotin-Modified aRNA



1. "Assemble a T7 IVT Master Mix and add 30 µL to each sample" on page 13
2. "Incubate for 2–14 hr at 40°C" on page 14
3. "Place the aRNA on ice briefly or freeze immediately" on page 14

aRNA Purification



1. "Prepare aRNA Binding Mix for the experiment" on page 14
2. "Add 60 µL aRNA Binding Mix and transfer sample to U-bottom plate" on page 14
3. "Add 120 µL ethanol to each sample and shake gently for 2 min" on page 14
4. "Capture the RNA Binding Beads and discard supernatant" on page 15
5. "Wash with 2 X 100 µL aRNA Wash Solution" on page 15
6. "Elute aRNA with 50 µL preheated aRNA Elution Solution" on page 15
7. "Prepare aRNA Binding Mix for the experiment" on page 14

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™ Premier RNA Amplification procedure. Instructions for the positive control reaction are provided in section "Positive control reaction" on page 19.

Materials provided with the kit and storage conditions

Cat. no. 4385821	Cat. no. AM1792	Cat. no. 4383452		
10 rxn kit	**30 rxn kit**	**100 rxn kit**	**Component**	**Storage**
11 µL	33 µL	110 µL	First Strand Enzyme Mix	–20°C
44 µL	132 µL	440 µL	First Strand Buffer Mix	–20°C
22 µL	66 µL	220 µL	Second Strand Enzyme Mix	–20°C
55 µL	165 µL	550 µL	Second Strand Buffer Mix	–20°C
66 µL	198 µL	660 µL	T7 Enzyme Mix	–20°C
220 µL	660 µL	2 x 1.1 mL	T7 Biotin IVT Mix	–20°C
10 µL	10 µL	10 µL	Control RNA (1 mg/mL HeLa total RNA)	–20°C
1.75 mL	1.75 mL	1.75 mL	Nuclease-free Water	any temp†
600 µL	1.8 mL	6 mL	aRNA Binding Buffer Concentrate	room temp
120 µL	360 µL	1.2 mL	RNA Binding Beads	4°C‡
10 mL	10 mL	30 mL	aRNA Wash Solution Concentrate • Add 100% ethanol as shown on the label before use.	room temp
5 mL	5 mL	15 mL	aRNA Elution Solution	4°C or room temp
1 mL	1 mL	1 mL	5X Array Fragmentation Buffer	room temp
10 ea.	20 ea.	– –	8-Strip PCR Tubes & Caps (0.2 mL)	room temp
1 ea.	2 ea.	2 ea.	U-Bottom Plate	room temp
–	–	4 ea.	PCR Plate	room temp
1 ea.	1 ea.	1 ea.	Reservoir	room temp
10 mL	10 mL	10 mL	Nuclease-free Water	any temp†

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

‡ Do not freeze.

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 or plates supplied with the kit
- Magnetic stand for 96-well plates
  Recommended: Magnetic Stand-96, Cat. no. AM10027
- Orbital shaker for 96-well plates such as the Barnstead/Lab-Line Titer Plate Shaker (available from major laboratory suppliers)
- (Optional) RNA controls for microarray analysis, such as ArrayControl™ RNA Spikes from Life Technologies (Cat. no. AM1780) or the GeneChip® Poly-A RNA Control Kit from Affymetrix (Cat. no. 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)

aRNA amplification procedure

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™ Premier 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 the first table are based on using total RNA from HeLa cells; use these recommendations as a starting point. The second table shows the corresponding recommended IVT incubation times.

Note: The RNA volume must be ≤ 5 µL.

Input RNA limits	
Recommendations	**Amount**
Recommended	100 ng
Minimum	20 ng
Maximum	500 ng

Recommended IVT incubation times	
RNA Amount	**IVT Incubation Time**
20–50 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™ Premier 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 (Cat. no. AM7000, AM7001).

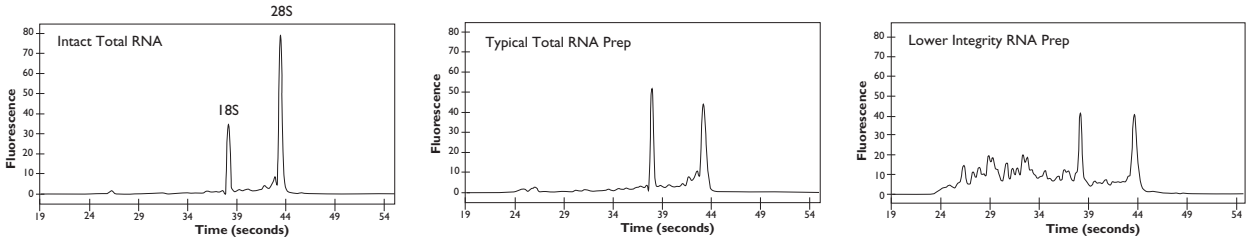
RNA integrity

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® instrument 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® instrument) show RNA samples with decreasing integrity that are all of sufficient quality to use as input for the MessageAmp™ Premier 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 2. on page 14). Refer to the second table in the section "Input RNA quantity and IVT reaction incubation time" on page 8 to help determine what incubation time to use.

- Use master mixes

  We strongly recommend preparing master mixes for each step of the MessageAmp™ Premier Kit procedure. This reduces the effects of pipetting error, saves time, and improves reproducibility.

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™ kit 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.

Equipment and reagent preparation

Prepare aRNA Wash Solution

- Add 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled aRNA Wash Solution Concentrate, as indicated on the label.
- Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as aRNA Wash Solution in these instructions. Store at room temperature.

Program the thermal cycler

Incubate all MessageAmp™ Premier 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 the following table.

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	

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 the table below. Include ≤5% overage to cover pipetting error.

Component	Amount
First Strand Buffer Mix	4 µL
First Strand Enzyme Mix	1 µL

Component	Amount
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 or wells.

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, Cat. no. AM1780 or Affymetrix GeneChip® Poly-A Control Kit, Cat. no. 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/plate 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 11).

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

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 the following table. Prepare master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error.

Component	Amount per reaction
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/plate 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 11).

   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/plate.

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.

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 the following table. Prepare master mix for all the samples in the experiment, including ≤ 5% overage to cover pipetting error.

Component	Amount per reaction
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/plate.

   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 11). The recommended incubation time is based on the amount of input RNA and is shown in the table below.

RNA Amount	IVT Incubation Time
20–50 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.

aRNA purification

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

Before beginning the aRNA purification:

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

1. Prepare *aRNA Binding Mix* for the experiment

   IMPORTANT! Prepare only the amount needed for all samples in the experiment plus ~10% overage to cover pipetting error.

   At room temperature, assemble aRNA Binding Mix in a nuclease-free tube for all the samples in the experiment following the instructions in the table below.

Component	Amount
RNA Binding Beads†	10 µL
aRNA Binding Buffer Concentrate	50 µL

† Mix the RNA Binding Beads by vortexing before dispensing.

2. Add 60 µL aRNA Binding Mix and transfer sample to U-bottom plate
   a. Add 60 µL aRNA Binding Mix to each sample.
   b. Transfer each sample to a well of a U-Bottom Plate.
3. Add 120 µL ethanol to each sample and shake gently for 2 min
   a. Add 120 µL 100% ethanol to each sample.
   b. Gently shake for ≥2 min to thoroughly mix (setting 4 on the Lab-Line Titer Plate Shaker). The aRNA in the sample will bind to the RNA Binding Beads during this incubation.

4. Capture the RNA Binding Beads and discard supernatant

   a. Move the plate to a magnetic stand and capture the magnetic beads, for ~5 min. When capture is complete, the mixture becomes transparent and the RNA Binding Beads will form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand used.

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

5. Wash with 2 X 100 µL aRNA Wash Solution

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

   a. Add 100 µL aRNA Wash Solution to each sample, and shake at moderate speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).

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

   b. Move the plate to a magnetic stand and capture the RNA Binding Beads as in the previous step.

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

   d. Repeat above substeps to wash a second time with 100 µL of aRNA Wash Solution.

   e. Move the plate to a shaker and shake the plate vigorously for 1 min to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker).

6. Elute aRNA with 50 µL preheated aRNA Elution Solution

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

      Vigorously shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker). Then check to make sure the RNA Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

   b. Move the plate to a magnetic stand, and capture the RNA Binding Beads.

   c. Transfer the supernatant, which contains the eluted aRNA, to a nuclease-free multiwell plate.

7. 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.

Evaluation and fragmentation of aRNA

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)

   $$A_{260} \text{ X dilution factor X } 40 = \mu g \text{ RNA/}$$

2. Assessing aRNA yield with RiboGreen® analysis

   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®analysis.

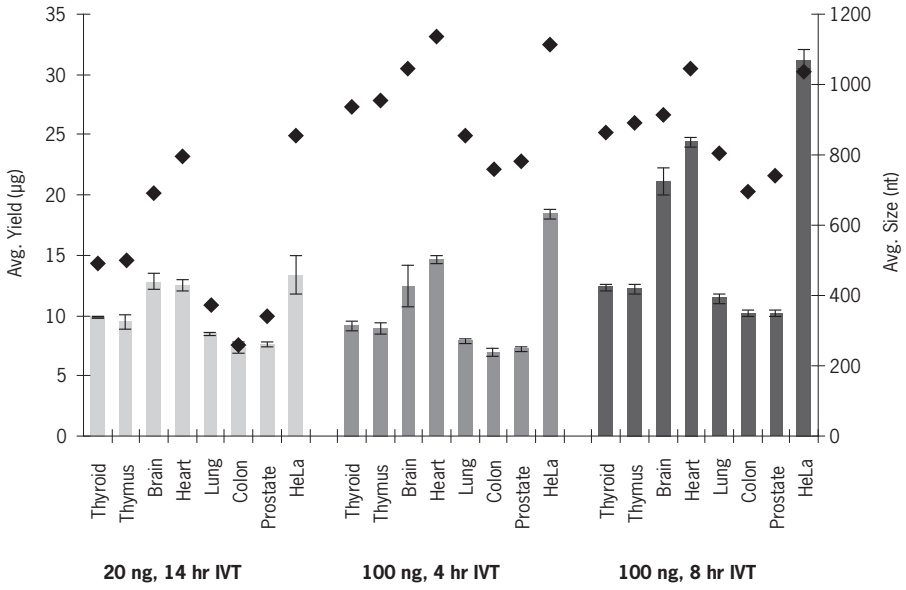
3. Expected yield

   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. Figure 3 shows yield and size data for aRNA produced with the kit from several different types of input RNA.

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.

Figure 3 Yield and Size of aRNA Synthesized Using the MessageAmp™ Premier RNA Amplification Kit. Triplicate RNA samples (20 or 100 ng) from HeLa cells or 7 tissues (Ambion® FirstChoice® Total RNA) were amplified and labeled with the MessageAmp Premier Kit using the indicated IVT reaction times. Shown are the average amplified RNA yields (bars) and median size (diamonds). Note that in some cases, 20 ng of input RNA provided enough labeled sample for GeneChip® array analysis. Increasing the amount of input RNA to 100 ng, provided enough labeled sample for GeneChip® array analysis even from tissues, such as thyroid and prostate, that are known to contain very little messenger RNA.



Analysis of aRNA size

The size distribution of aRNA can be evaluated using an Agilent® 2100 Bioanalyzer® instrument 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.

Expected aRNA size

We recommend analyzing aRNA size distribution using an Agilent® Bioanalyzer instrument 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.

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™ Premier 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.

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.

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.

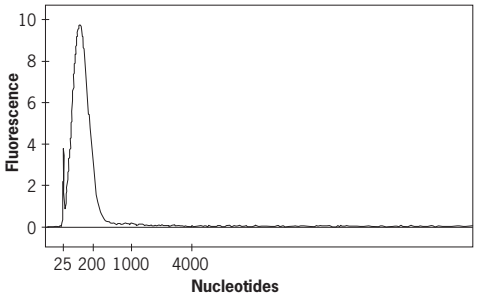
2. Incubate at 94°C for 35 min, then place in ice
   a. Incubate the fragmentation reaction at 94°C for 35 min.
   b. Place the reaction on ice immediately after the incubation.
3. (Optional) Evaluate a sample of the reaction on a bioanalyzer

   Analyze the size of the fragmentation reaction products by running a 300 ng sample of the reaction on an Agilent® Bioanalyzer instrument using an Agilent® RNA 6000 Nano Kit. Figure 4 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 4 Fragmented aRNA. Agilent® Bioananlyzer® instrument analysis of a 1 µL sample of a 30 µL fragmentation reaction containing 10 µg of aRNA.



Troubleshooting

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™ Premier reaction; follow the procedure starting at step 1. on page 11.

c. At step 2. on page 14, use an 8 hr incubation for the IVT reaction.

d. Continue with the procedure for making biotin-modified aRNA through section “aRNA purification” on page 14.

Expected results

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

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 (Cat. no. AM9780, AM9786).

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 (Cat. no. 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 “RNA integrity” on page 9 for more information).

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

Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 1–10% 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).

A Supplemental Information

Related products available from Life Technologies

FirstChoice® Total and Poly(A) RNA	Life Technologies 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® instrument, and they are precisely quantitated.
RNA Isolation Kits	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 Cat. no.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.
96-well Magnetic-Ring Stand Cat. no.AM10050	The 96-well Magnetic-Ring Stand features 96 powerful ring-shaped magnets arranged to cradle each well of a 96-well plate for quick, thorough bead capture. Captured magnetic beads form evenly distributed donut-shaped pellets with a large hole in the center. This capture pattern facilitates both supernatant removal and subsequent bead resuspension. The stand is suitable for high throughput applications conducted with multichannel pipettors or with robotic liquid handlers. However, because the pellets will be evenly distributed around the edge of the wells, it may require practice for efficient manual removal of supernatants.
Magnetic Stand-96 Cat. no.AM10027	The Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96 well Magnetic-Ring Stand (Cat. no. AM10050).

Quality control

Functional testing

The Control RNA is used in a MessageAmp Premier reaction following the instructions in section IV.A on page 19. 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® instrument. 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

A sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

A sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

A sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

A sample is incubated with protease substrate and analyzed by fluorescence.

B

Safety

General safety

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

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

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:
Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.).
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of

according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

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

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

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

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

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Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/sds**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

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

www.lifetechnologies.com

At the website, you can:

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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