PROTOCOL

ambion® | RNA
by life technologies™

MessageAmp™ II-Bacteria Kit

life technologies™

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MessageAmp™ II-Bacteria Kit

(Part Number AM1790)

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I. Introduction

IMPORTANT

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

A. Background

The MessageAmp™ II-Bacteria Kit for prokaryotic RNA amplification was developed to facilitate whole genome expression analysis from bacterial samples. The kit employs an in vitro transcription (IVT)-mediated linear amplification method optimized for use with bacterial RNA. In eukaryotic systems, RNA amplification is firmly established as the preferred method of sample preparation for gene expression analysis using most commercial microarray platforms, as well as for glass arrays and other noncommercial systems (Kacharmina et al. 1999, Pabon et al. 2001). MessageAmp II-Bacteria labeling requires orders-of-magnitude less input RNA, yet compares favorably in terms of reproducibility and dynamic range with standard labeling protocols such as direct labeling by reverse transcription or post-labeling reverse transcription (RT) reaction products.

Benefits of RNA amplification

RNA amplification was developed as a method to expand very small RNA samples so that there would be 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 2002 and Polacek 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 2002 and Polacek 2003).

MessageAmp II-Bacteria facilitates whole genome expression analysis in prokaryotes

Analysis of whole genome expression in eukaryotes with DNA microarrays has revolutionized the way biologists study gene function. Bacterial RNA amplification with the MessageAmp II-Bacteria Kit will facilitate similar studies in prokaryotes, even research involving rare or limited samples, such as species that are difficult to culture, environmental collections, small volume cultures, and bacterial RNA isolated from host cells. With over 800 microbial genomes now fully sequenced, and many more bacterial genome sequencing projects in progress, opportunities to analyze global expression analysis on pathogens, environmentally important microbes, and model organisms are rapidly expanding. Amplification of bacterial RNA will enable researchers to make better use of these new and exciting tools.

B. Product Description

The MessageAmp II-Bacteria Kit is a linear in vitro transcription-based RNA amplification system (Van Gelder et al. 1990) to produce amplified RNA (aRNA, also commonly called copied RNA or cRNA). Compared to methods for amplifying eukaryotic RNA, the major difference is that since bacterial mRNA does not have a stable poly(A) tail, it must be polyadenylated to be a suitable substrate for amplification. Therefore the first step in the procedure is polyadenylation using *E. coli* Poly(A) Polymerase (PAP) in an optimized reaction which ensures complete and representative polyadenylation of bacterial RNA molecules. Next, the tailed RNA is reverse transcribed in a reaction primed with an oligo(dT) primer bearing a T7 promoter. The MessageAmp II-Bacteria procedure employs ArrayScript™ reverse transcriptase, 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 resulting cDNA is then transcribed with T7 RNA Polymerase to generate hundreds to thousands of antisense RNA (aRNA) copies of each RNA in the sample. To maximize aRNA yield, Ambion® MEGAscript® in vitro transcription reagents are included in the kit. The IVT reaction can be configured to synthesize either labeled aRNA (e.g. biotin, Cy™Dyes, or amino allyl) or unlabeled aRNA that can subsequently be labeled by reverse transcription (for example with fluorescent or radioactive dNTPs). The aRNA can be labeled by either including a labeled nucleotide in the IVT reaction, or by reverse transcribing the aRNA after synthesis in a reaction containing a labeled nucleotide. The MessageAmp II-Bacteria Kit can thus be used to prepare both ^{33}P-labeled sense-strand cDNA (for nylon PCR arrays) and biotin- or fluorescent dye-labeled aRNA for glass slide microarrays or Affymetrix GeneChip® arrays.

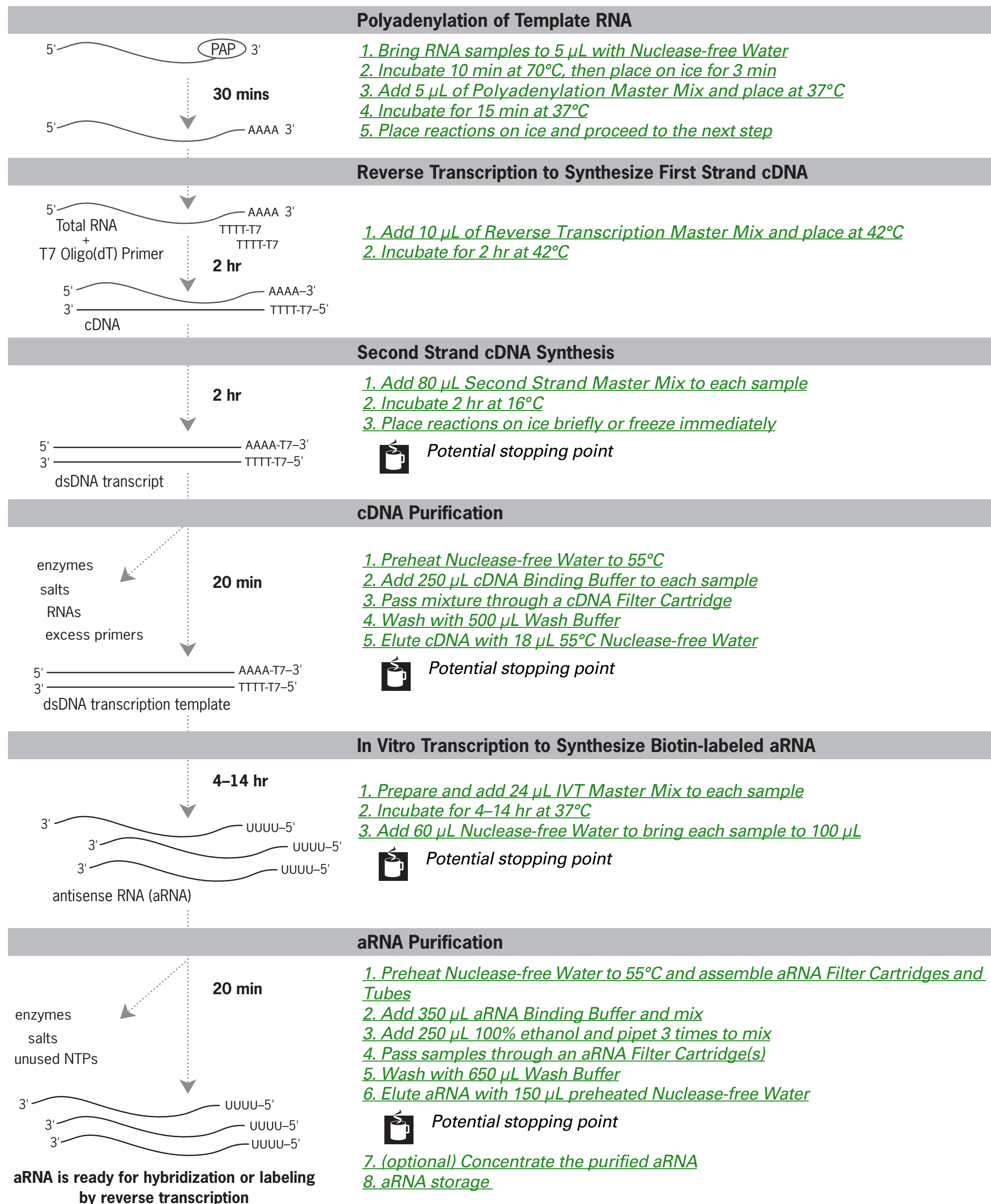
aRNA can be generated for any bacterial species, and input RNA prepared in many different ways can be used for amplification: total RNA, bacterial mRNA enriched using the Ambion MICROB*Express*™ Kit, or bacterial RNA enriched from host cell mixtures with the MICROB*Enrich*™ Kit. As little as 10 ng of enriched bacterial mRNA or 100 ng of total RNA can be successfully amplified and used for microarray analysis. This translates into a 10-fold to 500-fold reduction in the amount of RNA needed to probe a microarray, depending on the platform.

C. Procedure Overview

The MessageAmp II-Bacteria Prokaryotic RNA amplification procedure is depicted in Figure 1 on page 4.

- ***Polyadenylation*** utilizes *E. coli* Poly(A) Polymerase to polyadenylate (tag) the RNA sample, in preparation for oligo(dT)-mediated reverse transcription.
- ***Reverse Transcription to Synthesize First Strand cDNA*** is primed with the T7 Oligo(dT) VN anchored primer to synthesize cDNA with a T7 promoter sequence.
- ***Second Strand cDNA Synthesis*** converts the single-stranded cDNA with the T7 promoter primer into 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 from the dsDNA that inhibit in vitro transcription.
- ***In Vitro Transcription to Synthesize aRNA*** generates multiple copies of antisense aRNA from the double-stranded cDNA templates; this is the linear amplification step.
- ***aRNA Purification*** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the aRNA and facilitate subsequent enzymatic manipulations.

Figure 1. MessageAmp II-Bacteria Procedure Overview



D. Materials Provided with the Kit and Storage Conditions

This kit includes reagents for amplification of 20 bacterial RNA samples.

Poly(A) tailing, cDNA synthesis, and in vitro transcription

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

Amount	Component
22 µL	PAP
22 µL	10X Poly(A) Tailing Buffer
12 µL	Poly(A) Tailing ATP
22 µL	T7 Oligo(dT) VN Primer
22 µL	ArrayScript
22 µL	RNase Inhibitor
22 µL	10X First Strand Buffer
170 µL	dNTP Mix
210 µL	10X Second Strand Buffer
42 µL	DNA Polymerase
22 µL	RNase H
84 µL	T7 Enzyme Mix
84 µL	10X T7 Reaction Buffer
84 µL	T7 ATP
84 µL	T7 CTP
84 µL	T7 GTP
84 µL	T7 UTP
1.75 mL	Nuclease-free Water*
10 µL	Control RNA (1 mg/mL *E. coli* total RNA)

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

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

cDNA and aRNA purification

Store at temperatures shown below, and as shown on component containers.

Amount	Component	Storage
30 mL	Wash Buffer (Add 24 mL 100% ethanol)	4°C or room temp
7 mL	cDNA Binding Buffer	room temp*
9 mL	aRNA Binding 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 below room temperature. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 15 min and vortexing vigorously. Cool to room temperature before use.

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

E. Materials Not Provided with the Kit

Lab equipment and supplies

- 100% Ethanol (to prepare the Wash Buffer)
- Thermal cycler with a temperature-adjustable heated lid (recommended), or hybridization ovens or incubators set at 70°C, 42°C, 37°C, and 16°C. (See "Thermal cycler recommended" on page 15 for more information.)
- Heat block set at 55°C, for preheating the water for cDNA and aRNA purification
- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- Non-stick RNase-free 0.5 mL microcentrifuge tubes, for example, P/N AM12350
- RNase-free pipettors and tips, positive-displacement type recommended to increase the accuracy and precision of reaction inputs

Materials and equipment for RNA analysis

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

(optional) Reagents for synthesizing labeled aRNA

There are several ways to synthesize labeled aRNA for use in array hybridization. In most cases, the choice of array labeling moiety and strategy should be based on the recommendations of the manufacturers of the arrays and the array analysis detection system. Below we list modified nucleotides that have been used during development of the MessageAmp Kits.

Direct incorporation of modified NTPs during the IVT reaction

- Biotin-labeled UTP can be added to the in vitro transcription reaction to synthesize biotin-labeled aRNA. Biotin-11-UTP (P/N AM8451, 75 mM) is recommended because it gives good incorporation, has minimal effect on aRNA recovery during purification, and results in high signal on most commercial microarrays.
- 5-(3-aminoallyl)-UTP (P/N AM8437) can be incorporated into aRNA to provide an amine-reactive group for addition of label with any moiety bearing an N-hydroxysuccinimidyl ester (NHS) ester.
- Cyanine-dye–labeled CTP and UTP are available from PerkinElmer Life Sciences, Amersham Biosciences, and Enzo Biochemicals.

Incorporation of modified dNTPs by reverse transcription of aRNA made with the MessageAmp II-Bacteria Kit

It is important to keep in mind that aRNA is anti-sense and the resulting cDNA will be sense-strand. The microarray design will dictate which of these can be used.

- 5-(3-aminoallyl)-dUTP (P/N AM8439) can be added to reverse transcription reactions to produce amino allyl-modified cDNA from the aRNA made with this kit. The amino allyl-modified cDNA can then be labeled with any moiety bearing an N-hydroxysuccinimidyl (NHS) ester.
- Other labeled dNTPs that can be incorporated into cDNA by reverse transcription can also be used to label aRNA made with this kit.

F. Related Products

Product	Description
RiboPure™-Bacteria P/N AM1925	The RiboPure-Bacteria RNA Isolation Kit combines an efficient glass bead-mediated organic disruption step with glass fiber filter purification for high yields of exceptionally pure bacterial RNA.
FirstChoice® *E. coli* Total RNA P/N AM7940	This high quality *E. coli* total RNA has been precisely quantitated and shown to be intact by denaturing agarose gel electrophoresis, Northern analysis, reverse transcription, and capillary electrophoresis using the Agilent 2100 bioanalyzer. DNA is removed with a stringent DNase treatment.
MICROB*Express*™ Kit P/N AM1905	The MICROB*Express* Kit employs a novel technology to remove >95% of the 16S and 23S rRNA from total RNA of a broad spectrum of gram-positive and gram-negative bacteria.
MICROB*Enrich*™ Kit P/N AM1901	The MICROB*Enrich* Kit employs a novel technology to remove over 90% of mammalian RNA from complex mixtures of host-bacterial RNA samples. If desired the enriched bacterial RNA obtained can be further enriched for bacterial mRNA using the MICROB*Express* Kit.
Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452	Biotinylated UTPs are ideal for use as substrates for 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.
5-(3-aminoallyl)-UTP P/N AM8437	This 50 µM solution of amino allyl modified UTP can be used with the MessageAmp® II-Bacteria Kit to synthesize amine-reactive aRNA which can then be postlabeled with any amine-reactive label moiety.
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.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette see our web or print catalog	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. The Glass Array Hybridization Cassette is also offered, for incubation of glass microarray hybridization reactions.
RNA 6000 Ladder P/N AM7152	The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer.

II. Bacterial RNA Amplification Procedure

A. Important Parameters for Successful Amplification

The following suggestions are provided to help you obtain accurate and reproducible gene expression data. Carefully read all of the recommendations below and incorporate them into your lab workflow. Microarray data generation is extremely susceptible to variations in laboratory procedure, and the adage "bad data in, bad data out" is especially true for gene expression studies.

Suggestions for consistent results

Sample consistency

It is critical to use similar input RNA mass amounts, RNA type (total RNA or mRNA), and IVT incubation times when comparing data from different samples. Also, as with other RNA amplification methods, only amplified samples should be compared. Unamplified samples should not be compared directly with amplified samples. All amplification methods, or any labeling method for that matter, produce some level of bias in RNA representation. What is important for gene expression analysis is that the biases are reproducible within a method and that similar labeling methods are used on samples being compared.

Procedural consistency

Procedural consistency is very important for planning 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 which 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 procedures such as which tubes, tube racks, and incubators 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.

Harvesting bacteria for optimal RNA quality

To obtain the highest quality RNA, harvest actively dividing cells (logarithmic phase) for RNA isolation. RNA isolated from bacteria in the stationary phase typically exhibits increased degradation. For this reason, we strongly caution against harvesting cells that have grown into stationary phase for RNA isolation unless a specific experiment requires it.

The method used to harvest bacteria can also have a strong influence on RNA quality—especially the quality of mRNA. It is known that bacterial mRNAs exhibit a wide range of stabilities. For example, approximately 80% of all mRNAs in *E. coli* are known to have half-lives of 3–8 min (Bernstein et al. 2002). For this reason, it is important to collect cells in a manner that minimizes or eliminates the impact of harvesting conditions on both gene expression profiles and/or RNA quality. With this in mind, it is preferable to process small volumes of bacterial cultures (1–2 mL) and to use a brief centrifugation step (~1 min) to pellet the cells. The cell pellets can then be processed with one of the following treatments:

- Immediate cell lysis and RNA purification
- Rapid freezing in liquid nitrogen (a freeze-thaw treatment may aid lysis of some bacteria)
- Resuspension of cells in RNA*later*® Solution. For more information, go to www.invitrogen.com/site/us/en/home/support/technical-support.html

RNA isolation and purification recommendations

For the most sensitive array analysis, we recommend all of the following treatments/purification methods for RNA used as template in the MessageAmp II-Bacteria Kit:

RNA must be very pure and of high integrity

RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1. RNA must be suspended in high quality water, TE (10 mM Tris-HCl, 1 mM EDTA), or THE RNA Storage Solution (P/N AM7000, AM7001).

The integrity of the RNA sample, or the proportion that is full length, is another important component of RNA quality. Reverse transcribing partially degraded, tailed mRNAs will typically generate relatively short cDNAs that can potentially lack portions of the coding region. RNA integrity can be evaluated by microfluidics analysis using the Agilent 2100 bioanalyzer and an RNA LabChip Kit. Primarily full-length RNA will exhibit a ratio of 23S to 16S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. A metric developed by Agilent, the RIN analyzes information from both the rRNA bands, as well as information contained outside the rRNA peaks (potential degradation products) to provide a fuller picture of RNA degradation states. Search for "RIN" at the following web address for more information:

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 23S rRNA band appearing approximately twice as intense as the 16S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that microgram amounts of RNA must be sacrificed, and there is no easy way to objectively evaluate RNA integrity.

Subject RNA to glass fiber-based RNA purification (e.g. the RiboPure™-Bacteria Kit)

To prepare RNA for use in the MessageAmp II-Bacteria Kit, we strongly recommend including a glass fiber-based RNA purification step either as part of the RNA isolation strategy or as an additional clean-up step after RNA isolation. (e.g. with the MEGAclear™ Kit, P/N AM1908) unless the experimental design precludes it. The Ambion RiboPure™-Bacteria: Bacterial RNA Isolation Kit (P/N AM1925) was used extensively during the development of MessageAmp II-Bacteria and provided excellent RNA template for amplification. The RiboPure-Bacteria Kit does not include enzymatic pretreatments and thus does not alter expression profiles. Instead, RiboPure-Bacteria combines an efficient glass bead-mediated organic disruption step followed by a glass filter-based RNA purification for high yields of exceptionally pure bacterial RNA. The RiboPure-Bacteria Kit also includes DNA-*free*™ reagents for quick and simple DNase treatment of samples without organic extraction, alcohol precipitation, or column purification. Glass fiber purification increases the purity of RNA samples and dramatically reduces the amount of 5S rRNA and tRNA in samples (Figure 2 on page 12). RNA that is purified via glass fiber methods produces higher aRNA yields than RNA obtained with simple organic extraction methods, especially as more input RNA is used in the amplification reaction (Table 1 on page 12). This is believed to be a result of increased purity of glass fiber purified RNA; however, there is also evidence that 5S and tRNA are not efficient amplification substrates.

Table 1 shows empirical data for amplifications using different input amounts, RNA types, and IVT incubation times. Notice how the benefit of including a glass fiber purification step in the RNA preparation has a much greater effect on aRNA yield with 100 ng of input RNA compared with 10 ng of input RNA.

Table 1. Effect of IVT Incubation Time and RNA Isolation Method on aRNA Yield (avg. of triplicates)

RNA Isolation Method	Amount of total RNA	IVT incubation time 6 hours	IVT incubation time 14 hours
organic extraction	10 ng	34 µg	78 µg
organic extraction	100 ng	57 µg	130 µg
glass fiber purification	10 ng	31 µg	66 µg
glass fiber purification	100 ng	111 µg	198 µg

aRNA synthesized

DNase treat RNA

RNA should be treated with DNase to remove contaminating genomic DNA; we strongly recommend using only RNA that is free of genomic DNA in the MessageAmp II-Bacteria Kit for the best array analysis results. Contaminating genomic DNA can contribute to spurious amplification products.

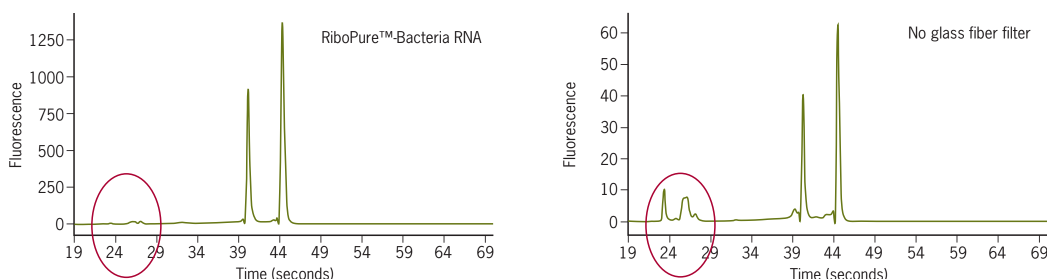


Figure 2. Glass Fiber Filter Purification Removes Most Small RNAs from Total RNA.

These electropherograms, produced with an Agilent 2100 bioanalyzer, demonstrate the effective removal of small RNAs (e.g. tRNAs and 5S rRNA) by a glass fiber filter-based RNA isolation method. The trace on the left shows *E. coli* total RNA isolated with the RiboPure™-Bacteria Kit. This kit uses a method where bead disruption and organic extraction are followed by glass fiber filter purification. The trace on the right shows *E. coli* total RNA isolated with a one-step disruption/organic extraction reagent for general purpose RNA isolation. A substantial amount of small RNA remains in the RNA prepared using the latter method. RNA isolation with RiboPure-Bacteria reduces tRNA and 5S rRNA relative to non-glass fiber filter-based methods by up to 75%.

Use bacterial mRNA instead of total RNA (e.g. MICROB*Express*™)

The MICROB*Express* Bacterial mRNA Enrichment Kit (P/N AM1905) employs a novel technology to remove >95% of the 16S and 23S rRNA from bacterial total RNA. The kit is suitable for mRNA purification from a broad spectrum of Gram-positive and Gram-negative bacteria. MICROB*Express*-enriched mRNA is the ideal template for RNA amplification and microarray analysis. Signal and signal/noise ratios are dramatically increased with the use of enriched mRNA, relative to total

RNA. A 10- to 100-fold increase in microarray sensitivity can be realized by amplifying RNA enriched for mRNA with the MICROB*Express* Kit. The use of enriched mRNA for all RNA amplification experiments is recommended, unless the experimental design precludes it. Figure 3 shows electropherograms of bacterial RNA before and after mRNA enrichment with the MICROB*Express* Kit.

Enrich bacterial RNA from host-bacterial cell RNA mixtures (e.g. MICROB*Enrich*™ Kit)

The Ambion MICROB*Enrich* Kit utilizes a technology similar to that employed in the MICROB*Express* Kit, removing >90% of host RNA from mixed RNA populations. Use of the MICROB*Enrich* Kit in conjunction with the MessageAmp II-Bacteria Kit will dramatically increase sensitivity of array analyses over non-enriched mixed RNA samples. With these tools, even very small samples or host cells infected at low levels can be subjected to rigorous gene expression analysis.

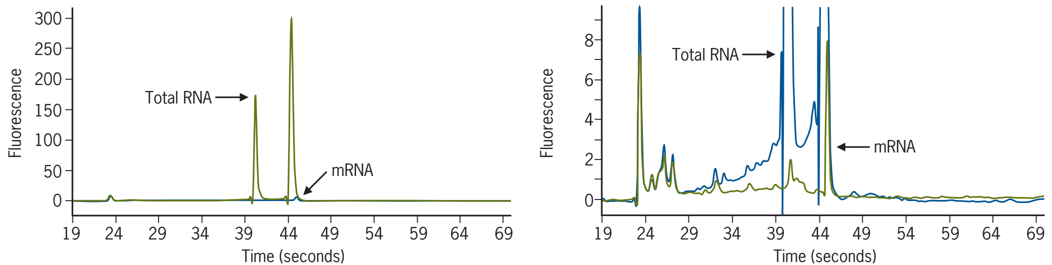


Figure 3. MICROB*Express* Kit Significantly Enriches Bacterial mRNA.

These electropherograms show a bacterial total RNA sample before and after enrichment for mRNA using the MICROB*Express* Kit. In this experiment, more than 95% of the bacterial 16S and 23S rRNA bands have been removed, leading to a selective enrichment of mRNA species. The trace on the right is identical to the one on the left, except that the relative fluorescence units have been rescaled to allow a closer examination of the MICROB*Express* enriched RNA.

Quantitate input RNA accurately

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-Bacteria amplification procedure. We recommend the NanoDrop spectrophotometer for rapid, accurate quantitation of nucleic acids. Traditional spectrophotometry and Invitrogen's RiboGreen RNA quantitation reagent can also be used for accurate RNA quantitation.

Recommended minimum and maximum amounts of input RNA

Table 2 on page 14 shows recommended minimum and maximum input RNA amounts for the MessageAmp II-Bacteria procedure. The minimum recommended input amounts are based on empirical data and were shown to be sufficient for expression analysis by microarray hybridization. Although it is possible to amplify less RNA than is shown in Table 2 (as was done in Table 1), with lower input RNA amounts,

the number of genes detected by microarray analysis may drop to unacceptable levels. Before using less input RNA than the amounts recommended in Table 2, consider conducting a pilot study to ensure that the results are acceptable (the results will vary based on RNA quality, genes of interest, and microarray platform used).

RNA amplification and expression analysis with eukaryotic RNA samples have been shown to be fairly impervious to different input RNA amounts. In-house scientists have seen that this is not necessarily the case with bacterial RNA amplification; comparisons of microarray results from amplified bacterial RNA produced using different amounts of input RNA have shown slightly increased variability on microarrays compared to using identical amounts of input RNA. Presumably the increase in variability is due to different levels of polyadenylation with different input RNA quantities. Therefore, always use similar RNA input amounts when comparing microarray data from different samples.

Table 2. Recommended Amounts of RNA to Use in the MessageAmp II-Bacteria Kit.

Input RNA Type	Recommended	Minimum	Maximum
Total RNA	500 ng	100 ng	1000 ng
MICROB*Express* enriched RNA	200 ng	10 ng	500 ng

Input RNA quantity and IVT reaction incubation time

Consider both the amount of sample RNA you have, how the RNA was prepared (e.g. total RNA vs. mRNA, RNA that was glass fiber purified vs. RNA not glass fiber purified, mixed RNA vs. bacterial RNA) and the amount of aRNA needed for your array analyses when planning MessageAmp II-Bacteria experiments. These factors will influence how much input RNA is used and how long to incubate the IVT reaction.

IMPORTANT

A 14 hr IVT reaction incubation is strongly recommended when using less than the recommended amount of input RNA shown in Table 2 or when modified nucleotides are included in the IVT reaction.

Table 3 shows experimental data obtained amplifying different mass amounts of bacterial total RNA and bacterial RNA enriched for mRNA, in either a 4 or 14 hour IVT MessageAmp II-Bacteria reaction.

Table 3. Effect of Input RNA, RNA type (Total RNA vs. mRNA) and IVT Time on aRNA Yield: Average of Triplicates

RNA Type	RNA Amount	IVT incubation time	
		4 hours	14 hours
		aRNA synthesized	
total RNA	100 ng	71 µg	157 µg
total RNA	200 ng	81 µg	174 µg
total RNA	500 ng	88 µg	187 µg
total RNA	1 µg	90 µg	181 µg
MICROB*Express* RNA	10 ng	23 µg	44 µg
MICROB*Express* RNA	50 ng	31 µg	65 µg
MICROB*Express* RNA	100 ng	31 µg	82 µg
MICROB*Express* RNA	200 ng	37 µg	96 µg

Reaction incubation times should be precise and consistent

The incubation times 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.

A range of 4–14 hr is given for the IVT reaction incubation time. Although a 4 hr IVT time may give enough aRNA for downstream applications, use a 14 hr IVT for most circumstances. Although differences in IVT incubation time among samples may have very little effect on array results, we recommend using a uniform IVT incubation time if aRNA yield from different samples will be compared or if you want to have equal amplification of different samples. Before using the shorter 4 hr IVT incubation, consider conducting a small pilot study comparing short vs. long IVT incubation times to ascertain whether 4 hr is sufficient.

Master mixes

Preparation of master mixes for the MessageAmp II-Bacteria procedure is strongly recommended. This approach reduces the effects of pipetting error and improves reproducibility. Using master mixes is especially important when aRNA yield from different samples will be compared.

Thermal cycler recommended

The MessageAmp II-Bacteria procedure is very sensitive to temperature; variable or inaccurate incubation temperatures can limit aRNA synthesis. It is also very important that condensation does not form in the reaction tubes during any of the incubations. Condensation changes the composition of reaction mixtures, which can greatly reduce yield.

- ***A thermal cycler with a temperature adjustable heated lid is recommended.***

  A calibrated thermal cycler, with a temperature-adjustable heated lid, is recommended, for the greatest temperature control and stability during MessageAmp II-Bacteria reaction incubations. Allow the thermal cycler to equilibrate to the required temperature before plac-

ing the tubes in the block for incubation. Follow the recommended settings for the lid temperatures. Too high a lid setting may inhibit the reaction; too low a setting may cause condensation.

NOTE

Even if you use a hybridization oven or incubator for most of the MessageAmp II-Bacteria reactions, a thermal cycler is strongly recommended for the 16°C second-strand synthesis reaction incubation (step II.E.2 on page 19). Turn off the heated lid if it cannot be adjusted to match the 16°C block temperature.

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

- ***Heat blocks or water baths are not recommended for MessageAmp II-Bacteria reaction incubations.***

Tubes: use 0.5 mL nonstick tubes

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

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

B. Prepare the Wash Buffer

Add 24 mL ACS-grade 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. Polyadenylation of Template RNA

Incubator needed

Thermal cycler with temperature-adjustable lid (program as described below).

1. Bring RNA samples to 5 µL with Nuclease-free Water

a. Place up to 1000 ng of total RNA (typically 100–500 ng) or up to 500 ng mRNA (typically 10 ng–200 ng) into a sterile RNase-free microcentrifuge tube. See Table 2 on page 14 for recommended amounts of input RNA to use in this procedure.

b. Add Nuclease-free Water to bring each sample to 5 µL. Vortex briefly to mix, then centrifuge to collect sample at the bottom of the tube.

2. Incubate 10 min at 70°C, then place on ice for 3 min

a. Program a thermal cycler for the denaturation step:

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

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

c. Remove the RNA samples from the thermal cycler and centrifuge briefly (~5 sec) to collect sample at the bottom of the tube. Place the mixture on ice for 3 min.

3. Add 5 µL of *Polyadenylation Master Mix* and place at 37°C

a. While the samples are incubating at 70°C, prepare ***Polyadenylation Master Mix*** in a nuclease-free tube at room temperature. Prepare enough master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error. Prepare the Polyadenylation Master Mix in the order shown:

Amount	Component
1.5 µL	Nuclease-Free Water
1.0 µL	10X Poly(A) Tailing Buffer
1.0 µL	RNase Inhibitor
0.5 µL	Poly(A)Tailing ATP
1.0 µL	PAP

b. ***Gently*** vortex to make a homogenous mixture without inactivating the enzyme, then centrifuge for ~5 sec to collect the master mix at the bottom of the tube.

c. Transfer 5 µL of Polyadenylation Master Mix to each RNA sample, mix thoroughly by gentle vortexing and follow with a quick spin to collect the reaction.

d. Program the thermal cycler for the poly(A) tailing reaction:

Temp	Time	Cycles
37°C (50°C lid)	15 min	1
4°C	hold	

e. Place the samples in the thermal cycler, equilibrated to 37°C, and start the run.

4. Incubate for 15 min at 37°C

Incubate the samples for 15 min at 37°C, then remove from the cycler and centrifuge briefly to collect the reaction at the bottom of the tube.

5. Place reactions on ice and proceed to the next step

Place the reactions on ice and proceed immediately to the reverse transcription.

IMPORTANT

Proceed immediately to the next step.

D. Reverse Transcription to Synthesize First Strand cDNA

Incubator needed

Thermal cycler programmed as follows:

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

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

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

a. Prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube at room temperature. Assemble enough master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error. Assemble the Reverse Transcription Master Mix in the order shown:

Amount	Component
3 μL	Nuclease-free Water
1 μL	T7 Oligo(dT) VN
1 μL	10X First Strand Buffer
4 μL	dNTP Mix
1 μL	ArrayScript

b. ***Gently*** vortex the tube to make a homogenous mixture without inactivating the enzyme, then centrifuge for ~5 sec to collect the master mix at the bottom of the tube.

c. Transfer 10 μL of Reverse Transcription Master Mix to each sample, mix thoroughly by gentle vortexing, and follow with a quick spin to collect the reaction.

d. Place the samples in the thermal cycler, and start the run.

2. Incubate for 2 hr at 42°C

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

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

IMPORTANT

Proceed immediately to the next step.

E. Second Strand cDNA Synthesis

Incubator needed

Thermal cycler programmed as follows:

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

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

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

a. On ice, prepare a ***Second Strand Master Mix*** by mixing the reagents listed in the following table in the order shown. Assemble enough master mix for all the samples in the experiment, including ≤5% overage to cover pipetting error.
On ice, assemble the Second Strand Master Mix in the order shown:

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. ***Gently*** vortex the tube to make a homogenous mixture without inactivating the enzymes, then centrifuge for ~5 sec to collect the master mix at the bottom of the tube.

c. Transfer 80 µL of Second Strand Master Mix to each sample, mix thoroughly by gentle vortexing so as not to inactivate the enzymes, and follow with a quick spin to collect the reaction.

d. Place the samples in the 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 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.F.5, during this 2 hr incubation.

3. Place reactions on ice briefly or freeze immediately

After the second strand synthesis incubation, proceed to section F. cDNA Purification (following), or immediately freeze reactions at –20°C. Do not leave the reactions on ice for long periods of time.

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.

F. 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 it could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

IMPORTANT

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

1. Preheat Nuclease-free Water to 55°C

Before beginning the cDNA purification, preheat at least 20 µL per sample of Nuclease-free Water to 55°C.

2. Add 250 µL cDNA Binding Buffer to each sample

Add 250 µL of cDNA Binding Buffer to each sample and mix thoroughly by gently vortexing.

3. Pass mixture through a cDNA Filter Cartridge

a. Pipet the cDNA sample/cDNA Binding Buffer 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.

4. Wash with 500 µL Wash Buffer

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

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 ethanol.

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

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

It is important to use Nuclease-free Water that is at 50°C to 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. To the center of the filter in the cDNA Filter Cartridge, apply 18 µL of preheated (55°C) Nuclease-free Water.

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. The double-stranded cDNA will now be in the eluate (~16 µL).

STOPPING POINT

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

G. In Vitro Transcription to Synthesize aRNA

Incubator needed

Thermal cycler programmed as follows:

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

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

Incorporating modified nucleotides

The IVT reaction can be set up to synthesize unmodified aRNA, or biotin-labeled UTP (or other modified nucleotides) can be incorporated into the aRNA during the IVT. ***Incubation of the IVT for the full 14 hours when modified nucleotides are used in the IVT reaction is recommended.***

1. Prepare and add 24 µL *IVT Master Mix* to each sample

a. At room temperature, prepare an ***IVT Master Mix*** by adding reagents to a nuclease-free microcentrifuge tube in the order listed in the following table. Prepare enough master mix for all samples in the experiment, including ≤5% overage to cover pipetting error.

Biotin labeled 40 µL rxn	Unmodified 40 µL rxn	Component
(16 µL)	(16 µL)	double-stranded cDNA (from step II.F.5 on page 21)
IVT Master Mix for a single reaction*		
4 µL	4 µL	T7 ATP
4 µL	4 µL	T7 CTP
4 µL	4 µL	T7 GTP
2.6 µL	4 µL	T7 UTP
1.4 µL	– –	Biotin-11-UTP†, 75 mM
4 µL	4 µL	T7 10X Reaction Buffer
4 µL	4 µL	T7 Enzyme Mix

* These are the current recommendations for preparing biotin-labeled aRNA. However, for continuity with previous experiments, the previous instructions are provided in section V.A starting on page 31.

† Biotin-16-UTP can be used instead of Biotin-11-UTP, if needed.

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 24 µL 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 contents in the bottom of the tube.

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

2. Incubate for 4–14 hr at 37°C

The minimum recommended incubation time is 4 hr and the maximum is 14 hr. A 14 hr IVT reaction incubation is strongly recommended to maximize aRNA yield.

A 14 hr IVT reaction is also recommended when using less than the recommended amount of input RNA shown in Table 2 on page 14 or when modified nucleotides are included in the IVT reaction.

3. Add 60 µL Nuclease-free Water to bring each sample to 100 µL

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

Place the diluted aRNA on ice if the aRNA purification step will be done immediately. Alternatively, the aRNA can be stored at –20°C.

STOPPING POINT

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

H. aRNA Purification

Incubator needed: heat block set at 55°C.

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

IMPORTANT

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

1. Preheat Nuclease-free Water to 55°C and assemble aRNA Filter Cartridges and Tubes

- Before beginning the aRNA purification, preheat a minimum of 150 µL per sample of Nuclease-free Water to 55°C.
- For each sample, place an aRNA Filter Cartridge into an aRNA Collection Tube and set aside for use in step 4.

2. Add 350 µL aRNA Binding Buffer and mix

a. Check to make sure that each IVT reaction was brought to 100 µL with Nuclease-free Water (step G.3 on page 22).

b. Add 350 µL of aRNA Binding Buffer to each aRNA sample. Mix thoroughly by gentle vortexing, and proceed to the next step immediately.

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

a. 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***.

b. 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 semiprecipitated state.

IMPORTANT

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

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

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

IMPORTANT

Slowly dispense the sample mixture to ensure all liquid is removed from the inside walls of the tip.

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 in the aRNA Collection Tube.

5. Wash with 650 µL Wash Buffer

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

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 solution 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 ethanol.

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

6. Elute aRNA with 150 µL preheated Nuclease-free Water

a. To the center of the filter, add 150 µL Nuclease-free Water that is preheated to 55°C.

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

c. Centrifuge for ~1.5 min at 10,000 x g, or until the solution is through the filter.

d. The aRNA will now be in the aRNA Collection Tube in ~150 µL of Nuclease-free Water.

e. Discard the aRNA Filter Cartridge.

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. If the sample was eluted with 150 µL Nuclease-free Water as suggested, this will be 15 µL of 5 M NH_4OAc.

b. Add 2.5 volumes of 100% ethanol (415 µL if the RNA was eluted in 150 µL). Mix well and incubate at –20°C for 30 min.

c. Microcentrifuge at top speed for 15 min at 4°C or room temperature. 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 re-spin the tube, and aspirate any fluid with a fine tipped pipette or syringe needle.

f. Air dry the pellet.

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

8. aRNA storage

Store aRNA at –70°C 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.

III. Assessing aRNA Yield and Quality

A. Quantitation

Assessing aRNA yield by UV absorbance

The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm. The NanoDrop 1000A Spectrophotometer is recommended because no dilutions and no cuvettes are needed. Follow the manufacturer's instructions.

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 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

NOTE

A_{260}/A_{280} ratios have traditionally been used to evaluate the purity of nucleic acid preparations, with high quality RNA having a ratio of 1.8-2.1. With aRNA synthesized using this kit, the amount of input RNA influences the A_{260}/A_{280} ratio. In general, there is an inverse relationship between input RNA mass and A_{260}/A_{280} ratio of the aRNA. In other words, with less input RNA, the ratio is higher. This is due to the length of the poly(A) tail added to the RNA during amplification; with less input RNA, each molecule receives a longer poly(A) tail which is converted to a poly(U) tail in the amplification product. The length of the poly(U) tract at the 5' end of the aRNA can affect the A_{260}/A_{280} ratio. This shift in ratio values is most apparent with very low input RNA (for example, 10 ng input). Under these conditions, expected values for A_{260}/A_{280} ratios range from 2.2 through 2.8.

Assessing aRNA yield with RiboGreen® dye

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

Expected yield

aRNA yields will be determined by several factors, including input RNA amount, RNA quality, RNA type (e.g. total RNA or mRNA), IVT reaction volume and incubation time, and aRNA elution method. To obtain reproducible results with the MessageAmp II-Bacteria Kit, all of these variables must be kept uniform. Table 3 on page 15 shows 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 (section III.A on page 26). To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA (from step II.H.6 on page 24). For more information, go to www.invitrogen.com/site/us/en/home/support/technical-support.html

Expected results

The size of the aRNA synthesized is determined by several factors including input RNA type (total RNA vs. mRNA), bacterial species and RNA isolation method, input RNA amount, and the incorporation of modified nucleotides (e.g. biotin). Figure 4 shows Agilent bioanalyzer electropherograms of aRNA derived from either 100 ng or 500 ng of high quality input total RNA or from 10 ng or 200 ng of RNA enriched for mRNA with the MICROB*Express* Kit. Note that the median aRNA size and the shape of the distribution curve can vary considerably depending on the factors mentioned above, but the average aRNA size generated from MICROB*Express* RNA should be ≥500 nt, and the average aRNA size generated from total RNA should be >900 nt.

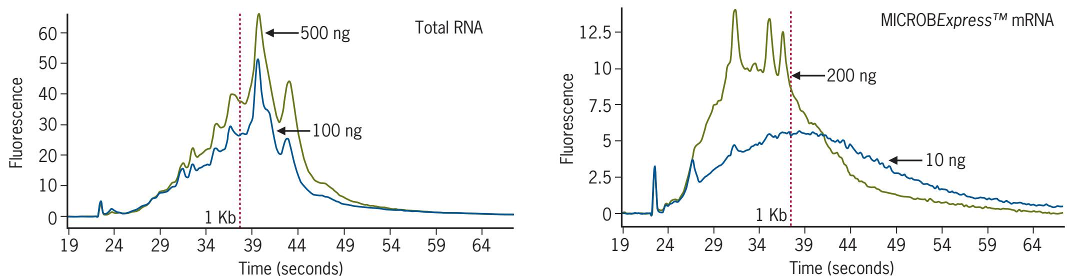


Figure 4. Profiles of aRNA Amplified from Total RNA and MICROB*Express*™ Kit-enriched RNA.

These electropherograms display the aRNA size distribution of aRNA generated with either total RNA (100 ng or 500 ng) or MICROB*Express* enriched mRNA (10 ng or 200 ng) from *E. coli*. Notice that aRNA generated from total RNA and mRNA differ in the overall shapes of their profiles. This is because total RNA will generate amplification products from rRNA. Notice also that when you amplify very small input amounts, such as 10 ng mRNA, the profile is very rounded and the size is slightly larger on average. This is due to the longer poly(A) tails generated with small amounts of input RNA. The vertical bar represents 1 Kb.

IV. Troubleshooting

A. Positive Control Reaction

Control RNA amplification instructions

To establish if the kit is working properly, a tube of Control RNA consisting of 1 mg/mL *E. coli* total RNA is provided.

1. Dilute the Control RNA 1:10 by adding 2 μL of Control RNA to 18 μL Nuclease-free Water.
2. Use 1 μL of the diluted Control RNA (100 ng) in an amplification reaction; follow the protocol starting at step II.C.1 on page 16.
3. At step II.G.2 on page 22, use a 6 hr incubation for the IVT reaction.
4. Continue with the procedure for making unmodified aRNA through section II.H.

Analysis of the positive control amplification

- After completing the aRNA purification, measure the A_{260} of the reaction product as described in section III.A starting on page 26. ***The positive control reaction should produce 100 μg of aRNA.***
- 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 ~900 nt***.

Troubleshooting the positive control reaction

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions.

Incubation temperature(s) were incorrect

Reaction 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 reaction temperature, use the lid with the heat turned off or do not use it to cover the reaction vessel(s).

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

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

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

Tubes, tips, or equipment were contaminated with nucleases

Using pipettes, tubes, etc. 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.

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 a sample 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.

Control RNA dilution was incorrect

Confirm that the Control RNA was diluted as described above.

There was a problem with gel electrophoresis

Agarose gels that are not completely denaturing, or that are not the proper percent agarose can provide inaccurate estimates of aRNA size. Fractionate a known RNA sample in an adjacent lane to effectively estimate the size of the aRNA.

B. Troubleshooting Low Yield and Small Average aRNA Size

Impure RNA samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA, or use the Ambion MEGAclear Kit (P/N AM1908) to further purify it before amplification.

Suboptimal sample RNA

For maximum amplification, use input RNA that meets the following criteria:

- Use intact RNA: use logarithmic phase bacterial cultures and work quickly to minimize RNA degradation and/or changes in gene expression due to the harvesting procedure (see section *II.A. Harvesting bacteria for optimal RNA quality* on page 9 for more information).
- Include a glass fiber-based purification in the RNA prep: RNA that has undergone glass fiber purification provides higher yields of aRNA when the recommended amount of input RNA is used in the amplification (Figure 2 on page 12).

- Quantitate RNA accurately: for reproducible array results, it is very important to use accurately quantitated input RNA. Quantitate RNA by spectrophotometry or using Molecular Probes RiboGreen RNA Quantitation Reagent.
- Use bacterial mRNA instead of total RNA: using RNA enriched for mRNA, such as that obtained using the Ambion MICROB*Express* Kit, can increase the sensitivity of array analysis 10- to 100-fold.
- Enrich mixed RNA for bacterial RNA: for analysis of bacterial RNA obtained from host-bacteria mixed cultures, we strongly recommend enriching the mixture for bacterial RNA using the MICROB*Enrich* Kit.

C. aRNA is Not Efficiently Reverse Transcribed

One of the most common ways of labeling aRNA after amplification is by reverse transcription to incorporate modified nucleotides. These troubleshooting suggestions are provided for problems encountered with reverse transcription of MessageAmp II-Bacteria reaction products.

The cDNA procedure relies on oligo(dT) priming

aRNA synthesized with the MessageAmp II-Bacteria Kit has a poly(U) tract near the 5' end but lacks a poly(A) tract at its 3' end. Thus any reverse transcription procedures that rely on oligo(dT) primers will not effectively convert aRNA to cDNA. Try using gene-specific or random primers.

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

If the aRNA contains ethanol carried over from the Wash Buffer, it can inhibit reverse transcription. Make sure that the filter is completely dry at step II.H.5.c on page 24, just before eluting the aRNA.

To remove ethanol from an aRNA sample, ethanol precipitate it following the instructions in II.H.7 on page 24. Be sure to include the double spin described in step II.H.7.e to remove the last traces of ethanol before air drying the aRNA pellet.

Absorbance readings are inaccurate

Confirm that your spectrophotometer is accurate by measuring the absorbance of an RNA or DNA sample of known concentration. Alternatively, assess the quantity of aRNA by a different method such as fractionating on an agarose gel adjacent to an RNA sample whose concentration is known and comparing the ethidium bromide staining or using a sensitive RNA dye like RiboGreen dye.

V. Appendix

A. IVT Reaction Using 10 mM Biotin-labeled CTP and UTP

This procedure is included for the convenience of customers who choose to continue using the former recommendations, for consistency with previous experiments.

Following is a list of biotin-labeled 10 mM CTP and UTP products recommended for this procedure.

- Biotin-11-CTP (10 mM): PerkinElmer Life Sciences is the preferred supplier of biotin-11-CTP (Cat #NEL 542); other biotin-labeled NTPs that may be used with this kit are available upon request. Alternatively, biotin-11-CTP is available from Enzo Biochemicals (Cat #42818).
- Biotin-16-UTP (10 mM) is available from Applied Biosystems (P/N AM8452).

IVT reaction volume

For the highest aRNA yield, conduct the IVT in a 40 µL final reaction volume. Instructions for a more economical 20 µL reaction with modified nucleotides are provided for users who want to reduce the reaction cost by using only half as much modified nucleotide. ***If you use a 20 µL IVT reaction volume, we strongly suggest incubating the IVT for the full 14 hours.***

1. Follow the procedure through cDNA purification

Follow the procedure detailed in section *II.B. Prepare the Wash Buffer* starting on page 16 through cDNA purification (step II.F.5 on page 21). Then substitute step 1 in section II.G on page 21 with the following instructions.

2. Mix modified NTPs with the cDNA and concentrate

For each sample, add biotin-labeled CTP and UTP to the eluted cDNA, and concentrate the mixture in a vacuum centrifuge concentrator until the volume is reduced to 18 µL for a 40 µL transcription reaction, or to 9 µL for a 20 µL transcription reaction, as described in the table below. This should take only a few minutes. ***Avoid drying the mixture to completion.***

Below is an example of the amount of biotin-labeled nucleotides commonly used to label sample for array analysis; however, we recommend that you follow the recommendations of the array manufacturer for the final concentration of modified nucleotides in amplification reactions.

40 µL rxn	20 µL rxn	Component
~16 µL	~16 µL	cDNA from step II.F.5 on page 21
7.5 µL	3.75 µL	10 mM biotin-11-CTP
7.5 µL	3.75 µL	10 mM biotin-16-UTP
18 µL	9 µL	concentrate to final volume

3. Prepare *IVT Master Mix*

a. At room temperature, prepare an ***IVT Master Mix*** by adding reagents to a nuclease-free microcentrifuge tube in the order listed below. Prepare enough master mix for all samples in the experiment, including ≤5% overage to cover pipetting error.

Biotin CTP/UTP IVT Master Mix		
40 µL rxn	20 µL rxn	Component
4 µL	2 µL	T7 ATP Soln
3 µL	1.5 µL	T7 CTP Soln
4 µL	2 µL	T7 GTP Soln
3 µL	1.5 µL	T7 UTP Soln
4 µL	2 µL	10X T7 Reaction Buffer
4 µL	2 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.

4. Add *IVT Master Mix* to each sample

a. Transfer IVT Master Mix to each sample following the guidelines below, mix thoroughly by gentle vortexing, and centrifuge briefly to collect the reaction components in the bottom of the tube.

40 µL rxn	20 µL rxn	
18 µL	9 µL	cDNA/modified nucleotide mixture (from step 2)
22 µL	11 µL	volume IVT Master Mix

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

5. Continue with the procedure

Continue with the MessageAmp II-Bacteria procedure starting at step II.G.2 on page 22.

B. References

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C. Quality Control

Functional testing

The Control RNA is used in a MessageAmp II-Bacteria reaction following the instructions in section IV.A on page 28. The aRNA yield is assessed by measuring the A_{260} on the NanoDrop ND1000 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.

D. Safety Information

WARNING

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

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

1. Chemical safety

WARNING

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

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

2. Biological hazard safety

WARNING

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

WARNING

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

In the U.S.:

- *U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety*

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

In the EU:

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

VI. Documentation and Support

A. Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

www.invitrogen.com/sds

or

www.appliedbiosystems.com/sds

B. Obtaining support

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

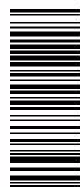
www.invitrogen.com

or

www.appliedbiosystems.com

At the website, you can:

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



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