PROTOCOL

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by life technologies™

MessageAmp™ II-96 Kit

PROTOCOL

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MessageAmp™ II-96 Kit

(Part Number AM1819)

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I. Introduction

IMPORTANT

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

A. Product Description and Background

The MessageAmp™ II-96 Kit is designed for amplification of RNA in a 96-well format. The procedure is based on the RNA amplification protocol developed in the laboratory of James Eberwine (Figure 1 on page 3; Van Gelder et al. 1990). It consists of reverse transcription of RNA using an oligo(dT) primer bearing a T7 promoter and ArrayScript™ reverse transcriptase (RT), which is engineered to produce higher yields of first strand cDNA than wild type enzymes. ArrayScript RT catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second strand synthesis and cleanup to become a template for in vitro transcription with T7 RNA Polymerase. To maximize aRNA yield, Life Technologies proprietary MEGAscript® in vitro transcription (IVT) technology is used in the kit to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. (In this protocol the antisense amplified RNA is referred to as aRNA, in scientific literature it is also commonly called cRNA.) The IVT can be configured to synthesize either biotin-labeled aRNA or unlabeled aRNA that can subsequently be labeled by reverse transcription (for example with fluorescently labeled dNTPs).Theresulting aRNA is suitable for use on most commercially available microarray gene expression systems.

Benefits of RNA amplification

RNA amplification was originally developed as a method to expand RNA samples of very limited amounts to produce enough material for array hybridization (Yue et al. 2001). Several groups have conducted studies to determine whether amplification of RNA introduces bias, and they report that any bias is minimal (Li et al. 2004, Feldman et al. 2002, and Polacek et al. 2003). Additionally, among the benefits of amplification is a more reproducible expression profile from a wide range of RNA inputs. Some researchers conclude that amplification actually improves the reliability of array results regardless of whether it is needed for sample expansion (Feldman et al. 2002 and Polacek et al. 2003). RNA amplification has become the standard method for preparing RNA samples for array analysis (Kacharmina et al. 1999, Pabon et al. 2001).

The MessageAmp II-96 advantage

Optimized protocols

All reagents and protocols have been optimized to produce high quality aRNA with maximum yield and reproducibility. The simple, rapid aRNA purification procedure prepares the aRNA for downstream applications such as reverse transcription or post-synthesis labeling reactions.

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

Built-in flexibility

The NTPs for IVT are provided in separate tubes so that modified nucleotides, such as biotinylated UTP or CTP, or cyanine 3/cyanine 5 CTP and UTP, can be used in the reaction to synthesize labeled aRNA.

Ready for automation

This protocol was designed with both manual and robotic liquid handling systems in mind. It can easily be adapted for use on a robotic platform; downloadable protocols for the Biomec 3000 and NX workstations are available on our website at:

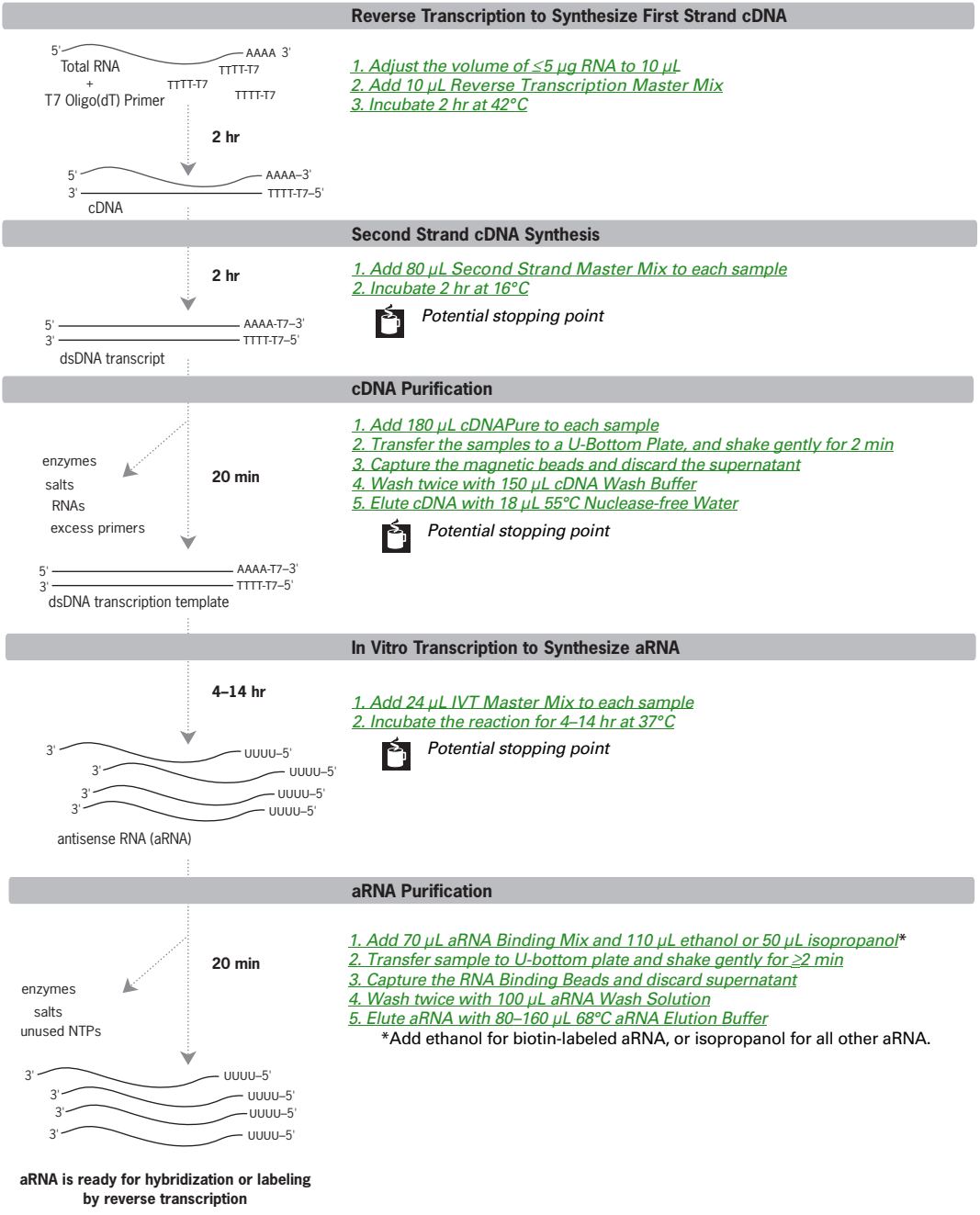
www.invitrogen.com/ambion/techlib/automation

Compatible with second round amplification

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

1. For the first round amplification, use the MessageAmp II Kit to make unmodified aRNA. (You will need more than one kit to process more than 20 samples.)
2. For the second round of amplification, use the MessageAmp II-96 Kit components plus the Second Round Primers from the MessageAmp II Kit, and follow instructions from both protocols as detailed below:
   - For second round reverse transcription and second strand cDNA synthesis, follow the instructions in the MessageAmp II protocol. (You will need Second Round Primers from more than one MessageAmp II Kit to process more than 20 samples.)
   - For second round cDNA purification, follow either the MessageAmp II Kit or the MessageAmp II-96 protocol, depending on your preference and available kit components.
   - For the second round amplification IVT reaction, follow the instructions in the MessageAmp II-96 Kit protocol.

Figure 1. Procedure Overview



B. Materials Provided with the Kit and Storage

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

cDNA synthesis and in vitro transcription reagents

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

Amount	Component
110 µL	T7 Oligo(dT) Primer
110 µL	ArrayScript™ RT
110 µL	RNase Inhibitor
220 µL	10X First Strand Buffer
880 µL	dNTP Mix
220 µL	DNA Polymerase
110 µL	RNase H
10 µL	Control RNA (1 µg/µL HeLa total RNA)
1.1 mL	10X Second Strand Buffer
440 µL	T7 Enzyme Mix
440 µL	T7 10X Reaction Buffer
440 µL	T7 ATP Solution
440 µL	T7 CTP Solution
440 µL	T7 GTP Solution
440 µL	T7 UTP Solution

cDNA and aRNA purification components

Amount	Component	Storage
20 mL	Nuclease-free Water	any temp*
20 mL	cDNAPure†	4°C ‡
1.1 mL	RNA Binding Beads	4°C‡
40 mL	cDNA Wash Buffer Concentrate Add 32 mL 100% ethanol before use	room temp
25 mL	aRNA Wash Solution Concentrate Add 20 mL 100% ethanol before use	room temp
450 µL	Bead Resuspension Solution	room temp
10 mL	aRNA Elution Buffer	room temp
5.5 mL	aRNA Binding Buffer Concentrate	room temp
2	U-Bottom Plate	room temp
4	PCR Plate	room temp
1	Reservoir	room temp

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

C. Materials Not Provided with the Kit

Lab equipment and supplies

- ACS grade (or higher quality) alcohol:
  – 100% ethanol
  – 100% isopropanol
- Thermal cycler with adjustable-temperature heated lid (recommended), hybridization oven, or fixed temperature air incubators set at 70°C, 42°C, 37°C, and 16°C. (See "Procedural notes" on page 9 for more information.)
- Vortex mixer
- Orbital shaker for 96-well plates, such as the Barnstead/Lab-Line Titer Plate Shaker (available from Fisher or VWR)
- Magnetic stand for 96-well plates (we recommend the Ambion® 96-well Magnetic-Ring Stand, P/N AM10050, for its high-strength magnets and quality design)
- Microcentrifuge
- RNase-free pipettors and tips, positive-displacement type recommended to increase the accuracy and precision of reaction inputs
- Aluminum sealing foil or tape for sealing reactions in 96-well plates during incubations

(Optional) Materials and equipment for RNA analysis

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

(Optional) Biotin labeled UTP

Biotin labeled UTP, for making biotinylated 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.

D. Related Products

96-well Magnetic-Ring Stand P/N AM10050	The Ambion 96-well Magnetic-Ring Stand features 96 powerful ring-shaped magnets arranged to cradle each well of a 96-well plate for quick, thorough bead capture. Captured magnetic beads form evenly distributed donut-shaped pellets with a large hole in the center. This capture pattern facilitates both supernatant removal and subsequent bead resuspension. The stand is suitable for high throughput applications conducted with multichannel pipettors or with robotic liquid handlers. However, because the pellets will be evenly distributed around the edge of the wells, it may require practice for efficient manual removal of supernatants.
Magnetic Stand-96 P/N AM10027	The Ambion Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96-well Magnetic-Ring Stand (P/N AM10050).
MagMAX™-96 Total RNA Isolation Kit P/N AM1830	The MagMAX™-96 Total RNA Isolation Kit is a magnetic bead based total RNA purification system designed for rapid high throughput processing of cells in 96-well plates. High yield and high quality total RNA can be obtained from 100 to 500,000 cultured eukaryotic cells. The kit can also be used for total RNA isolation from small tissue samples.
MagMAX™-96 for Microarrays Total RNA Isolation Kit P/N AM1839	The MagMAX-96 for Microarrays Total RNA Isolation Kit is designed for rapid, high throughput isolation of total RNA from mammalian cells and tissues in 96-well plates. The procedure employs the robust and reliable lysis/denaturant, TRI Reagent® and Ambion MagMAX magnetic bead-based RNA purification technology to provide high yields of pure, intact RNA that can be used directly for quantitative reverse transcriptase PCR (qRT-PCR) and microarray analysis. The kit can also be used to process fewer than 96 samples at one time.
RNAqueous®-96 Automated Kit P/N AM1812	The RNAqueous-96 Automated Kit employs a simple and rapid procedure to purify total RNA from source material without using organic solvents (such as phenol). It is formatted specifically for high throughput RNA isolation on robotic platforms.
ArrayControl™ Spikes P/N AM1780	The ArrayControl Spikes are a set of eight control RNA transcripts designed for the normalization and validation of glass microarray experiments. The Spikes range in size from 750 to 2000 bases and each transcript has a 30-base 3' poly(A) tail. The precisely quantitated RNA Spikes are designed to be added to your RNA sample before labeling, to serve as internal controls for sample labeling and hybridization efficiency.
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.

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.
Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452	Ambion biotinylated UTPs are ideal for use as substrates in vitro transcription reactions, and can be utilized by a variety of RNA polymerases, including T7, T3, and SP6 RNA polymerases. Biotinylated RNA can be used in place of radioactively labeled RNA in many applications with detection via one of a variety of streptavidin-based methods.
RNA Fragmentation Reagents P/N AM8740	Amplified RNA is commonly fragmented prior to hybridization on oligonucleotide microarrays to improve the hybridization kinetics and signal produced on oligonucleotide microarrays. Ambion RNA Fragmentation Reagents include a 10X Fragmentation Reagent and a Stop Solution.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette See web or print catalog for P/Ns	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. The Glass Array Hybridization Cassette is also available, for incubation of glass microarray hybridization reactions.

II. aRNA Amplification Procedure

A. Planning the Experiment

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

RNA quantity and IVT incubation time

The MessageAmp II-96 procedure can accommodate a wide range of input RNA amounts, but for reproducible and comparable results, use a fixed amount of input RNA for all experiments. Typically ***100 ng–5 µg of total RNA*** should be used in a MessageAmp II-96 amplification reaction. ***The recommended amount of RNA per reaction is 1 µg of total RNA.***

The amount of input RNA needed will depend on how much aRNA is required for your experiments, and how long the IVT amplification is incubated.

Using 1 µg or more total RNA and a 4 hr IVT incubation typically produces enough aRNA for several microarray hybridizations. When amplifying RNA samples of limited amounts (e.g. ~250 ng or less), however, incubating the IVT reaction for 14 hr will maximize the amount of aRNA produced.


NOTE

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

Other input RNA requirements

RNA purity

RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range 1.7–2.1.

RNA integrity

The integrity of the RNA sample, or the proportion of full-length mRNA, can have a significant impact on the amplification reaction. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that lack the sequence upstream of the break in the RNA molecule.

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

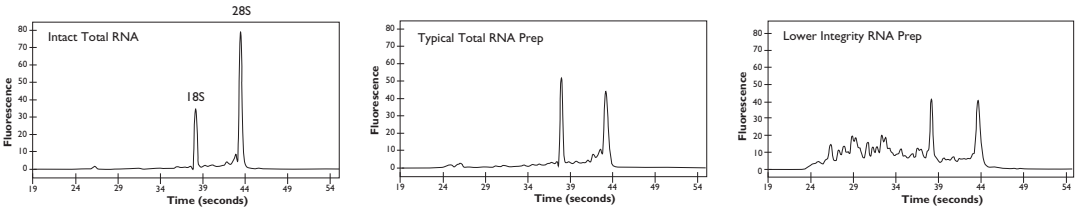


Figure 2. Bioanalyzer Images of Increasingly Degraded Total RNA

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

Procedural notes

Thermal cycler recommended

The MessageAmp II-96 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 plate wells 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-96 reaction incubations. Allow the thermal cycler to equilibrate to the required temperature before placing the plates 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-96 reactions, a thermal cycler is strongly recommended for the 16°C second-strand synthesis reaction incubation (step II.D.2 on page 13). 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.

- ***Heat blocks or water baths are not recommended for MessageAmp II-96 reaction incubations.***

Orbital shaker settings

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

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

Master mixes

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

B. Reagent Preparation

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

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

Store at room temperature.

2. Add 20 mL 100% ethanol to the aRNA Wash Solution Concentrate

Add 20 mL 100% ethanol (ACS grade or better) to the bottle labeled aRNA Wash Solution Concentrate. Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as ***aRNA Wash Solution*** in these instructions.

Store at room temperature.

3. Prepare *aRNA Binding Mix* for the experiment

IMPORTANT

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

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

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

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

* Mix the RNA Binding Beads by vortexing before dispensing.

b. Next, add aRNA Binding Buffer Concentrate

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

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

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

C. Reverse Transcription to Synthesize First Strand cDNA

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. Adjust the volume of ≤5 µg RNA to 10 µL

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

b. Add Nuclease-free Water to a final volume of 10 µL.

2. Add 10 µL *Reverse Transcription Master Mix*

a. Prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube at room temperature. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

Reverse Transcription Master Mix (for a single 20 µL reaction)	
Amount	**Component**
1 µL	Nuclease-free Water
1 µL	T7 Oligo(dT) Primer
2 µL	10X First Strand Buffer
4 µL	dNTP Mix
1 µL	RNase Inhibitor
1 µL	ArrayScript

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

c. Transfer 10 µL of the Reverse Transcription Master Mix to each RNA sample, mix by gently pipetting up and down 3–4 times. Cover the reactions with aluminum sealing foil.
To facilitate the transfer process, you can distribute the master mix into 8 wells of a PCR plate and use an 8-channel pipette to transfer the master mix.

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

3. Incubate 2 hr at 42°C

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

Place the plate on ice and immediately proceed to the second strand cDNA synthesis (next).

D. 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 adding the following reagents to a reservoir in the order listed below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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

b. Mix gently by rocking the reservoir back and forth.

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

2. Incubate 2 hr at 16°C

Place the plate in the 16°C thermal cycler, start the run, and incubate for 2 hr.

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

3. Place reactions on ice briefly or freeze immediately

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

STOPPING POINT

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

E. cDNA Purification

Before beginning the cDNA purification:

- Remove the cDNAPure from the refrigerator and allow it to equilibrate to room temperature for 30 min before use.
- Shake the container of cDNAPure to fully resuspend the magnetic beads before use. cDNAPure is a slurry of DNA binding magnetic beads in binding buffer. The magnetic beads will settle over time.

- Preheat a minimum of 20 µL per sample of Nuclease-free Water to 55°C.

IMPORTANT

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

1. Add 180 µL cDNAPure to each sample

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

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

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

NOTE

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

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

3. Capture the magnetic beads and discard the supernatant

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

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

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

4. Wash twice with 150 µL cDNA Wash Buffer

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

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

NOTE

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

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

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

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

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

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

a. Elute the cDNA from the magnetic beads by adding 18 µL preheated (55°C) Nuclease-free Water to each sample.

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

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

d. Carefully transfer 16 µL of the supernatant, which contains the eluted cDNA, to a new PCR Plate.


NOTE

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

F. 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.

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

a. Assemble the ***IVT Master Mix*** at room temperature in a nuclease-free tube. Use the reaction components for the type of aRNA that you want to synthesize. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

IVT Master Mix (for a single 40 µL reaction)		
Biotin labeled	Unmodified	Component
4 µL	4 µL	T7 ATP Soln
4 µL	4 µL	T7 CTP Soln
4 µL	4 µL	T7 GTP Soln
2.6 µL	4 µL	T7 UTP Soln
1.4 µL	– –	Biotin-11-UTP*, 75 mM
4 µL	4 µL	10X T7 Reaction Buffer
4 µL	4 µL	T7 Enzyme Mix

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

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

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

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

Place the plate in the thermal cycler and start the run.

The minimum recommended incubation time is 4 hr. The maximum incubation time is 14 hr.

G. aRNA Purification

Before beginning the aRNA purification:

Preheat the aRNA Elution Buffer to 68°C.

1. Add 70 µL aRNA Binding Mix and 110 µL ethanol or 50 µL isopropanol

Biotin-labeled aRNA requires slightly different conditions for optimal interaction with the RNA Binding Beads than unmodified aRNA or aRNA synthesized with fluorescent dye- or amino allyl-nucleotides. Follow the instructions below appropriate for the type of aRNA to be synthesized.

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

b. ***Biotin-labeled aRNA:*** Add 110 µL of 100% ethanol to each sample. ***Unmodified aRNA, amino allyl aRNA, and CyDye-modified aRNA:*** Add 50 µL of 100% isopropanol to each sample.

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

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

3. Capture the RNA Binding Beads and discard supernatant

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

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

4. Wash twice with 100 µL aRNA Wash Solution

a. Add 100 µL aRNA Wash Solution (prepared as described in step II.B.2 on page 10) to each sample, and shake at moderate speed for 1 min.

NOTE

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

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

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

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

e. Move the plate to a shaker and shake the plate vigorously for 1 min to evaporate residual ethanol from the beads.
Do not overdry the beads as this may make them difficult to resuspend in the next step.

5. Elute aRNA with 80–160 µL 68°C aRNA Elution Buffer

a. Elute the purified aRNA from the RNA Binding Beads by adding 80–160 µL preheated (68°C) aRNA Elution Buffer to each sample.

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

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

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

e. Store purified aRNA at –20°C overnight or at –80°C for long-term storage.
For long-term storage, keep the aRNA at –80°C and minimize repeated freeze-thawing. Splitting samples into 5–20 µg aliquots is a good way to prevent multiple freeze-thaws.

III. aRNA Evaluation and Preparation for Array Hybridization

A. aRNA Quantitation

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 because no dilutions or 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 using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

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

Assessing aRNA yield with the RiboGreen® assay

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

B. Analysis of aRNA Size with a Bioanalyzer

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

Expected results

Using 1 µg of high quality input total RNA, the expected aRNA profile is a distribution of sizes from 250–5500 nt with a peak centered at 1000–1500 nt (see Figure 3 on page 20). Reaction products may be smaller when less input RNA or lower quality input RNA is used.

C. Preparing Labeled aRNA for Hybridization

1. Concentrate the labeled aRNA if necessary

Typically, microarrays are hybridized with 25–100 µL hybridization solution. Since the labeled aRNA is eluted in about 40 µL, it may need to be concentrated to use it for microarray hybridization. We recommend vacuum drying the labeled aRNA in the dark (if appropriate) until the volume is reduced to 1–10 µL (to keep out ambient light cover the lid of the vacuum drier with aluminum foil, if necessary).

Bring the volume of the aRNA to 10 µL with either hybridization buffer or Nuclease-free Water and calculate the aRNA concentration based on the amount of RNA coupled to dye or the A_{260} reading.

2. Fragment labeled RNA for hybridization to oligonucleotide microarrays

For microarrays printed with oligonucleotides, the labeled aRNA must typically be fragmented for proper hybridization. Any RNA hydrolysis method that produces RNA fragments in the 60–200 nucleotide size range can be used. We recommend Ambion RNA Fragmentation Reagents (P/N AM8740) for this procedure. Follow the procedure associated with the fragmentation method used.

3. Dilute the labeled aRNA into the hybridization solution

aRNA amount

The amount of aRNA to use for hybridization will depend on your microarray type and will have to be optimized for maximum sensitivity and minimal background.

Most arrays require 1–10 µg of labeled aRNA.

Choice of hybridization solution

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

IV. Troubleshooting

A. Positive Control Reaction

Control RNA amplification instructions

To establish if the reaction conditions are optimal, a tube of Control RNA is provided.

1. Use 1 µL (1 µg) of the Control RNA in a MessageAmp II-96 reaction; follow the protocol starting at step II.C.1 on page 12.
2. At step II.F.2 on page 16, use a ***14 hr*** incubation for the IVT reaction.
3. Continue with the procedure for making unmodified aRNA through section II.G.

Analysis of the positive control amplification reaction

Quantitative analysis

After completing the aRNA purification, measure the A_{260} of the reaction product as described in section III. on page 18; the positive control reaction should produce ≥90 µg of aRNA.

Qualitative analysis

Also, run 100–200 ng of the reaction products on a bioanalyzer as described in section III.B on page 18; the expected aRNA profile is a distribution from 250–5500 nt with a peak centered at 1000–1500 nt (Figure 3).

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

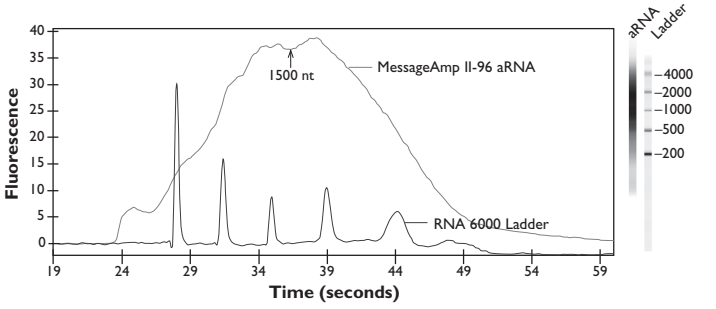


Figure 3. Bioanalyzer Electropherogram of MessageAmp II-96 aRNA.

A sample of the aRNA produced from the MessageAmp II-96 positive control reaction was analyzed on an Agilent 2100 Bioanalyzer.

B. Factors that Affect Both the Positive Control and Experimental Samples

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions. These suggestions also apply to problems with amplification of experimental RNA.

Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective RNA amplification.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.
- If a thermal cycler is used for incubation, check the accuracy of the adjustable temperature lid.

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

Condensation occurs when the cap of the reaction vessel is cooler (e.g. room temperature) than the bottom of the tube. As little as 1–2 µL of condensate in an IVT reaction changes the concentrations of the nucleotides and magnesium which can reduce yields.

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

Nuclease-contaminated tubes, tips, or equipment

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

Absorbance readings were inaccurate

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

Incomplete resuspension or dispersion of magnetic beads

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

1. Be sure to resuspend RNA Binding Beads just before each step in preparing the aRNA Binding Mix (section II.B.3 on page 11).
2. Make sure that magnetic bead mixtures are fully resuspended before adding them to cDNA or aRNA samples for purification procedures (steps II.E.1 on page 14, and II.G.1 on page 16).

3. For efficient elution of purified reaction products, make sure that magnetic beads are fully resuspended in the elution solution (steps II.E.5 on page 15 and II.G.5 on page 17).
4. Avoid overdrying magnetic beads before eluting the RNA (in steps II.E.4.e on page 15 and II.G.4.e on page 17) because this may make the beads more difficult to resuspend. If the beads are inadvertently overdried, increase the mixing time (to 10 min) during the elution step to allow the beads to rehydrate.

Loss of the cDNAPure and/or the RNA Binding Beads

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

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

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

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

C. Troubleshooting Low Yield and Small Average aRNA Size

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

Impure RNA samples

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

Lower than expected input RNA concentration

Take another A_{260} reading of your RNA sample or try using more RNA in the aRNA amplification procedure.

RNA sample integrity is poor

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See section *II.A. RNA integrity* on page 8 for more information).

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

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

D. Bead Carryover in Eluted Sample

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

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

V. Appendix

A. References

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B. Quality Control

Functional testing

The Control RNA is used in a MessageAmp II reaction following the instructions in section IV.A on page 20. The aRNA yield is assessed by measuring the A_{260} on the Nanodrop ND1000A spectrophotometer. The median size of the aRNA is assessed using the mRNA smear assay on the Agilent 2100 bioanalyzer.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

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

C. Safety Information

WARNING

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

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

1. Chemical safety

WARNING

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

- *Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.*
- *Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).*
- *Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).*
- *Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.*
- *Handle chemical wastes in a fume hood.*

- *Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)*
- *After emptying a waste container, seal it with the cap provided.*
- *Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.*
- *Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.*
- ***IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.*

2. Biological hazard safety

WARNING

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

WARNING

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

In the U.S.:

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

In the EU:

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

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:

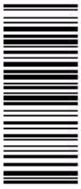
www.invitrogen.com

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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