MICROB*Enrich*™ Kit

(Part Number AM1901)

Protocol

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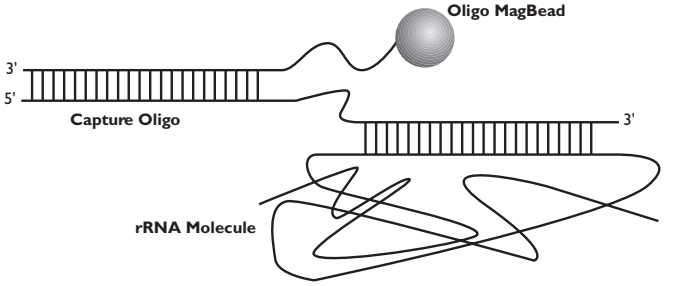
I. Introduction

A. Background

MICROB*Enrich*™ is designed to rapidly enrich bacterial RNA from mixtures containing human, mouse, or rat RNA; and bacterial RNA. The kit employs a novel, modified capture hybridization procedure, similar to that used in the Ambion® MICROB*Express*™ Kit (Figure 1), to deplete the mammalian RNA from the mixture.

Figure 1. Hybridization Capture of rRNA



The process employs optimized reagents and conditions to capture and remove 18S rRNA and 28S rRNA from up to 100 µg of a purified RNA mixture. In addition, the procedure will simultaneously remove polyadenylated mRNAs along with the rRNAs. MICROB*Enrich* was designed to remove >90% of human, mouse, or rat RNA from complex host-bacteria RNA mixtures.

The MICROB*Enrich* procedure is rapid and simple. Purified RNA is incubated with the Capture Oligonucleotide Mix in Binding Buffer for 60 minutes. Magnetic beads, derivatized with an oligonucleotide that hybridizes to the capture oligonucleotide and to the polyadenylated 3' ends of eukaryotic mRNAs, are then added to the mixture and allowed to hybridize for 15 minutes. The magnetic beads, with 18S rRNA, 28S rRNA, and polyadenylated mRNAs attached, are pulled to the side of the tube with a magnet. The enriched bacterial RNA in the supernatant is moved to a fresh tube. The magnetic beads are briefly washed, and finally the RNA is precipitated with ethanol. The resulting RNA will contain total bacterial RNA, and other small RNAs (5S, tRNAs) from the eukaryotic RNA population. If desired, the bacterial mRNA can be further enriched by subsequently using the MICROB*Express* Bacterial mRNA Purification Kit.

MICROB*Enrich* is designed so that small RNAs (including tRNA and 5S rRNA) remain in the enriched bacterial RNA population if they were present in the RNA mixture treated in the procedure. Typically RNA

isolated using one-step reagents and classic guanidinium/phenol procedures contains appreciable amounts of these small RNAs. Glass fiber-based RNA isolation methods remove about 75% of these small RNAs.

B. Materials Provided with the Kit

The MICROB*Enrich* Kit contains reagents for the depletion of a total of 500 µg of mammalian RNA from mixtures of bacterial and mammalian RNA. Because of the diverse nature of bacteria-host cell interactions, the experiments used to study them use widely variable numbers of bacteria and host cells. Hence the quantities of RNA isolated from such cell mixtures will also be widely variable. These factors make it difficult to design a kit that fits the needs of all users.

Ambion designed the MICROB*Enrich* mammalian RNA depletion kit to be flexible and scalable. From 5–100 µg of human, mouse, or rat RNA can be depleted in a single reaction in one tube. Reagents are supplied for 20 separate reactions each with up to 25 µg of host cell RNA.

If depletion reactions use <25 µg of host cell RNA as input, then the kit reagents will be depleted before a total of 500 µg of RNA can be treated. For example if the kit is used for 20 reactions, each with 5 µg host cell RNA (the minimum recommended amount), the kit reagents will be completely depleted after treating only 100 µg of host cell RNA.

Amount	Component	Storage
27 µL	Control RNA (1 mg/mL)	–20°C
210 µL	Capture Oligo Mix	–20°C
100 µL	Glycogen (5 mg/mL)	–20°C
1 mL	3 M NaOAc (sodium acetate)	–20°C
1.375 mL	Oligo MagBeads	4°C*
9 mL	Binding Buffer	4°C
2.4 mL	Wash Solution	4°C
2.8 mL	Regeneration Solution 1	4°C
5.5 mL	Regeneration Solution 2	4°C
1.375 mL	Resuspension Solution	4°C
5 mL	Nuclease-free Water	any temp†
42	1.5 mL tubes	room temp
21	Collection Tubes (2 mL)	room temp

* ***Do not freeze***

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

C. Materials Not Provided with the Kit

- RNA mixture containing human, mouse, or rat RNA; and bacterial RNA (see *II.A. Input RNA Requirements* on page 5)
- Magnetic stand (e.g. Ambion P/N AM10026)
- RNA precipitation reagents
  – 100% ethanol (ice-cold)
  – 70% ethanol (ice-cold)
- 37°C and 70°C heat block or water bath incubators
- Microcentrifuge capable of RCF of 10,000 x g
- Vortex mixer

D. Related Products Available from Applied Biosystems

Single Place Magnetic Stand P/N AM10026	Ambion's magnetic stand accommodates 1.5 mL tubes; it is used to capture magnetic beads in solutions.
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.
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.
RNA Isolation Kits see our web or print catalog	Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits.
MEGAclear™ P/N AM1908	MEGAclear purifies RNA from transcription, and other enzymatic reactions yielding high quality RNA free of unincorporated nucleotides, enzymes and buffer components with close to 100% recovery of input RNA.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.
RNase-free Tubes & Tips see our web or print catalog	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
Electrophoresis Reagents see our web or print catalog	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.
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.

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.
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. Ambion also offers the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.

II. MICROB*Enrich* Procedure

A. Input RNA Requirements

RNA source organisms compatible with MICROB*Enrich*

The MICROB*Enrich* procedure is designed to work with complex mixtures of purified mammalian and bacterial total RNA. The procedure was optimized with human, mouse, and rat total RNA mixed with *E. coli* total RNA, and removes >90% of the eukaryotic 18S and 28S rRNAs, and polyadenylated mRNAs present in these mixtures. MICROB*Enrich* will work with any bacterial species.

Host RNA:Bacterial RNA ratio determination

Prior knowledge of the ratio of host:microbe RNA in the mixture will often be incomplete. If the bacterial RNA is expected to represent a significant portion of the RNA mixture, Ambion suggests that users evaluate the total RNA sample with the RNA 6000 LabChip kit and an Agilent bioanalyzer, or by denaturing agarose gel electrophoresis to obtain an estimate of the mass ratio of eukaryotic and prokaryotic RNAs present. In some samples of total RNA from host-microbe interaction studies, the mass of host RNA will far exceed that of the microbial RNA. If this is the case, it may be assumed that 100% of the sample is eukaryotic RNA for purposes of calculating the amounts of Capture Oligo Mix and Oligo MagBeads to be used (step II.B.2 and II.C.1).

Accurate quantitation of input RNA is important

It is important to accurately quantitate RNA so as not to overload the MICROB*Enrich* system. Ambion recommends using a high quality, calibrated spectrophotometer, or the RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes). *MICROBEnrich* does not remove DNA from RNA. If the input RNA contains DNA contamination, it will contribute to the A_{260} and may interfere with host cell RNA depletion.

The RNA must be in a solution with ≥1 mM EDTA

The RNA mixture will be heat denatured at 70°C in step II.B.3 on page 8 to prepare it for hybridization with the Capture Oligo Mix. Degradation of the RNA can potentially occur at this step due to RNA hydrolysis in the presence of divalent cations such as magnesium salts. To prevent this, make sure that the RNA solution has at least 1 mM EDTA to chelate any divalent cations that may be present. If you know that your RNA has significant amounts of divalent cations, change the RNA solution by precipitation (see *RNA precipitation instructions* on page 7) or by using an RNA clean-up system such as MEGAclear. Resuspend the RNA in TE or THE RNA Storage Solution because these solutions contain effective chelating agents.

Total RNA isolation method and small RNAs

The MICROB*Enrich* procedure was optimized with RNA prepared using Ambion RNAqueous® (P/N AM1912), and RiboPure™-Bacteria (P/N AM1925) RNA isolation products. The kit will work well, however, with any high quality RNA preparation.

MICROB*Enrich* does ***not*** remove small RNAs (including tRNA and 5S rRNA) from the enriched bacterial RNA population. ***Therefore to maximize bacterial RNA enrichment, use RNA that does not contain small structural RNAs as starting material for the procedure***. Typically RNA isolated using glass fiber-based isolation methods (e.g. Ambion RNAqueous and RiboPure-Bacteria Kits) contains only about 25% of the 5S rRNAs and tRNAs present in RNA purified using one step reagents (e.g., TRI Reagent®), and classic guanidinium/phenol procedures (e.g. TōTALLY RNA™). Ambion MEGAclear™ Kit will remove approximately 75% of small structural RNA species from total RNA. This is shown with *E. coli* RNA in Figure 2.

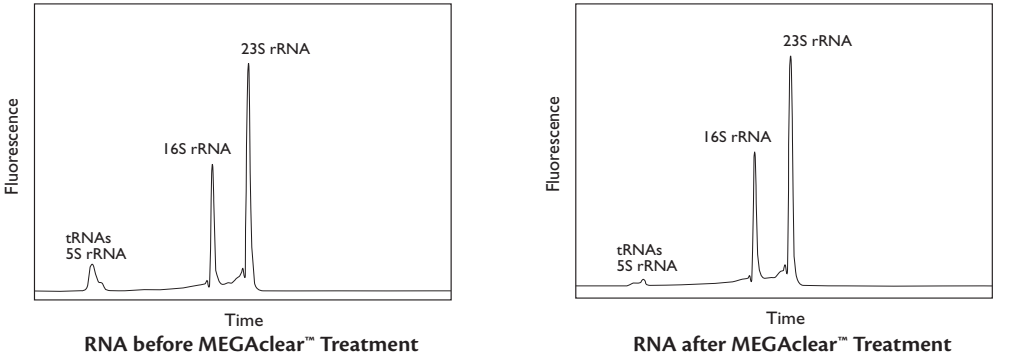


Figure 2. Total *E. coli* RNA and the Same RNA After Further Purification with MEGAclear™.

200 ng of a single prep of *E. coli* RNA was either directly analyzed on an Agilent 2100 bioanalyzer using the RNA 6000 Nano LabChip Kit, or was further purified using the Ambion MEGAclear Kit to remove small RNAs before loading onto the bioanalyzer. The data show that MEGAclear removes most of the 5S and tRNA from total RNA.

Precipitate RNA if necessary for further purification or to concentrate it

Excess salt

Total RNA prepared with a method that includes a solid phase extraction such as RNAqueous, RiboPure, or RiboPure-Bacteria can be used immediately after elution because such samples are unlikely to have high levels of salt. On the other hand, RNA isolated by methods that include only organic extractions, for example using TRI Reagent or TōTALLY RNA, may have a substantial amount of residual salt. If RNA from these types of procedures has been precipitated only a single time, we recommend doing a second alcohol precipitation and a 70% ethanol wash to remove residual salt before starting the MICROB*Enrich* procedure (see below for instructions).

A_{260}:A_{280} should be >1.7

When quantifying the RNA mixture, check the A_{280} of the RNA solution, and calculate the A_{260}:A_{280} ratio to get an idea of the purity of the RNA. Ambion advises that the A_{260}:A_{280} ratio be >1.7. If a n RNA sample does not meet this criteria it is advisable to phenol/chloroform extract the sample and reprecipitate, or to use a solid-phase clean-up technique like the MEGAclear Kit.

The volume of RNA should be less than 30 µL

The recommended maximum amount of RNA per reaction is 100 µg and the recommended maximum volume is 30 µL.

MICROB*Enrich* is a scalable procedure; it can be used with 5–100 µg of an RNA mixture per reaction. The kit configuration is based on a ≥25 µg RNA sample size, however, so if each of the 20 reactions are conducted with <25 µg RNA, less than full 500 µg of mixed RNA can be treated with the kit.

RNA precipitation instructions


NOTE

*The reagents for this RNA precipitation are **not** supplied with the kit.*

1. Precipitate the RNA by adding the following and mixing well:
   - 0.1 volume 5 M ammonium acetate or 3 M sodium acetate
   - (optional) 5 µg glycogen
     The glycogen acts as a carrier to increase precipitation efficiency from dilute RNA solutions; it is unnecessary for solutions with ≥200 µg RNA/mL)
   - 2.5–3 volumes 100% ethanol
2. Leave the mixture at –20°C overnight, or quick-freeze it in ethanol and dry ice, or in a –70°C freezer for 30 min.
3. Recover the RNA by centrifugation at ≥12,000 x g for 30 min at 4°C.
4. Carefully remove and discard the supernatant. The RNA pellet may not adhere tightly to the walls of the tubes, so we suggest removing the supernatant by gentle aspiration with a fine-tipped pipette.
5. Centrifuge the tube briefly a second time, and aspirate any additional fluid that collects with a fine-tipped pipette.
6. Add 1 mL ice cold 70% ethanol, and vortex the tube.
7. Re-pellet the RNA by centrifuging for 10 min at 4°C. Remove the supernatant carefully as in steps 4 and 5 above.
8. Dissolve the RNA in ≥30 µL TE (10 mM Tris-HCl pH 8, 1 mM EDTA) or THE RNA Storage Solution.

B. Anneal RNA and Capture Oligonucleotide Mix


NOTE

The most accurate way to evaluate the bacterial RNA enrichment of samples at the end of the procedure, is to include a mock reaction sample where the Capture Oligo Mix is not included, but otherwise the sample is subjected to the entire MICROBEnrich procedure. (See section III.A on page 14 for more information.)

1. Add 5–100 µg RNA to 300 µL Binding Buffer

a. Pipet 300 µL Binding Buffer into a 1.5 mL tube provided with the kit.

b. Add the RNA mixture (5–100 µg RNA in a maximum volume of 30 µL) to the Binding Buffer.

c. Close the tube, and tap or vortex gently to mix.

2. Add 2 µL Capture Oligo Mix for every 5 µg of RNA

a. Add 2 µL of Capture Oligo Mix for 5 µg RNA in Binding Buffer (i.e. use 10 µL Capture Oligo Mix for every 25 µg RNA).

b. Close the tube and tap or vortex gently to mix, and microfuge briefly to get the mixture to the bottom of the tube.

3. Heat to 70°C for 10 min

Incubating the mixture at 70°C for 10 min denatures secondary structures in RNA, including the 18S and 28S rRNAs, and polyadenylated mRNAs. This heat denaturation helps to facilitate maximal hybridization of the capture oligonucleotides to the mammalian RNA.

4. Anneal at 37°C for 1 hr

The 37°C, 1 hour incubation allows the capture oligonucleotides to hybridize to homologous regions of the 18S and 28S rRNAs. The Binding Buffer is optimized to function specifically and efficiently at this temperature.

Prepare the Oligo MagBeads as described in the next section during this incubation.

C. Prepare the Oligo MagBeads

IMPORTANT

The Oligo MagBeads are in a 1% (10 mg/mL) suspension. Vortex the tube just before pipetting to be sure they are well suspended.

1. Withdraw 25 µL Oligo MagBeads for every 5 µg of input RNA to a 1.5 mL tube

For each 5 µg of input RNA remove 25 µL Oligo MagBeads to a 1.5 mL tube (use 125 µL of Oligo MagBeads for 25 µg RNA).

NOTE

Record the volume of Oligo MagBeads placed in each tube; this volume will be used for the subsequent washing and equilibration steps.

2. Capture the Oligo MagBeads and carefully remove and discard the supernatant

a. Capture the Oligo MagBeads by placing the tube on a magnetic stand. Leave the tube on the stand until all of the Oligo MagBeads are arranged inside the tube near the magnet. This will take ~3 min with the Ambion Single Place Magnetic Stand (it may take longer with a weaker magnet).

b. Carefully remove the supernatant by aspiration, leaving the beads in the tube, and discard the supernatant.

3. Wash the Oligo MagBeads with an equal volume of Nuclease-free Water

a. Add Nuclease-free Water to the captured Oligo MagBeads; use a volume of Nuclease-free Water equal to the original volume of the Oligo MagBeads (the volume at step C.1 on page 9—before magnetic capture).

b. Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing.

c. As described in step C.2, recapture the Oligo MagBeads with a magnetic stand, and carefully aspirate and discard the Nuclease-free Water leaving the beads in the tube.

4. Equilibrate the Oligo MagBeads with an equal volume of Binding Buffer

a. Add Binding Buffer to the captured Oligo MagBeads; use a volume of Binding Buffer equal to the original volume of the Oligo MagBeads.

b. Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing.

c. As described in step C.2 on page 9, recapture the Oligo MagBeads with a magnetic stand, and carefully aspirate and discard the Binding Buffer leaving the beads in the tube.

d. Store the Oligo MagBeads on ice until ~5 min before they will be used (in step D.2 on page 10), then remove them from ice to warm up to room temperature.

D. Capture the Mammalian RNA and Recover the Enriched Bacterial RNA

1. Heat the Wash Solution to 37°C

The preheated Wash Solution will be used in step 4 on page 10.

2. Add RNA/Capture Oligonucleotide Mix to prepared Oligo MagBeads and incubate 15 min at 37°C

a. After incubating the RNA and Capture Oligo Mix for 1 hr at 37°C (step B.4 on page 8) remove the tubes to room temperature, microcentrifuge briefly, and immediately add the contents to the washed and equilibrated Oligo MagBeads (from step C.4.d on page 9).

b. Very gently vortex or tap the tube to mix and microfuge very briefly to get the mixture to the bottom of the tube.

c. Incubate 15 min at 37°C.
During this step the 18S rRNAs, 28S rRNAs, and poly(A) RNAs are captured on the Oligo MagBeads.

3. Capture the Oligo MagBeads, and recover the supernatant containing the enriched bacterial RNA

a. Capture the Oligo MagBeads by placing the tube on the Magnetic Stand. Leave the tube on the stand until all of the Oligo MagBeads are arranged inside the tube near the magnet. This will take ~3 min using the Ambion Single Place Magnetic Stand.

b. Aspirate the supernatant which contains the enriched bacterial RNA, being careful not to dislodge the Oligo MagBeads. Transfer it to a Collection Tube on ice.
If a very small amount of Oligo MagBeads are accidentally carried over to the supernatant, they can be removed at the end of the procedure.

4. Recover any remaining bacterial RNA from the Oligo MagBeads by washing them with 100 µL Wash Solution at 37°C and recovering the wash

a. Add 100 µL Wash Solution that has been prewarmed to 37°C to the captured Oligo MagBeads.

b. Remove the tube from the magnetic stand, and resuspend the beads by brief, gentle vortexing.

c. Incubate 5 min at 37°C.

d. As in step D.3, recapture the Oligo MagBeads, and carefully recover the supernatant. Pool this supernatant with the RNA already in the Collection Tube and proceed immediately either to the precipitation described in step E.1 (below) or to the MICROB*Express* procedure (see step 5 below).
Do not discard the Oligo MagBeads. Place the used beads on ice and prepare them for a second use as described in section *II.F. Oligo MagBead Regeneration* on page 12. Regenerate the beads just after completing the procedure; this should be done on the same day they were used.

5. Bacterial mRNA Enrichment with MICROB*Express*:

To further enrich the bacterial mRNA component using the MICROB*Express* kit, proceed directly to section *II.G. Transition to the MICROBExpress Enrichment Procedure* on page 13. Skip the precipitation described in section II.E below, and store the bacterial RNA on ice until you are ready to start the MICROB*Express* procedure.

E. Precipitate and Resuspend the Enriched Bacterial RNA

Proceed with the following precipitation only if the enriched bacterial RNA will ***not*** undergo the MICROB*Express* procedure. To further enrich the bacterial mRNA by using MICROB*Express*, skip this precipitation, and go to section *II.G. Transition to the MICROBExpress Enrichment Procedure* on page 13.

1. Ethanol precipitate the enriched bacterial RNA

a. Add the following to the pooled bacterial RNA from step D.4.d on page 10 (the volume should be ~400–470 µL), and briefly vortex to mix.
   - 1/10th volume 3 M Sodium Acetate (40–47 µL)
   - 4 µL Glycogen (5 mg/mL)

b. Add 2.5 volumes ice cold 100% ethanol, and vortex to mix thoroughly.

c. Precipitate at –20°C for at least 1 hr.

d. Centrifuge for 30 min at ≥10,000 X g (typically ~13,000 rpm in a microcentrifuge) and carefully decant and discard the supernatant.

e. Do a 70% ethanol wash as follows:

   i. Add 750 µL ice cold 70% ethanol and vortex briefly.

   ii. Centrifuge for 5 min at ≥10,000 X g. Discard the supernatant.

f. Do a second 70% ethanol wash as in step e.

g. Briefly re-spin the tube after discarding the second 70% ethanol wash. Carefully remove any remaining supernatant with a pipettor, being careful not to dislodge the pellet.

h. Air dry the pellet for 5 min. Do not air dry the pellet for more than 5 min.

2. Resuspend the enriched bacterial RNA in an appropriate buffer

a. Resuspend the RNA pellet to the desired concentration in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) or in a buffer appropriate for your downstream application (THE RNA Storage Solution, TE Buffer, 1 mM EDTA or Nuclease-free Water are among the possible choices). We typically resuspend the RNA enriched from mixtures of 25 µg mammalian RNA and 2 µg of bacterial RNA in 50 µL for analysis on the Agilent bioanalyzer, or in 25 µL for gel analysis.

b. Rehydrate the RNA for 15 min at room temperature. Vortex the sample vigorously if necessary to resuspend the RNA. Collect the sample by brief centrifugation.


NOTE

If the pellet will not go into solution after 15 min at room temp and vigorous vortexing, heat the sample to 70°C for 5 min; this should help resuspend the pellet. Do ***not*** *heat RNA samples that are in a solution lacking EDTA because non-chelated divalent cations can cause hydrolysis of RNA at high temperatures.*

3. Remove residual Oligo MagBeads if necessary

If the RNA solution has a brownish color, there are probably a small amount of Oligo Magbeads remaining in the sample. To remove them, put the tube on the magnetic stand for ~3 min and move the enriched RNA solution to a new RNase-free tube.

The yield of RNA from a mixture of 25 µg eukaryotic total RNA and 2 µg prokaryotic RNA should be 3–5 µg. Yield will vary depending on the ratio of mammalian:bacterial RNA in the mixture, the RNA isolation technique used to obtain the RNA mixture, and RNA integrity.

F. Oligo MagBead Regeneration

To enrich for bacterial RNA from 500 µg of input mixed RNA, the Oligo MagBeads must be regenerated and used a second time. If the supplied Oligo MagBeads are used only once and discarded, there will only be enough Oligo MagBeads in the kit to treat 250 µg of input RNA. Ambion has determined that the regeneration and reuse of Oligo MagBeads does not decrease the efficiency of RNA capture.

Used Oligo MagBeads contain bound rRNA, capture oligonucleotides, and mRNA. Regeneration removes all these components from the beads and prepares them for a ***single*** reuse.

1. At room temp, incubate Oligo MagBeads in 2 volumes Regeneration Solution 1 for 1 hr

a. At room temp, add 2 volumes Regeneration Solution 1 to the Oligo MagBeads (50 µL Regeneration Solution 1 per 25 µL original volume of Oligo MagBeads withdrawn at step C.1 on page 9).

b. Vortex well until the beads are thoroughly resuspended, quick spin, and incubate at room temperature for 1 hour.

c. Capture the Oligo MagBeads completely on a magnetic stand, then carefully remove and discard the supernatant.

2. Wash Oligo MagBeads twice with 2 volumes Regeneration Sol'n 2

a. Add 2 volumes Regeneration Solution 2 to the Oligo MagBeads (50 µL Regeneration Solution 2 per 25 µL original volume of Oligo MagBeads withdrawn at step C.1 on page 9).

b. Remove the tube from the magnetic stand and vortex well until beads are thoroughly resuspended. Quick spin and capture the Oligo MagBeads completely using the magnetic stand. Remove the supernatant and discard.

c. Do a second wash with 2 volumes of Regeneration Solution 2 (i.e. repeat steps 2.a and 2.b).

3. Resuspend the Oligo MagBeads in their original volume of Resuspension Solution

Resuspend the Oligo MagBeads in an equal volume of Resuspension Solution (25 µL Resuspension Solution per 25 µL original volume of Oligo MagBeads withdrawn at step C.1 on page 9).

Store Oligo MagBeads at 4°C until further use. Regenerated Oligo MagBeads are in a 1% suspension; they can be used exactly as they were used the first time.

G. Transition to the MICROB*Express* Enrichment Procedure

To further enrich the bacterial mRNA by using the MICROB*Express* Kit, the following steps will provide a seamless transition from the MICROB*Enrich* procedure.

If there may be >10 µg bacterial RNA in your sample:

MICROB*Express* is designed to purify mRNA from ≤10 µg bacterial total RNA. If you think there may be more than 10 µg bacterial RNA present in the sample then precipitate the RNA (following the instructions in section II.E on page 11), quantitate the RNA by reading the A_{260}, and resuspend in TE to ≤10 µg/µL.

If there is ≤10 µg bacterial RNA in your sample:

1. Start the MICROB*Express* procedure at step *II.B.2 Add 4 µL Capture Oligo Mix*. (Step II.B.1, adding Binding Buffer, is unnecessary because the RNA is already in a suitable binding buffer.)
2. Continue with the MICROB*Express* procedure through to the end of section II.D following the instructions as written. (The volumes do ***not*** need to be adjusted even though the sample volume will be higher when the input RNA comes directly from the MICROB*Enrich* procedure.)

NOTE

Since the volume of mRNA at the end of the procedure will be higher than a typical MICROBExpress reaction, the volumes shown in parentheses for precipitating the RNA in step II.E.1 of the MICROBExpress procedure will be incorrect—but the proportions listed will be accurate.

III. Assessing Yield and Quality of Enriched mRNA

A. Evaluating Depletion of Host Cell RNA

To accurately evaluate the effectiveness of the enrichment procedure, compare MICROB*Enrich* enriched RNA with the amount of mixed RNA from which it was enriched. Evaluate the samples by denaturing agarose gel electrophoresis (section III.C on page 15) or with an Agilent 2100 bioanalyzer (section III.D on page 17).

1. Comparison with a mock MICROB*Enrich* reaction

The best way to evaluate the effectiveness of host cell RNA depletion is to perform a mock MICROB*Enrich* reaction by subjecting your starting RNA to the entire procedure leaving out the Capture Oligo Mix. Then compare equal fractions of RNA enriched from the mock and normal MICROB*Enrich* treatment.

Precipitate both RNAs as described in section II.E on page 11, and resuspend them in the same volume. Compare equal volumes of each sample (mock and normal) by denaturing agarose gel electrophoresis or with an Agilent bioanalyzer.

2. Comparison with untreated starting RNA

If you do not have enough starting RNA to do a mock reaction, MICROB*Enrich* enriched RNA may be compared with the total RNA from which it was enriched—as in the following example:

The MICROB*Enrich* Control RNA is at a concentration of 1 µg/µL, therefore a 27 µL sample contains 27 µg of RNA. Rat RNA comprises 25 µg and *E. coli* RNA comprises 2 µg of the Control RNA. The bacterial RNA represents 2 µg/27 µL or 0.074 µg/mL.

Before starting the procedure, reserve 1 µL of the Control RNA. The remaining solution now contains 26 µg RNA/26 µL. The bacterial RNA is still at 0.074 µg/µL in this sample.

0.074 µg/µL x 26 µL = 1.924 µg bacterial RNA

Perform the MICROB*Enrich* procedure on the 26 µL of RNA. Precipitate the enriched bacterial RNA and resuspend in 26 µL: the same volume as the starting material. Because the procedure only depletes mammalian RNA, the mass of bacterial RNA present in the sample remains unchanged (1.924 µg), and since the sample was resuspended in its original volume, the concentration of bacterial RNA should also remain unchanged (1.924 µg/26 µL = 0.074 µg/mL).

NOTE

The enriched RNA can be resuspended in a volume different than the starting RNA volume, however, to accurately evaluate the effectiveness of eukaryotic RNA depletion, be sure to compare equivalent fractions of enriched and starting RNA.

The effectiveness of mammalian RNA depletion is evaluated by comparing the 1 µL of the reserved Control RNA with 1 µL of the enriched RNA. Each sample should contain approximately the same amount of bacterial RNA.

B. Quantitation of RNA

1. Assessing RNA yield by UV absorbance

The concentration of an RNA solution can be determined by diluting an aliquot of the preparation (usually a 1:50 to 1:100 dilution) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and reading the absorbance in a spectrophotometer at 260 nm. Be sure to zero the spectrophotometer with the TE used for sample dilution.

An A_{260} of 1 is equivalent to 40 µg RNA/mL. The concentration (µg/mL) of RNA is therefore calculated as follows:

A_{260} X dilution factor X 40 µg/mL

The typical yield from 25 µg of high quality total RNA is 5–10 µg. The RNA is resuspended in 50 µL, thus the concentration will be 100–200 ng/µL.

An example of RNA concentration determination from an A_{260} reading is shown below:

1 µL of the prep is diluted 1:50 into 49 µL of TE

The A_{260} = 0.1

RNA concentration = 0.1 X 50 X 40 µg/mL = 200 µg/mL or 200 ng/µL

Since there are 49 µL of the prep remaining after using 1 µL to measure the concentration, the amount of remaining RNA is 49 µL X 200 ng/µL = 9.8 µg

2. Assessing RNA yield with RiboGreen®

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

C. Denaturing Agarose Gel Electrophoresis

Many mRNAs form extensive secondary structure. Because of this, it is best to use a denaturing gel system to size-fractionate RNA. Either formaldehyde-or glyoxal-based denaturing gel systems can be used to evaluate your RNA, Ambion offers the NorthernMax® line of reagents for agarose gel analysis of RNA.

Be sure to include a positive control RNA on the gel so that unusual results can be attributed to a problem with the gel or a problem with the RNA under analysis. RNA molecular weight markers (such as Ambion Millennium™ Markers P/N AM7150, AM7151), an RNA sample known to be intact, or both, can be used for this purpose. It is also a good idea to include a sample of the starting RNA that was used in the enrichment procedure.

Expected appearance of denatured RNA on agarose gels

If a mock MICROB*Enrich* reaction was performed, as described in section III.A.1 on page 14, equal fractions of the mock and experimental samples should be compared on the gel. If you do not have enough starting RNA to do a mock reaction follow the example in section III.A.2 on page 14 for comparison with untreated starting RNA.

In RNA enriched by the MICROB*Enrich* procedure, the host cell 18S and 28S rRNA bands will be absent or very faint. Bands corresponding to prokaryotic 16S and 23S rRNAs may be visible, depending on the mass of prokaryotic RNA in the starting material. If the input RNA was purified using a glass fiber filter method, small RNAs ≤200 bases (5S and tRNAs) may be faint or undetectable. RNA recovered from MICROB*Enrich* that is subsequently enriched for bacterial mRNA with the MICROB*Express* procedure, may show a smear of RNA ranging from <0.5 kb and larger.

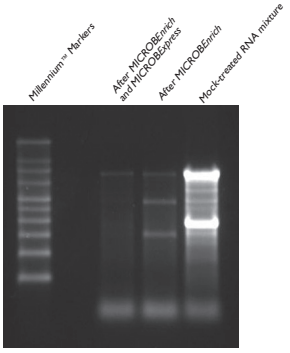


Figure 3. RNA Before and After Enrichment MICROB*Enrich*™ and MICROB*Express*™.

Rat liver total RNA (25 µg) and E. coli total RNA (2 µg) were mixed and subjected to one of the following:

- a mock MICROB*Enrich* procedure in which the Capture Oligo Mix was left out of the reaction
- the MICROB*Enrich* procedure
- the MICROB*Enrich* procedure with further mRNA enrichment using the MICROBExpress Kit.

RNA was precipitated and an equal fraction of each sample was subjected to denaturing formaldehyde agarose gel electrophoresis

D. Agilent 2100 Bioanalyzer Analysis of Enriched mRNA

1. Evaluating rRNA removal with the RNA 6000 Nano LabChip® Kit

The Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® Kit provide a particularly effective method for evaluating rRNA removal with the MICROB*Enrich* Kit. Follow the instructions for RNA analysis provided with the RNA 6000 Nano LabChip Kit. At Ambion, we have found that this system performs best by loading 1 µL of a 50–250 ng/µL RNA solution.

To compare RNA samples before and after MICROB*Enrich* bacterial RNA enrichment, follow the recommendations in section *III.A. Evaluating Depletion of Host Cell RNA* on page 14.

2. Expected Results

In enriched RNA samples, the 18S and 28S rRNA peaks will be absent or very small (Figure 4). The peak calling feature of the software may even fail to identify these peaks at all. A peak corresponding to 5S and tRNAs may be present depending on the technique used to isolate the input total RNA.

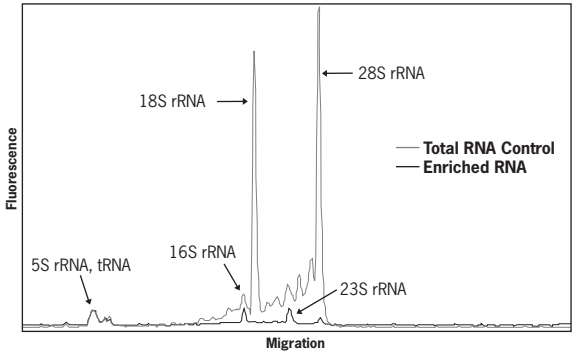


Figure 4. RNA Before and After Enrichment with the MICROB*Enrich*™ Kit.

Rat liver total RNA (25 µg) and *E. coli* total RNA (2 µg) were mixed and subjected to the MICROB*Enrich* procedure. A separate sample of mixed total RNA was subjected to a mock MICROB*Enrich* procedure in which the Capture Oligo Mix was left out of the reaction. RNA was precipitated and an equal fraction of each sample was run on the Agilent 2100 bioanalyzer.

IV. Troubleshooting

A. Positive Control Reaction

Instructions for using the Control RNA

The control RNA is a mixture of 25 µg rat RNA and 2 µg *E. coli* RNA at 1 µg/µL.

a. Purify bacterial RNA from 10 µL of the Control RNA, and do a mock MICROB*Enrich* reaction with a second 10 µL of the Control RNA.

Set up the RNA/Capture Oligo Mix annealing mixture as follows:

Control reaction	Mock reaction	Component
10 µL	10 µL	Control RNA
300 µL	300 µL	Binding Buffer
4 µL	– –	Capture Oligo Mix

Carry both the control reaction and the mock reaction through the RNA enrichment procedure starting at step II.B.3 on page 8 through step II.E.2 on page 11.

Resuspend both reaction products in 20 µL of the Nuclease-free Water supplied with the kit. If either RNA solution is slightly brown in appearance, remove the residual Oligo MagBeads by placing the tube on a magnetic stand and transferring the RNA solution to a fresh nuclease-free 1.5 mL tube.

b. Evaluate the mock and control reactions either by agarose gel electrophoresis or with the RNA LabChip Kit on a bioanalyzer.

i. Agarose gel electrophoresis instructions

Measure the A_{260} of the enriched bacterial RNA from the positive control reaction, and calculate its concentration following the instructions in section III.B on page 15.

Run 0.5–1 µg of the control RNA on a denaturing agarose gel (section III.C on page 15) and compare it with an equal volume of the mock treated RNA (since they were resuspended in the same volume, this will give an accurate idea of the degree of bacterial RNA enrichment in the control reaction).

ii. Bioanalyzer instructions

Run 1 µL of the mock and control reactions using the RNA LabChip Kit on a bioanalyzer following the manufacturer's instructions.

Expected result of the positive control reaction

The MICROB*Enrich* procedure should remove ≥90% of 18S and 28S rRNAs from the control RNA.

If the control reaction fails, contact our Technical Service Department.

B. Low Final Yield of RNA

The mass yield of enriched RNA from 25 µg total RNA is typically 5–10 µg and may vary depending on the quality of the input RNA, and whether or not it contained large amounts of 5S and tRNA.

1. Incorrect quantitation of RNA

It is very important that the total RNA used as starting material be accurately quantified before starting the procedure for optimal results. Accurate quantitation is important for many downstream procedures as well.

Ambion recommends using a well calibrated spectrophotometer or the RiboGreen RNA Quantitation Assay and Kit (Molecular Probes). The Control RNA (1 mg/mL) provided with the kit can be used as a standard with either technique.

2. Salt concentration of input RNA is high

The RNA must be in a low salt solution to properly hybridize to the Capture Oligo Mix. Total RNA prepared using methods that include solid-phase extraction such as RNAqueous and RiboPure can be used immediately after elution because such samples are unlikely to have high levels of salt. On the other hand, RNA isolated by methods based on the classic Chomzynski and Sacchi technique, or using one-step reagents, may have a substantial amount of residual salt. If RNA from these types of procedures has been precipitated only a single time, we recommend doing a second alcohol precipitation and a 70% ethanol wash to remove residual salt before starting the MICROB*Enrich* procedure. (See section *II.A. RNA precipitation instructions* on page 7 for a procedure.)

3. RNA is degraded

See section *IV.D. RNA Degradation* on page 21.

4. Inefficient precipitation of RNA

For optimal RNA recovery precipitate the mRNA as described in step II.E.1 on page 11 using 1/10th volume 3M sodium acetate and 2.5volumes of 100% ethanol. Note that the addition of glycogen to the precipitation mixture is essential for complete recovery of nucleic acids from the MICROB*Enrich* Binding Buffer.

Be sure to precipitate the RNA for at least 1 hour at –20°C; precipitating for a full hour will dramatically improve RNA yield over shorter precipitations. The speed and time of centrifugation is also important for good precipitation of nucleic acids; centrifuge at ≥10,000 x g for 30 min.

5. RNA pellet is not completely resuspended

It may be difficult to completely resuspend the RNA pellet at the end of the procedure (step II.E.2 on page 11). Be sure to air dry the pellet for 5 min only, and allow the RNA to rehydrate for at least 15 min. Vortex the tube vigorously; if the pellet is still not dissolved, and the solution used for resuspension contains EDTA, heat the mixture for 5 min at 70°C and vortex again.

C. Host Cell Ribosomal RNA Contamination

1. Procedure was not followed accurately

Each step of the MICROB*Enrich* procedure was scrutinized during product development to identify the optimal conditions for removal of host cell rRNA. To maximize removal of host cell ribosomal RNA follow the procedure exactly. Following is a list of some of the most crucial parts of the procedure:

a. Initial quantitation of input RNA was incorrect

The MICROB*Enrich* enrichment procedure has been carefully optimized for use with 100 µg or less RNA. If more RNA is used, removal of host cell RNA will not proceed to completion, thus the initial RNA quantitation is essential for proper functioning of the kit. The MICROB*Enrich* kit is scalable (5–100 µg of RNA). RNA, Capture Oligo Mix and Oligo MagBeads must all be scaled accordingly. If these reagents are not used in the proper ratios, the kit will function suboptimally.

To verify that your RNA quantitation method is accurate, test it against a standard curve made with dilutions of an RNA of known concentration (e.g. RNA markers or commercially prepared RNA).

b. RNA was not denatured completely before hybridization to the Capture Oligo Mix

It is important that the RNA is completely heat denatured by following the instructions in step II.B.3 on page 8. If the rRNA is incompletely denatured, hybridization of the Capture Oligo Mix will not be efficient. This will result in substandard enrichment. Also, be sure that the water bath used for the incubation is set to 70°C and allow the incubation to proceed for the full 10 minutes.

c. Hybridization conditions are incorrect

Ensure that the hybridizations in steps II.B.4 on page 8 and II.B.2 on page 8 are done at the proper temperature and for the full time.

d. Wash conditions are incorrect

The wash step is important for removing bacterial RNA trapped in the rRNA:Capture Oligonucleotide complex. If this step is done improperly it can result in increased host cell rRNA contamination. Be sure to use the volumes, and time and temperature of incubation, in step II.D.4 on page 10.

e. Oligo MagBeads were not regenerated

Regenerate the Oligo MagBeads on the same day as the procedure is completed, and store the slurry in the Resuspension Solution.

2. Carryover of excess Oligo MagBeads

With relatively weak magnets, it is common to have some magnetic bead contamination in the resuspended enriched RNA sample. ***Oligo MagBeads will carry contaminating host cell RNA and can potentially affect downstream applications.*** Be careful not to dislodge Oligo Mag-

Beads when removing supernatants. It may be useful to tilt the tube bottom toward the magnet to hold the beads in place. After precipitation and resuspension, if the RNA appears brownish in color, put the sample in the magnetic stand for at least 3 min and carefully remove the supernatant to a new RNase-free tube. The solution should appear clear after this step indicating that all of the Oligo MagBeads have been removed.

3. Mammalian host is incompatible with MICROB*Enrich*

This kit is designed to enrich bacterial RNA from complex mixtures of human, mouse, and rat RNA. At the time of publication of this Protocol, host cell RNA from other species can not be removed with the MICROB*Enrich* Kit. Check our web site or technical service department for updates.

D. RNA Degradation

1. Input total RNA is degraded

To see whether the RNA used in the MICROB*Enrich* procedure was degraded from the start, evaluate a 0.5–5 µg sample of the input RNA mixture on a denaturing agarose gel (see section III.C starting on page 15). Total RNA should produce rRNA bands that appear sharp and well-defined (Figure 3 on page 16). In high quality RNA samples, the 28S rRNA band will be 1.5–2 fold brighter than the 18S rRNA band.

2. Practice RNase-free technique

All of the typical precautions against RNase contamination should be observed. Gloves should be worn at all times and changed frequently to avoid the introduction of RNases. Bags containing the centrifuge tubes and the solution tubes and bottles should be kept closed when they are not in use to avoid contamination with dust. Any tubes or solutions not supplied with the kit, which will contact the RNA, should be bought or prepared so that they are free from RNases.

For more information on avoiding RNase contamination see Ambion Technical Bulletins 159 and 180 (available on our web site at http://www.ambion.com/techlib/tb/tb_159.html and http://www.ambion.com/techlib/tb/tb_180.html).

3. Degradation caused by the heat denaturation step

RNA degradation can occur at elevated temperatures when there are divalent cations in the solution. If your RNA solution does not contain at least 1 mM EDTA, contaminating divalent cations could cause RNA hydrolysis during the heat denaturation step (step II.B.3 on page 8).

E. Poor Performance of Enriched RNA in Downstream Applications

1. Residual salt remaining in RNA preparation

High salt concentration in the enriched RNA sample may negatively affect downstream applications. Follow the precipitation procedure in step II.E.1 on page 11 exactly. Include two 70% ethanol washes for consistent performance of the RNA in applications such as cDNA labeling. An optional phenol/chloroform extraction and ethanol precipitation can be done to ensure that the RNA is free of contaminating protein and salt.

2. Carryover of excess Oligo MagBeads

Excessive contamination with Oligo MagBeads in the final sample can affect downstream applications. As described in step II.E.3 on page 12, remove any remaining Oligo MagBeads from the enriched mRNA by placing the tube on the magnetic stand for at least 3 min and carefully removing the supernatant to a new RNase-free tube. The solution should appear clear after this step indicating that all of the Oligo MagBead have been removed.

3. RNA degradation

See section *IV.D. RNA Degradation* on page 21.

4. Low A_{260}:A_{280} Ratio

If the A_{260}:A_{280} ratio of the enriched mRNA is <1.7 it can indicate a sample with excess protein or other contaminating substances. Excessive protein contamination of total RNA preparations is not uncommon, especially when using a procedure based on organic extraction, and some of this protein may be carried over to the enriched RNA. Excess carryover of Oligo MagBeads may also cause a low A_{260}:A_{280} ratio. See *IV.C. Host Cell Ribosomal RNA Contamination* on page 20 for Oligo MagBead removal.

V. Appendix

A. References

Morrissey DV, Collins ML (1989) Nucleic acid hybridization assays employing dA-tailed capture probes. Single capture methods. *Molecular and Cellular Probes* **3**:189–207.

Morrissey DV, Lombardo M, Eldredge JK, Kearney KR, Groody EP, Collins ML (1989) Nucleic acid hybridization assays employing dA-tailed capture probes. I. Multiple capture methods. *Anal Biochem* **181**:345–359.

Hunsacker WR, Badri H, Lombardo M, Collins ML (1989) Nucleic acid hybridization assay employing dA-tailed capture probes. II. Advanced multiple capture methods. *Anal Biochem* **181**:360–370.

B. Quality Control

Functional Testing
The Control RNA consisting of 25 µg rat RNA and 2 µg *E. coli* RNA is used in a MICROB*Enrich* mRNA purification experiment. The procedure is shown to remove >90% of the rat RNA from the mixture, through analysis by capillary electrophoresis using 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.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.

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

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

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