PROTOCOL

ambion® | RNA
by life technologies™

MICROB*Express*™ Kit

life technologies™

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Part Number 1905M Rev. C
01/2011

MICROB*Express*™ Kit

(Part Number AM1905)

Protocol

I. Introduction

IMPORTANT

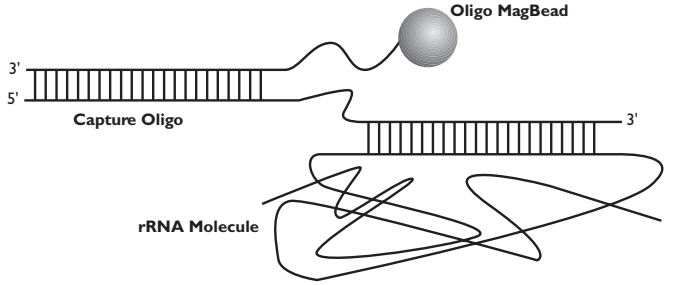
Before using this product, read and understand the "Safety Information" in the appendix in this document.

A. Background

MICROB*Express*™ is designed to rapidly enrich bacterial mRNA from purified total RNA by removing the 16S and 23S ribosomal RNAs (rRNA). The kit employs a modified capture hybridization approach (Figure 1), to remove these abundant rRNAs from the mixture.

The MICROB*Express* method is a novel modification to conventional sandwich capture hybridization protocols that were developed for the capture and detection of specific nucleic acid molecules (Morrissey and Collins 1989, Morrissey et al. 1989, Hunsacker et al. 1989). The process employs optimized reagents and conditions to capture and remove essentially all rRNA (16S rRNA, 23S rRNA) from up to 10 µg of purified total RNA.

Figure 1. Hybridization Capture of rRNA



The MICROB*Express* procedure is rapid and simple. Purified RNA is incubated with the Capture Oligonucleotide Mix in Binding Buffer for 15 minutes. Magnetic beads, derivatized with an oligonucleotide that hybridizes to the capture oligonucleotide, are then added to the mixture and allowed to hybridize for 15 minutes. The magnetic beads, with 16S and 23S rRNAs attached, are pulled to the side of the tube with a magnet. The enriched 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 mRNA, tRNA, 5S rRNA, and other small RNAs.

MICROB*Express* is designed so that small RNAs (including tRNA and 5S rRNA) remain in the enriched mRNA population if they were present in the total RNA treated in the procedure. Typically total 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. To remove even more of the small structural RNAs and further enrich for mRNA, an optional glass fiber filter-based purification (e.g., the Ambion MEGAclear™ Purification Kit) can be performed after completing the MICROB*Express* procedures. Glass fiber-based isolation and cleanup prior to and following MICROB*Express* results in the most highly enriched bacterial mRNA.

B. Materials Provided with the Kit

The MICROB*Express* Kit contains material for 20 mRNA purification reactions from up to 10 µg of total RNA.

Amount	Component	Storage
30 µL	Control RNA (1 mg/mL)	–20°C
92 µL	Capture Oligo Mix	–20°C
200 µL	Glycogen (5 mg/mL)	–20°C
805 µL	3 M Sodium Acetate	–20°C
1.15 mL	Oligo MagBeads	4°C
7 mL	Binding Buffer	4°C
2.4 mL	Wash Solution	4°C
1.75 mL	Nuclease-free Water	any temp*
50	1.5 mL tubes	room temp
25	Collection Tubes	room temp

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

C. Materials Not Provided with the Kit

- Total RNA (see *II.A. Input RNA Requirements* on page 4)
- 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

Single Place Magnetic Stand P/N AM10026	Our magnetic stand accommodates 1.5 mL tubes; it is used to capture magnetic beads in solutions.
MICROB*Enrich*™ P/N AM1901	The MICROB*Enrich* Kit employs a novel technology to remove over 90% of mammalian RNA from complex mixtures of host-bacterial RNA samples. If desired the enriched bacterial RNA obtained can enriched for bacterial mRNA using the MICROB*Express* Kit
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 product 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 product catalog	Ambion RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. For more information, see our product catalog at www.invitrogen.com/ambion.
Electrophoresis Reagents see our product catalog	We offer gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. For available products, see our product catalog at www.invitrogen.com/ambion.
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 product 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. we also offer the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.

II. MICROB*Express* Procedure

A. Input RNA Requirements

RNA source organisms compatible with MICROB*Express*

The MICROB*Express* mRNA purification procedure is designed to work with purified total RNA from many different bacteria, including both Gram positive and Gram negative species. The procedure was optimized with total *E. coli* RNA, and has been found to remove 90–99% of the rRNA from the following Gram positive and Gram negative bacterial species:

- *E. coli*
- *Bacillus subtilis*
- *Staphylococcus aureus*
- *Mycobacterium bovis*

As new information on species compatibility becomes available, it is added to a frequently updated list of compatible organisms. For available products, see our product catalog at www.invitrogen.com/ambion.

Use only high quality RNA in the MICROB*Express* procedure

RNA integrity is critically important for the success of the MICROB*Express* procedure, as it is for many downstream applications. Even moderate levels of RNA degradation can lead to inefficient removal of rRNA by the capture reagents. This will result in recovery of mRNA that is not as highly enriched as it could be. Several factors such as bacterial species, cellular RNase activity, growth phase, and RNA isolation method, can all have an effect on RNA quality. Evaluate the integrity of RNA you want to use in the MICROB*Express* procedure by gel electrophoresis or Agilent bioanalyzer (sections III.C and III.D). Verify the integrity of your RNA by comparing the intensity of the 23S and 16S rRNA signals; in high quality RNA samples the 23S rRNA band will be 1.5–2 fold brighter than the 16S rRNA band. We do not recommend using RNA that fails to meet this quality standard with the MICROB*Express* Kit.

Accurate quantitation of input RNA is important

It is extremely important to accurately quantitate RNA so as not to overload the MICROB*Express* system. If more than the recommended 10 µg of total bacterial RNA is used in the MICROB*Express* procedure, rRNA removal will be incomplete. We recommends using a high quality, calibrated spectrophotometer, or the RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes). MICROB*Express* does not remove DNA from RNA. If the input RNA contains DNA contamination, it will contribute to the A_{260} and may interfere with mRNA depletion.

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

The total RNA will be heat denatured at 70°C in step II.B.3 on page 7 to prepare it for hybridization with the Capture Oligo Mix. Degradation of the total 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 6) 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*Express* 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*Express* does ***not*** remove small RNAs (including tRNA and 5S rRNA) from the enriched mRNA population. ***Therefore to maximize mRNA enrichment, use total RNA that does not contain small structural RNAs as starting material for the procedure***. Typically total 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 total 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. Including an optional glass fiber-based purification (e.g. MEGAclear) after the MICROB*Express* procedure will further enrich the mRNA by removing 75% of the residual small RNAs. This will provide an exceptionally clean sample for downstream applications. Please contact our Technical Service Department to discuss any questions you have about options for total RNA isolation and clean-up in relation to the MICROB*Express* procedure.

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 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 two 70% ethanol washes to remove residual salt before starting the MICROB*Express* procedure (see below for instructions).

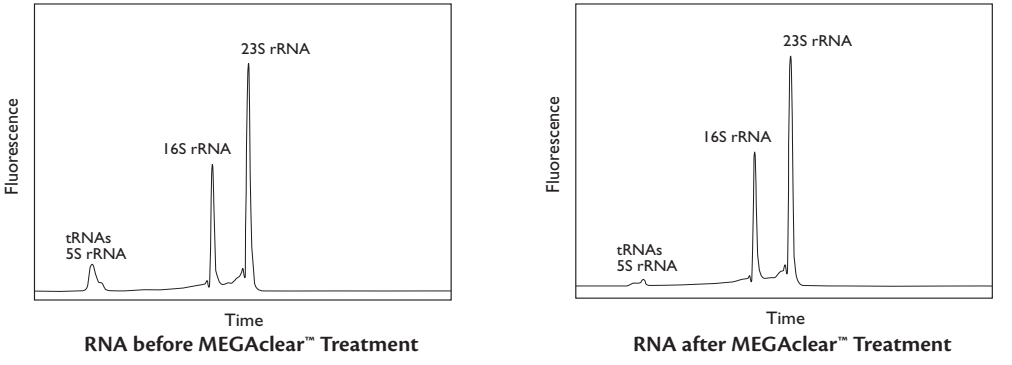


Figure 2. Total *E. coli* RNA and the Same RNA After Further Purification with MEGAclear™.

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.

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

When quantifying the total RNA, 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. We advise that the A_{260}:A_{280} ratio be >1.7. If a total 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 15 µL

The recommended maximum amount of RNA per reaction is 10 µg and the recommended maximum volume is 15 µL.

It is possible that 1 µg or less RNA can be used in MICROB*Express*; we have used as little as 2 µg of starting total RNA per reaction without modifying the reaction conditions.

RNA precipitation instructions

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.

NOTE

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

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. Repeat steps 6 and 7.
9. Dissolve the RNA in ≥15 µL TE (10 mM Tris-HCl pH 8, 1 mM EDTA) or THE RNA Storage Solution.

B. Anneal RNA and Capture Oligonucleotide Mix

The most accurate way to evaluate the mRNA 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 MICROBExpress procedure. (See section III.A on page 12 for more information.)

1. Add 2–10 µg total RNA to 200 µL Binding Buffer

a. Pipet 200 µL Binding Buffer into a 1.5 mL tube provided with the kit.

b. Add total RNA (2–10 µg RNA in a maximum volume of 15 µL) to the Binding Buffer.

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

2. Add 4 µL Capture Oligo Mix

a. Add 4 µL of Capture Oligo Mix to the RNA in Binding Buffer.

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 16S and 23S rRNAs. This heat denaturation helps to facilitate maximal hybridization of the rRNAs to the capture oligonucleotides.

4. Anneal at 37°C for 15 min

The 37°C, 15 min incubation allows the capture oligonucleotides to hybridize to homologous regions of the 16S and 23S 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.


NOTE

Originally we suggested a 1 hr incubation at this step. Further MICROBExpress optimization experiments have shown that nearly complete binding of capture oligonucleotide to rRNAs occurs within 15 min. A 1 hr incubation is still an option, however, it will result in only a very modest increase in rRNA removal.

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 50 µL Oligo MagBeads per sample to a 1.5 mL tube

For each RNA sample, remove 50 µL Oligo MagBeads to a 1.5 mL tube. Oligo MagBeads for up to 10 RNA samples (500 µL) can be processed in a single 1.5 mL tube.


NOTE

Record the volume of Oligo MagBeads withdrawn at this step; 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 8—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 8, recapture the Oligo MagBeads with a magnetic stand, and carefully aspirate and discard the Binding Buffer leaving the beads in the tube.

5. Resuspend the Oligo MagBeads in an equal volume of Binding Buffer, and bring the slurry to 37°C

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

b. Remove the tube from the magnetic stand, and resuspend the beads by gently tapping the tube or very gentle vortexing.

c. Place the Oligo MagBead slurry in a 37°C incubator, and allow the temperature to equilibrate to 37°C before proceeding.

D. Capture the rRNA and Recover the Enriched mRNA

1. Heat the Wash Solution to 37°C

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

2. Add 50 µL prepared Oligo MagBeads to the RNA/Capture Oligo Mix and incubate at 37°C for 15 min

a. Gently vortex the tube of washed and equilibrated Oligo MagBeads from step C.5 to resuspend them, and add 50 µL of Oligo MagBeads to the RNA/Capture Oligo Mix from step B.4 on page 7.

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 oligonucleotide sequence on the Oligo MagBeads anneals to the Capture Oligonucleotides, and the Capture Oligonucleotides remain hybridized to the 16S and 23S rRNAs. The hybridization "sandwich" of Oligo MagBead: Capture Oligonucleotide: rRNA is formed at this step. (This is illustrated in Figure 1 on page 1.)

3. Capture the Oligo MagBeads, and move the mRNA to a Collection Tube

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 mRNA, 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 mRNA 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 in the 37°C Wash Solution.
This wash step recovers mRNAs that were inadvertently trapped in the rRNA:Capture Oligonucleotide hybrids.

c. 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 to the precipitation described in step E.1 (below).

E. Precipitate and Resuspend the Enriched mRNA

1. Ethanol precipitate the enriched mRNA

a. Add the following to the pooled mRNA from (the volume should be ~350 µL), and briefly vortex to mix.
- 1/10th volume 3 M Sodium Acetate (35 µL)
- 1/50th volume Glycogen (5 mg/mL),
  the final concentration will be 100 µg/mL (7 µL)

b. Add 3 volumes ice cold 100% ethanol (1175 µL), 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 mRNA in an appropriate buffer

a. Resuspend the RNA pellet in 25 µL 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).

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 mRNA solution to a new RNase-free tube.

Enriched mRNA yield from 10 µg of high quality total RNA is typically 1–2.5 µg.

4. (optional) Glass fiber filter-based purification

Including an optional glass fiber-based purification of the enriched mRNA (e.g. using the Ambion MEGAclear Kit) will further enrich the mRNA by removing 75% of any residual small RNAs (including tRNA and 5S rRNA).

Follow the instructions provided with your glass fiber-based purification product.

III. Assessing Yield and Quality of Enriched mRNA

A. Evaluating rRNA Depletion

To accurately evaluate the effectiveness of the enrichment procedure, compare MICROB*Express* enriched mRNA with the amount of total RNA from which it was enriched. Evaluate the samples by denaturing agarose gel electrophoresis (section III.C on page 13) or with an Agilent 2100 bioanalyzer (section III.D on page 14).

1. Comparison with a mock MICROB*Express* reaction

The best way to evaluate the effectiveness of rRNA depletion is to perform a mock MICROB*Express* 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*Express* treatment.

Precipitate both RNAs as described in section II.E on page 10, 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*Express* enriched RNA may be compared with the total RNA from which it was enriched—as in the following example:

> A 10 µL total RNA sample is at a concentration of 1 µg/µL. Before the MICROB*Express* procedure, reserve 1 µL of the RNA. The sample now contains 9 µg RNA/9 µL. Perform the MICROB*Express* procedure on the 9 µL RNA sample. Resuspend the reaction product in the original starting volume (9 µL), thus 1 µL of the MICROB*Express* enriched RNA represents the amount of RNA purified from 1 µg of starting RNA. Compare 1 µL of the enriched RNA with 1 µL of the reserved total RNA to evaluate rRNA depletion.

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 1:20 to 1:50 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 10 µg of high quality total RNA is 1–2.5 µg. The RNA is resuspended in 25 µL, thus the concentration will be 40–100 ng/µL. If a glass fiber-based purification is performed after the MICROB*Express* procedure, the mRNA yield will be even lower (<1 µg).

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} = 0.05

RNA concentration = 0.05 X 50 X 40 µg/mL = 100 µg/mL or 100 ng/µL

Since there are 24 µL of the prep remaining after using 1 µL to measure the concentration, the amount of remaining RNA is 24 µL X 100 ng/µL = 2.4 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, We offer 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*Express* reaction was performed, as described in section III.A.1 on page 12, 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 12 for comparison with untreated starting RNA.

In enriched mRNA samples, a smear of mRNA ranging from ~0.5 kb upward may be visible. The 16S and 23S rRNA bands will be absent or very faint (Figure 3). A band corresponding to 5S and tRNAs will be present if the total RNA used in the MICROB*Express* procedure

contained these small RNAs. If the input total RNA was purified by a glass fiber filter method, the band representing the 5S and tRNAs may be faint.

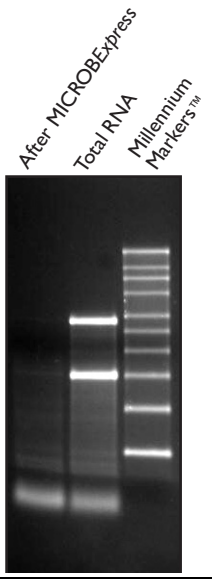


Figure 3. RNA Before and After mRNA Enrichment with the MICROB*Express*™ Kit

16S and 23S rRNAs were removed from 10 µg of total *E. coli* RNA using the MICROB*Express* Kit. A second 10 µg sample of total RNA was subjected to a mock MICROB*Express* procedure in which the Capture Oligo Mix was left out of the reaction (Total RNA). 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*Express* Kit. Follow the instructions for RNA analysis provided with the RNA 6000 Nano LabChip Kit. We have found that this system performs best with 50–250 ng/µL RNA solutions; usually loading 1 µL of a typical enriched RNA sample gives good results.

To compare RNA samples before and after MICROB*Express* mRNA enrichment, follow the recommendations in section *III.A. Evaluating rRNA Depletion* on page 12.

2. Expected Results

In enriched mRNA samples, the 16S and 23S 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 whether these RNAs were

present in the total RNA used in the MICROB*Express* procedure. Typically, mRNA enriched from a prep prepared by a glass fiber filter isolation method will have a smaller 5S and tRNA peak than RNA obtained with other methods. The size and shape of the 5S rRNA/tRNA peak is unchanged by the MICROB*Express* procedure.

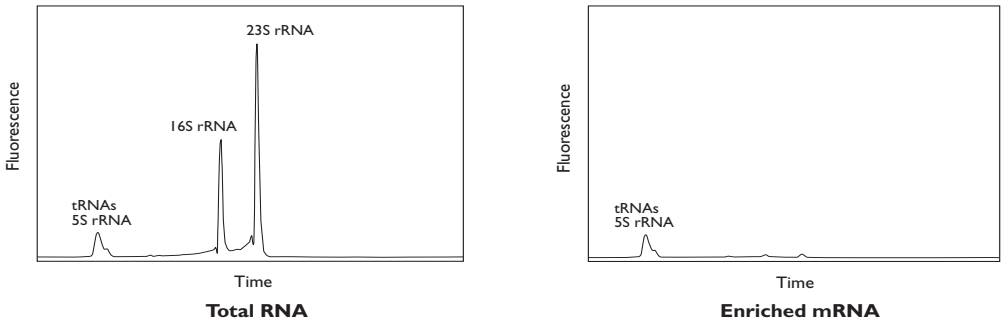


Figure 4. E. coli RNA Before and After mRNA Enrichment with the MICROB*Express*™ Kit.

mRNA was enriched from 10 µg of total RNA from *E. coli* using the MICROB*Express* Kit. The electropherograms above were generated by running 200 ng of the RNA before and after mRNA enrichment on a Agilent 2100 bioanalyzer using the RNA LabChip Kit.

IV. Troubleshooting

A. Positive Control Reaction

Instructions for using the Control RNA

a. Purify mRNA from 10 µL of the Control RNA.
Starting with 10 µL of the Control RNA, follow the MICROB*Express* procedure starting at section II.B, step 1 on page 7. Continue with the mRNA enrichment through section II.E, step 2 on page 10. Resuspend the enriched mRNA in the Nuclease-free Water supplied with the kit. If the 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 tube.

b. Evaluate the enriched mRNA either by agarose gel electrophoresis or with the RNA LabChip Kit on a bioanalyzer.
Measure the A_{260} of the enriched mRNA from the positive control reaction, and calculate its concentration following the instructions in section III.B on page 12.

i. Agarose gel electrophoresis instructions
Run 0.5–1 µg of enriched RNA on a denaturing agarose gel (section III.C on page 13) and compare with mock treated RNA or untreated control RNA as described in section III.A on page 12.

ii. Bioanalyzer instructions
Run 100–150 ng of the enriched RNA using the RNA LabChip Kit on a bioanalyzer following the manufacturer's instructions. Compare enriched RNA with mock treated RNA or untreated control RNA as described in section III.A on page 12.

Expected result of the positive control reaction

The MICROB*Express* procedure should remove at least 90%, and typically removes 95–99% of 16S and 23S rRNAs from the control RNA.

If the control reaction fails, consider repeating it, paying close attention to the instructions. If it fails a second time, contact our Technical Service Department.

B. Low Final Yield of RNA

The mass yield of enriched RNA from 10 µg total RNA is typically 1–2.5 µg and may vary depending on the quality of the input RNA, and whether or not it contained 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.

We recommend using a well calibrated spectrophotometer or the RiboGreen RNA Quantitation Assay and Kit (Molecular Probes). The Control RNA 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-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 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*Express* procedure. (See section *II.A. RNA precipitation instructions* on page 6 for a procedure.)

3. RNA is degraded

See section *IV.D. RNA Degradation* on page 19.

4. Inefficient precipitation of RNA

For optimal RNA recovery precipitate the mRNA as described in step II.E.1 on page 10 using 1/10th volume 3M sodium acetate and 3 volumes of 100% ethanol. Note that the addition of glycogen to the precipitation mixture at a final concentration of 100 µg/µL is essential for complete recovery of nucleic acids from the MICROB*Express* 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. mRNA 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 10). 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.

6. mRNA is very pure after post-MICROB*Express* glass fiber-based purification

MICROB*Express* enriched mRNA samples that have been further purified by glass fiber filter-based purification methods, (e.g. MEGAclear) typically contain nearly pure mRNA. As such, the RNA yield from these samples will be very low, typically less than 1 µg. In order to accurately quantitate these low concentration samples it may be necessary to use spectrophotometer dilutions of 1 in 20, or to use more sensitive RNA quantitation methods, such as RiboGreen assay (Molecular Probes).

C. Ribosomal RNA Contamination

1. Procedure was not followed accurately

Each step of the MICROB*Express* procedure was scrutinized during product development to identify the optimal conditions for removal of rRNA. If there is ribosomal RNA contamination in your enriched mRNA, check to make sure that the procedure was followed 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*Express* enrichment procedure has been carefully optimized for use with 10 µg or less total RNA. If more RNA is used, removal of rRNA will not proceed to completion, thus the initial RNA quantitation is essential for proper functioning of the kit. This is especially important when using RNA isolated with procedures that yield highly intact RNA with low recovery of tRNA and 5S rRNA (e.g. RiboPure-Bacteria). These RNA samples contain proportionately more 16S and 23S rRNA per mass amount than RNA that contains small structural RNAs in addition to rRNA and mRNA.

To verify that your RNA quantitation method is accurate, test it against a standard curve made with dilutions of either the Control RNA provided with the kit or another 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 7. 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 7 and II.B.2 on page 7 are done at the proper temperature and for the full time.

d. Wash conditions are incorrect

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

2. Carryover of excess Oligo MagBeads

With relatively weak magnets, it is common to have some magnetic bead contamination in the resuspended enriched mRNA sample. ***Oligo MagBeads will carry contaminating rRNA and can potentially affect downstream applications.*** Be careful not to dislodge Oligo MagBeads 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 mRNA appears brownish in color, put the sample in the magnetic stand for at least 2 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. Bacterial species incompatible with MICROB*Express*

Although the MICROB*Express* procedure should perform well with most Eubacterial RNA, the rRNA from some species will not be effectively removed with the kit reagents. This may be due to interspecific or rRNA operon specific polymorphisms within the Capture Oligo binding sites. If the control reaction works and all troubleshooting options are ineffective, contact Technical Service for recommendations.

D. RNA Degradation

NOTE

It is difficult to identify degraded enriched mRNA since high quality mRNA appears as a smear when run on a gel.

1. Input total RNA is degraded

To see whether the RNA used in the MICROB*Express* procedure was degraded from the start, evaluate a 0.5–5 µg sample of the input total RNA on a denaturing agarose gel (see section III.C starting on page 13). Total RNA should produce rRNA bands that appear sharp and well-defined (Figure 3 on page 14). In high quality RNA samples, the 23S rRNA band will be 1.5–2 fold brighter than the 16S 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, go to www.invitrogen.com/site/us/en/home/support/technical-support.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 7).

E. Poor Performance of Enriched RNA in Downstream Applications

1. Residual salt remaining in RNA preparation

High salt concentration in the enriched mRNA sample may negatively affect downstream applications. Follow the precipitation procedure in step II.E.1 on page 10 exactly. Include two 70% ethanol washes for consistent performance of the RNA in applications such as cDNA labeling. An optional glass fiber filter-based purification can be performed 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 11, remove any remaining Oligo MagBeads from the enriched mRNA by placing the tube on the magnetic stand for at least 2 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 19.

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 mRNA. Excess carryover of Oligo MagBeads may also cause a low A_{260}:A_{280} ratio. See *IV.C. Ribosomal RNA Contamination* on page 18 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 is used in a MICROB*Express* mRNA purification experiment. The procedure is shown to remove greater than 95% of 16S and 23S rRNA from 10 µg of Control RNA.

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

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

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

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.*

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

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

B. Obtaining support

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

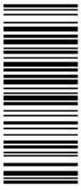
www.invitrogen.com

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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