Demonstrated Protocol: Bacterial Ribosomal RNA (rRNA) Depletion Workflow for RNA-Seq

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Overview

This demonstrated protocol uses the RiboMinus™ Pan-Prokaryote Probe Mix to selectively and efficiently capture and remove the highly abundant 5S, 16S, and 23S ribosomal RNA (rRNA) species from total RNA isolated from a spectrum of bacterial organisms. The resulting rRNA-depleted RNA can then be used in a variety of downstream applications, including the generation of whole transcriptome RNA-Seq libraries for sequencing with Ion GeneStudio™ S5 Systems.

Demonstrated protocols have been successfully performed by Thermo Fisher Scientific research and development (Fig. 1) but are not formally verified. There are no technical specifications for demonstrated protocols. Users assume all risk when using these protocols.

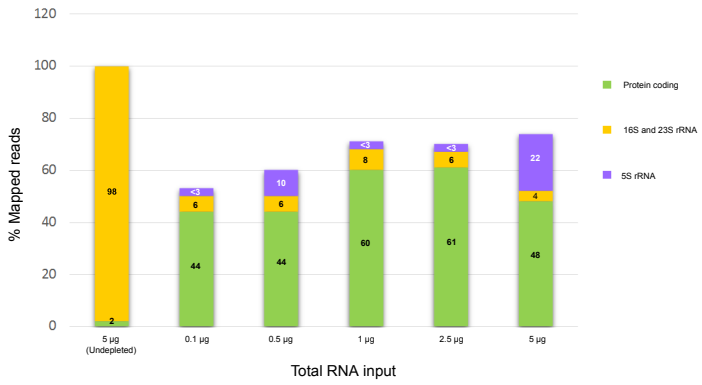


Fig. 1 ***E. coli*** **rRNA depletion performance with RiboMinus™ Pan-Prokaryote Probe Mix**

Required materials

Unless otherwise indicated, materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier..

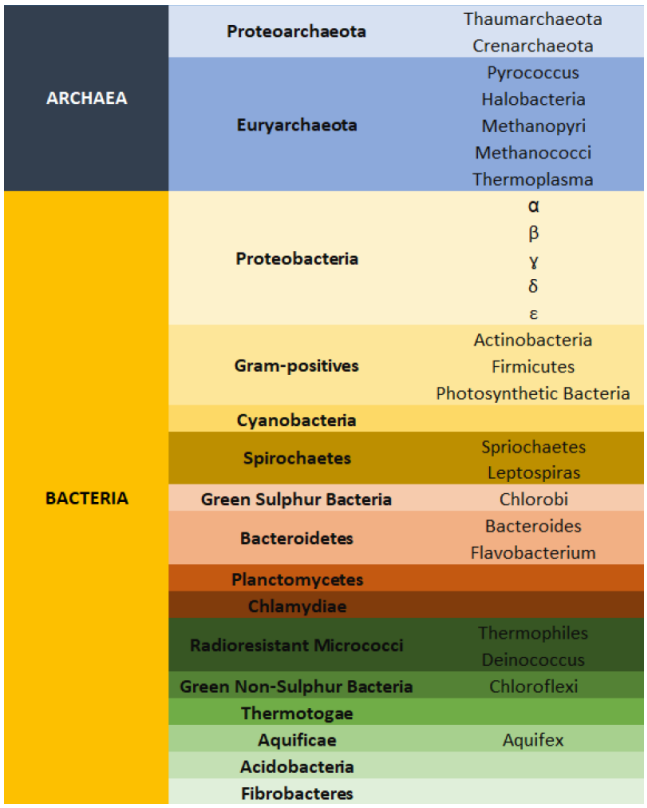
Item description	Cat. No.
RiboMinus™ Eukaryote System v2 *or* RiboMinus™ Eukaryote Kit v2	A15026 A15020
RiboMinus™ Pan-Prokaryote Probe Mix[1]	—
Non-stick RNase free Microentrifuge Tubes (1.5-mL and 2-mL)	MLS
DynaMag™-2 Magnet	12321D
Nuclease-free Water	AM9938
100% ethanol	MLS

[1] See "Obtaining the RiboMinus™ Pan-Prokaryote Probe Mix".

Obtaining the RiboMinus™ Pan-Prokaryote Probe Mix

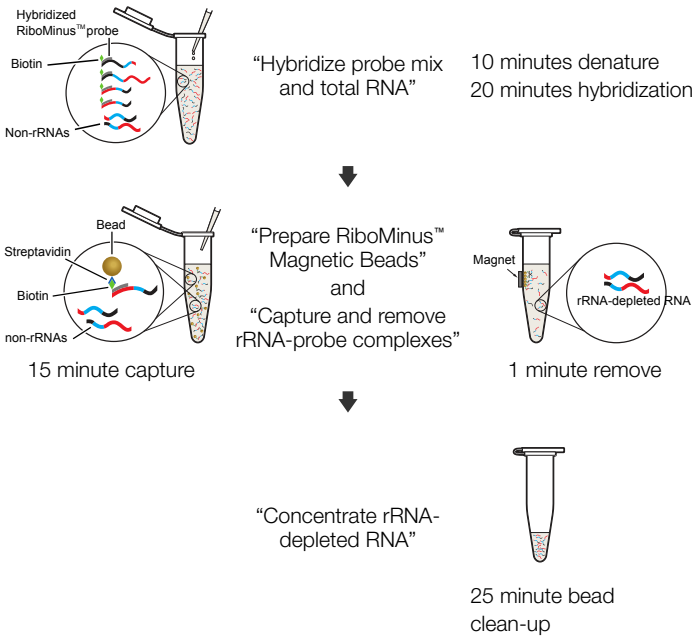
Send requests for the RiboMinus™ Pan-Prokaryote Probe Mix to **techsupport@thermofisher.com** or your field application specialist (FAS). Include your name, institution, and mailing address to receive Pan-Prokaryote probes for 5S, 16S, and 23S rRNA in a ready-to-use pool sufficient for 12 depletion reactions by mail.

Species compatible with the RiboMinus™ Pan-Prokaryote Probe Mix	
Acinetobacter baumannii	*Moorella thermoacetica*
Campylobacter jejuni	*Pseudomonas putida*
Clostridioides difficilereplace	*Salmonella typhimurium*
Escherichia coli	*Staphylococcus aureus*
Fusobacterium nucleatum	*Thermoanaerobacter kivui*
Helicobacter pylori	*Vibro cholerae*
Janthinobacterium sp.	



The RiboMinus™ Pan-Prokaryote Probe Mix includes 3' biotinylated DNA oligos designed to target 5S, 16S, and 23S rRNA at the phyla level of conserved regions of 76 pan-prokaryote species.

Workflow



Protocol for depletion of ribosomal RNA from total RNA samples

This protocol describes how to deplete of ribosomal RNA from 100–5,000 ng of total RNA samples using the RiboMinus™ Pan-Prokaryote Probe Mix with modules from the RiboMinus™ Eukaryote System.

Prepare 1X Hybridization Buffer

1. Pre-heat 2X Hybridization Buffer at 50°C for at least ~15 minutes with a heat block or water bath to bring any precipitated salts into solution.
2. Prepare 200 µL of 1X Hybridization Buffer for each RNA sample by diluting 2X Hybridization Buffer with an equal volume of nuclease-free water.

Hybridize probe mix and total RNA

1. Mix the components in the order listed in the following table in a sterile PCR strip tube or 96-well plate.

Component	Volume
2X Hybridization Buffer	50 µL
RiboMinus™ Pan-Prokaryote Probe Mix	1 or 3 µL [1]
Total RNA (100–5,000 ng)	*x* µL
Nuclease-free water	up to 100 µL

[1] Use 1 µL of RiboMinus™ Pan-Prokaryote Probe Mix for samples with 100 ng to <1,000 ng total RNA input, or 3 µL for samples with 1,000–5,000 ng total RNA input.

2. Mix by gentle vortexing, then centrifuge briefly.
3. Incubate the plate/strip in a thermal cycler using the following conditions.

   Note: Set ramp rate at 100%, and heated lid to default at 105°C.

Step	Temperature	Time
Denaturation	10 minutes	70°C
Hybridization	20 minutes	37°C

   Note: Do not place samples in cold water. Do not allow samples to cool to room temperature.
4. During incubation for hybridization, proceed to "Prepare RiboMinus™ Magnetic Beads".

After the hybridization step is complete, leave the reactions at 37°C, until ready to add to sample to prepared RiboMinus™ Magnetic Beads.

Prepare RiboMinus™ Magnetic Beads

1. Resuspend the bottle (blue cap) of RiboMinus™ Magnetic Beads thoroughly by vortexing.
2. For each sample, add 500 µL of bead suspension into a sterile, RNase-free 1.5-mL microcentrifuge tube.
3. Place the tubes with the bead suspension on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
4. Remove the tubes from magnetic stand.
5. Wash the beads with 500 µL nuclease-free water by dispensing the water down the side of the tube where the beads are collected.
6. Place the tubes on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
7. Repeat steps 5–7 one more time.
8. Resuspend the beads in 200 µL of 1X Hybridization Buffer.
9. Place the prepared beads in a 37°C heat block for at least 5 minutes. The beads can stay at 37°C until ready to use with the hybridized RNA/probe mix.

Capture and remove rRNA-probe complexes

1. Briefly centrifuge the RNA/probe mix in the PCR plate/strip tube to collect the contents at the bottom.
2. Transfer each RNA/probe mix (100 µL) sample to a tube containing prepared RiboMinus™ Magnetic Beads (200 µL). Mix well by pipetting or by low speed vortexing.
3. Incubate tubes at 37°C for 15 minutes using a heat block or water bath.
4. Centrifuge the tubes briefly, then place the tubes on a magnetic stand for 1 minute or until the solution clears.
5. Transfer the supernatant (300 µL) containing the rRNA-depleted RNA to a new tube.

Concentrate rRNA-depleted RNA

1. Mix the components in the order listed in the following table in a sterile , RNase-free 2-mL microcentrifuge tube.

Component	Volume
Nucleic Acid Binding Beads (white cap)	10 µL
Binding Solution Concentrate	400 µL

2. Mix thoroughly by pipetting up and down.
3. Add the entire volume (300 µL) of bead supernatant containing the rRNA-depleted RNA to the microcentrifuge tube, then mix thoroughly by pipetting up and down.
4. Add 1 mL of 100% ethanol to the microcentrifuge tube. Ensure the cap is securely closed, then mix well by inverting the tube.

   Note: The mixture should look homogeneous at this point.
5. Incubate at room temperature for 5 minutes. If any sample is retained in the cap, centrifuge the tube briefly to collect the contents at the bottom before proceeding.
6. Place tube onto a magnetic stand for 3–5 minutes or until the solution clears. Aspirate and discard the supernatant without disturbing the beads.
7. Remove the tube from the stand, and wash the beads by dispensing 300 µL of prepared Wash Solution down the side of the tube where the beads are collected.
8. Replace the tube on the magnetic stand, allow solution to clear. Aspirate and discard the supernatant. Carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the bead pellet.
9. Keep the tube on magnetic stand for 2 minutes to allow the beads to air dry, then remove the tube from the stand.

10. Add 12–50 µL of pre-heated (70°C) nuclease-free water to the beads and incubate for 1 minute at room temperature to elute the RNA.

    Note: Use an elution volume suitable for your downstream application.

11. Place the tube on the magnetic stand and carefully collect the supernatant in a new microcentrifuge tube.

IMPORTANT! The supernatant contains the eluted rRNA-depleted RNA (RiboMinus™ RNA).

Analysis of rRNA-depleted RNA (RiboMinus™ RNA)

- The purified RiboMinus™ RNA is easily quantitated using UV absorbance at 260 nm or with the Qubit™ Fluorometric Quantitation Platform along with the Qubit™ RNA Assay Kit. Expect 3–6% recovery of your total RNA starting input.
- To verify the rRNA depletion, use a Bioanalyzer™ assay or perform agarose gel electrophoresis on the sample. The efficiency for RNA depletion in RiboMinus™ RNA, RNA degradation, and RNA concentration can be effectively analyzed using the Agilent™ 2100 Bioanalyzer™ with the Agilent™ RNA 6000 Pico Kit or the RNA 6000 Nano Kit (see Figure 2, Figure 3).
- Analysis for low-input applications is optional (these are samples starting with 100 ng to <1000 ng input of total RNA into the RiboMinus™ protocol). Where materials are limiting for low input applications, it is recommended that all the RiboMinus™ RNA be used for your downstream application.

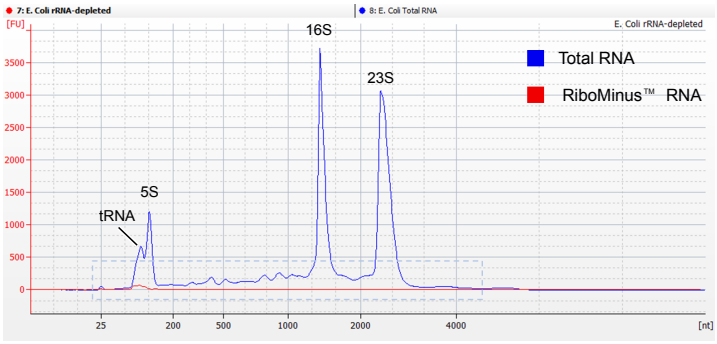


Fig. 2 Agilent™RNA 6000 Pico Assay shows an overlay of RiboMinus™ RNA with *E. coli* total RNA. The RiboMinus™ RNA sample was diluted 1:5 and 1 µL was ran along with 1 µL of a 1:5 dilution of *E. coli* total RNA Samples. 5 µg total RNA input into the depletion reaction.

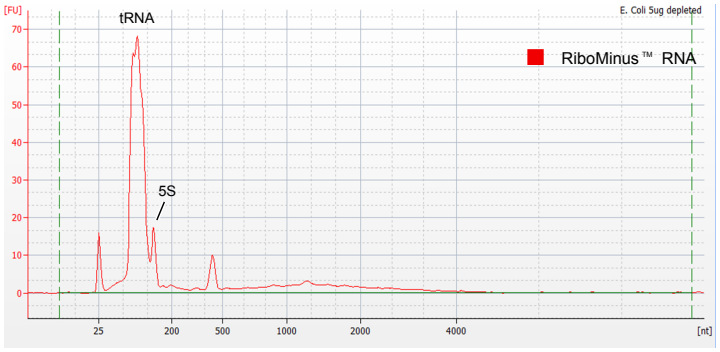


Fig. 3 Agilent™RNA 6000 Pico Assay detailed trace of RiboMinus™ RNA shows absence of rRNA peaks.

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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