First Day: Ribo-Zero rRNA Removal

RNA Input Recommendations

The Ribo-Zero rRNA Removal Kit supports rRNA depletion from 1–5 µg total RNA samples. For the Epidemiology kit, input of 500 ng–2.5 µg and total RNA samples is supported.

DNA-Free RNA

The RNA samples must be free of contaminating DNA, which can cause inaccurate RNA quantification, interfere with rRNA removal, and negatively affect library prep and sequencing.

Before beginning the Ribo-Zero protocol, treat the sample with Baseline-ZERO DNase and then purify the treated RNA.

RNA Quantification

Use a fluorometric method to quantify the amount of total RNA in the sample. Accurate RNA quantification is necessary for determining the volume of removal solution and the maximum volume in which the total RNA sample can be dissolved when preparing to treat it with removal solution.

Dilute the sample in molecular biology-grade water. Do not exceed the recommended input amount of total RNA, which is:

- 5 µg for most Ribo-Zero kits.
- 2.5 µg for the Ribo-Zero (Epidemiology) kit.

Compare rRNA content before and after a Ribo-Zero rRNA Removal Kit reaction to assess the depletion of rRNA in total RNA. The success of rRNA removal depends on factors such as species, sample quality, and sequence homology between the rRNA sample and removal solution, especially with 5S rRNA removal. Use the RNAMatchMaker at epibio.com/rnamatchmaker to verify sample and kit sequence homology sequence homology.

FFPE-RNA Samples

You can use RNA extracted from highly degraded total RNA, such as that obtained from formalin-fixed paraffin-embedded (FFPE), tissue with Ribo-Zero kits. However, the quality of FFPE RNA is highly variable due to the tissue-fixation procedure, sample age, storage conditions, fixation reversal process, and other issues. Therefore, Illumina® cannot guarantee success with every FFPE RNA sample.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Core Kit Temperature

- ***Do not freeze the core kit or put the contents on ice.*** Freezing the magnetic beads damages them, decreasing kit performance.
- Always use the magnetic beads at room temperature. Store at 2°C to 8°C when not in use.

Automation

- Illumina supports Illumina liquid handling robotic platforms and protocols only. Contact the manufacturer of your liquid handling instrument for hardware, software, protocol, and technical support.
- When using an automated liquid handling instrument with magnetic beads, do not use conductive tips.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each tube***.

Capping the Tubes

- Always cap the tubes before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps

Tube Transfers

- When transferring volumes between tube strips, transfer the specified volume from each tube of a strip to the corresponding tube of the other strip.
- If beads are aspirated into the pipette tips, dispense the sample back into the tube on the magnetic stand and wait until the liquid is clear (~2 minutes).

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the tube, and to prevent sample loss.

RNA Removal Worfklow

The following figure illustrates the Ribo-Zero workflow. Safe stopping points are marked between steps.

Figure 1 Ribo-Zero Workflow

Wash Magnetic Beads

Wash the magnetic beads using 1 of 2 methods:

- Individual washing for 6 or fewer samples, as described in *Individual Washing* on page 9.
- Batch washing for 7 or more samples, as described in *Batch Washing* on page 10.

Individual Washing

Consumables

- 1.5 ml RNase-free microcentrifuge tubes with caps
- Magnetic beads (225 µl per sample)
- RNase-free water (225 µl per sample)
- Magnetic Bead Resuspension Solution (65 µl per sample)
- [Optional] RiboGuard RNase Inhibitor (1 µl per sample)

About Reagents

- Dispense magnetic beads slowly to avoid air bubbles.
- RiboGuard RNase Inhibitor is recommended to prevent RNA degradation from RNase contaminants.

Preparation

1 Prepare the following consumables.

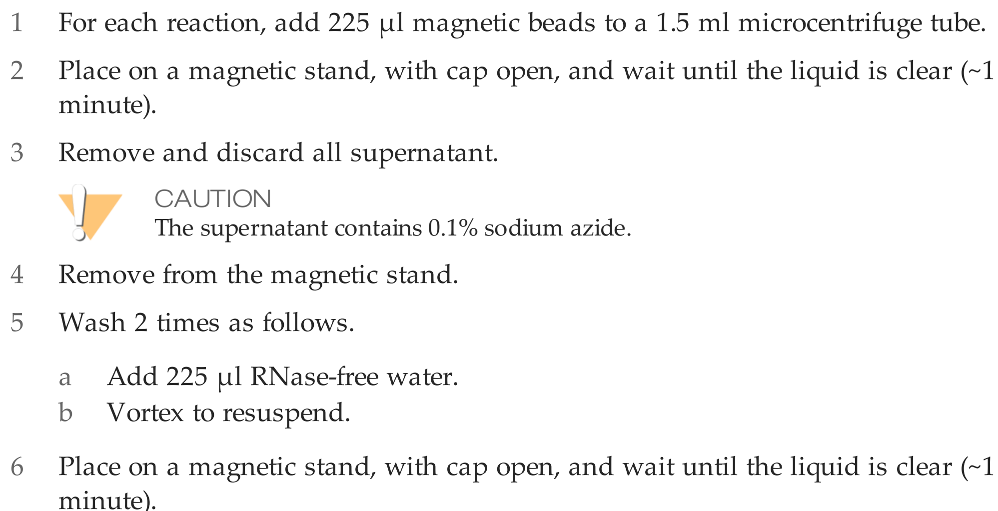
Item	Storage	Instructions
Magnetic beads	2°C to 8°C	Bring to room temperature. Vortex to mix.
Magnetic Bead Resuspension Solution	2°C to 8°C	Bring to room temperature.
RNase-free water	2°C to 8°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
[Optional] RiboGuard RNase Inhibitor	-85°C to -65°C	Thaw on ice. Invert to mix, and then centrifuge briefly.

Procedure

1 For each reaction, add 225 µl magnetic beads to a 1.5 ml microcentrifuge tube.

2 Place on a magnetic stand, with cap open, and wait until the liquid is clear (~1 minute).

3 Remove and discard all supernatant.



CAUTION
The supernatant contains 0.1% sodium azide.

4 Remove from the magnetic stand.

5 Wash 2 times as follows.

a Add 225 µl RNase-free water.
b Vortex to resuspend.

6 Place on a magnetic stand, with cap open, and wait until the liquid is clear (~1 minute).

7 Remove and discard all supernatant.

8 Remove from the magnetic stand.

9 Add 65 µl Magnetic Bead Resuspension Solution.

10 Vortex to resuspend.

11 [Optional] Add 1 µl RiboGuard RNase Inhibitor, and then pipette to mix.

12 Set aside at room temperature.

Hybridize Probes

In this step, probes in the removal solution hybridize to rRNA present in the sample. Before probe hybridization, the sample must be purified and free of gDNA contamination.

Consumables

- 0.2 ml or 0.5 ml RNase-free microcentrifuge tubes with caps
- RNase-free water
- Ribo-Zero Reaction Buffer (4 µl per sample)
- Ribo-Zero Removal Solution (8–10 µl per sample)

About Reagents

- Thorough mixing is important at this step.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
Ribo-Zero Reaction Buffer	-85°C to -65°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
Ribo-Zero Removal Solution	-85°C to -65°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
RNase-free water	2°C to 8°C	Thaw on ice. Invert to mix, and then centrifuge briefly.

2 Set a heat block or thermal cycler to 68°C.

Procedure

1 Use the following table to determine the appropriate volumes of RNA sample and Ribo-Zero Removal Solution for use in step 2.

Table 1 RNA and Reagent Volumes

Total RNA Input for Epidemiology Kit	Total RNA Input for Other Ribo-Zero Kits	Max Total RNA per Sample	Removal Solution per Sample
500 ng to 1.25 µg	1–2.5 µg	28 µl	8 µl
> 1.25–2.5 µg	> 2.5–5 µg	26 µl	10 µl

2 For each sample, combine the following volumes in a 0.2 ml or 0.5 ml microcentrifuge tube. Pipette to mix.
   - RNase-free water (x µl)
   - Ribo-Zero Reaction Buffer (4 µl)
   - RNA sample (y µl)
   - Ribo-Zero Removal Solution (8–10 µl)

   The total volume per sample is 40 µl.

3 Place on the preheated heat block or thermal cycler and incubate for 10 minutes.

4 Remove from heat, and then centrifuge briefly.

5 Incubate at room temperature for 5 minutes.

Remove rRNA

This step combines the probe-hybridized samples with the washed magnetic beads, which bind to the probes. The recovered RNA sample is depleted of rRNA.

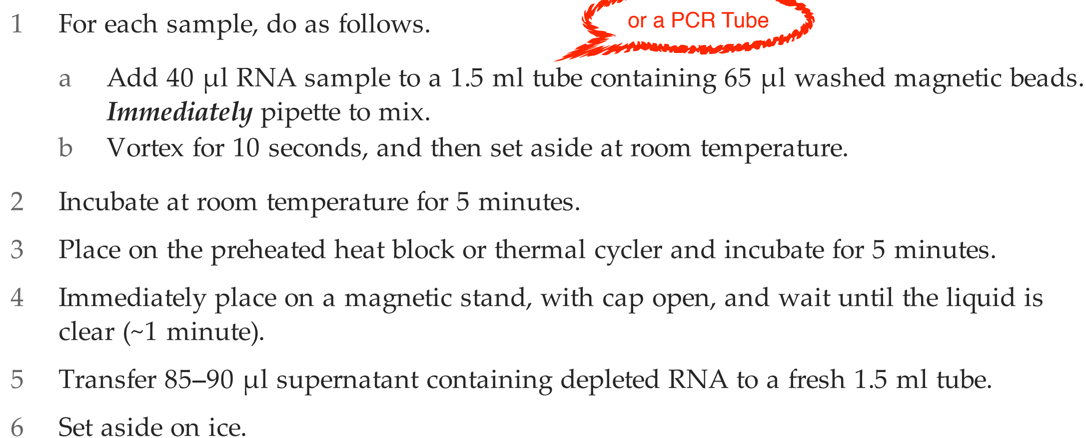
Consumables

- 1.5 ml RNase-free microcentrifuge tubes with caps

Preparation

1 Set a heat block or thermal cycler to 50°C.

Procedure



1 For each sample, do as follows.
   a Add 40 µl RNA sample to a 1.5 ml tube containing 65 µl washed magnetic beads. ***Immediately*** pipette to mix.
   b Vortex for 10 seconds, and then set aside at room temperature.

2 Incubate at room temperature for 5 minutes.

3 Place on the preheated heat block or thermal cycler and incubate for 5 minutes.

4 Immediately place on a magnetic stand, with cap open, and wait until the liquid is clear (~1 minute).

5 Transfer 85–90 µl supernatant containing depleted RNA to a fresh 1.5 ml tube.

6 Set aside on ice.

SAFE STOPPING POINT

If you are stopping, cap the tubes and store at -25°C to -15°C overnight or at -85°C to -65°C for up to 30 days.

First Day: Clean up Depleted RNA with RNA Clean & Concentrator-5 Kit

Make sure guidelines are followed to ensure the RNA isolation procedure is performed in an RNase-free environment.

Buffer Preparation

Before starting, add 48 ml 100% ethanol (52 ml 95% ethanol) to the 12 ml **RNA Wash Buffer** concentrate (R1013, R1015) or 96 ml 100% ethanol (104 ml of 95% ethanol) to the 24 ml **RNA Wash Buffer** concentrate (R1014, R1016).

Protocol

All centrifugation steps should be performed at 10,000 – 16,000 *x g.*
RNA species ≥17 nt will be recovered.
For DNA-free RNA, perform DNase I treatment prior or during the clean-up protocol (page 4).

1. Add 2 volumes **RNA Binding Buffer** to each sample[1] and mix.

   Example: Mix 100 µl buffer and 50 µl sample.

2. Add an equal volume of ethanol (95-100%) and mix.

   Example: Add 150 µl ethanol.

3. Transfer the sample[2] to the **Zymo-Spin™ IC Column** in a **Collection Tube** and centrifuge for 30 seconds. Discard the flow-through.
4. Add 400 µl **RNA Prep Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
5. Add 700 µl **RNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
6. Add 400 µl **RNA Wash Buffer** to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube (not provided).
7. Add 15 µl **DNase/RNase-Free Water** directly to the column matrix and centrifuge for 30 seconds.

   Alternatively, for highly concentrated RNA use ≥6 µl elution.

   The eluted RNA can be used immediately or stored at -70°C.

Notes:

[1] Adjust the sample volume to 50 µl (minimum).

[2] To process samples >800 µl, **Zymo-Spin™** columns may be reloaded.