Protocol for ribosomal RNA depletion with riboPOOLs (probes)

Materials provided:

- riboPOOL (freeze-dried)
- Nuclease-free water

Product description:

riboPOOLs efficiently remove abundant ribosomal RNAs (rRNAs) from total or fragmented RNA prior to downstream analysis by Next-Generation Sequencing or other methods.

riboPOOLs are provided either as probes or in a complete kit. ***This protocol is for the use of riboPOOL probes alone with reagents from other commercial vendors (as listed below).*** For riboPOOL kits, please refer to our riboPOOL Kit manual provided.

riboPOOLs are available for diverse species and can be tailor-made towards ribosomal RNA or other abundant RNAs from any species. Combination riboPOOLs are also available for rRNA depletion from samples containing multiple species (e.g. for metatranscriptomics or pathogen-infected tissue). riboPOOLs consist of complex mixtures of single-stranded 3'-biotinylated DNA probes designed to specifically hybridize with cytoplasmic and mitochondrial rRNAs, enabling their removal with streptavidin-conjugated magnetic beads. The workflow with riboPOOLs can be completed in ~70 min (with one-step depletion and silica column clean-up), is enzyme-free, and compatible with high-throughput automation. As it does not rely on polyA-selection, it can be used to detect non-polyadenylated RNA including non-coding RNA, histones and prokaryotic RNA with uniform transcript coverage.

Additional materials and equipment required for rRNA depletion protocol with riboPOOL probes (not provided):

- Streptavidin-coated magnetic beads, such as:
  - Dynabeads MyOne Streptavidin C1 from Thermo Fisher (#65001) *or*
  - Hydrophilic Streptavidin Magnetic Beads from New England Biolabs, NEB (#S1421S)
- Sterile, low-binding 1.5 ml or 2 ml tubes and low-retention tips for minimal surface binding of RNA and beads
- Magnetic tube rack
- Temperature-controlled mixer or thermal cycler
- RNase inhibitor (optional)
- Common laboratory equipment – benchtop centrifuge, vortex, pipettes
- Personal protection equipment – lab coat, gloves

Buffers (not provided):

All buffers should be made in DEPC-treated or nuclease-free water.

Buffer Name	Components	Minimum volume required per sample (µl)	Volume for 20 reactions (µl)
Hybridization Buffer (HB)	10 mM Tris-HCl (pH 7.5) 1 mM EDTA 2 M NaCl	5.5	110
Depletion Buffer (DB)	10 mM Tris-HCl (pH 7.5) 1 mM EDTA 1 M NaCl	286	5720
*Bead Resuspension Buffer	0.1 M NaOH 0.05 M NaCl	220	4400
*Bead Wash Buffer	0.1 M NaCl	110	2200

*These buffers are only required when using Dynabeads from Thermo Fisher.

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Additional notes:

- riboPOOLs work best for high quality RNA samples though can also be used for degraded samples.
- RNA input amount may range from 100 ng to 5 µg.
- RNA input should be DNA-free.
- Due to abundance of rRNA, expect to lose ≥ 90% of initial input. *Expected yield for 1 µg of input RNA is < 80 ng.*
- During protocol, avoid leaving tubes with RNA at room temperature over an extended period of time.
- To agitate beads, flick tube gently till solution becomes homogenous. Alternatively, vortex tube at medium speed.
- Take necessary precautions to avoid RNase contamination.

Protocol:

1. ***Resuspension of riboPOOL***
   a. Centrifuge tube containing riboPOOL at 11 000 *x g* for 30s before opening.
   b. Resuspend riboPOOL in nuclease-free water provided to a final concentration of 100 µM (e.g. 50 µl for 5 nmol).

2. ***Hybridization of riboPOOL to RNA***
   a. To 14 µl of RNA sample (100 ng - 5 µg of total RNA), add:
   If sample volume is > 14 µl, adjust HB volume accordingly to 0.25X total volume. Total volume however should not exceed 40 µl.
      i. 1 µl of resuspended riboPOOL
      ii. 5 µl of **Hybridization Buffer**
      iii. RNase inhibitor (optional)
      Follow manufacturer's instructions for volume required and ensure enzyme is active at 68°C. RNase inhibitor may also be introduced during bead preparation.
   b. Vortex well and spin down droplets.
   c. Incubate at 68°C for 10 min to denature RNA.
   d. Allow to **cool slowly** from 68°C to 37°C for optimal hybridization.
   To do this, shut off the heating block and let temperature fall naturally to 37°C. If using temperature-controlled ramping, cool at 3°C/min.

3. ***Preparation of beads***

Ribosomal RNA depletion with riboPOOLs can either be performed in two steps to increase efficiency with less beads, or in one step to save time but with double the volume of beads. To compare results, refer to riboPOOL Application Note. Please follow the respective workflows according to your preference for a one-step or two-step depletion.

One-step depletion

If using Dynabeads MyOne Streptavidin C1, Thermo Fisher #65001:

a. Resuspend the beads by carefully vortexing tube at medium speed.
b. Transfer **160 µl** of bead suspension (10 mg/ml) per sample into a fresh tube.
*To prepare multiple samples, aliquot bead suspension for up to 6 samples (i.e. **960 µl**) in a single tube.*
c. Place tube on magnetic rack and wait for 1 min.
Beads may stick to sides of tube making solution appear brown but aspirated solution should be clear.

Two-step depletion

If using Dynabeads MyOne Streptavidin C1, Thermo Fisher #65001:

a. Resuspend the beads by carefully vortexing tube at medium speed.
b. Transfer **80 µl** of bead suspension (10 mg/ml) per sample into a fresh tube.
*To prepare multiple samples, aliquot bead suspension for up to 6 samples (i.e. **480 µl**) in a single tube.*
c. Place tube on magnetic rack and wait for 1 min.
Beads may stick to sides of tube making solution appear brown but aspirated solution should be clear.

d. Aspirate and discard all supernatant.

e. Add 100 µl per sample (i.e. 600 µl for 6 samples) of **Bead Resuspension Buffer** and agitate the tube well to resuspend beads. Place on magnetic rack (1 min), aspirate and discard supernatant.

f. Repeat step 3e.

g. Add 100 µl per sample of **Bead Wash Buffer** to resuspend beads. Place on magnetic rack (1 min), aspirate and discard supernatant.

h. Resuspend beads in **80 µl** per sample (i.e. **480 µl** for 6 samples) of **Depletion Buffer**.

If using Hydrophilic Streptavidin Magnetic Beads, NEB #S1421S:

a. Resuspend the beads by carefully vortexing tube at medium speed.

b. Transfer **600 µl** of bead suspension (4 mg/ml) per sample into a fresh tube.
*To prepare multiple samples, aliquot bead suspension for up to **3** samples (i.e. 1800 µl) in a single 2 ml tube.*

c. Place tube on magnetic rack (1 min), aspirate and discard supernatant.

d. Add 100 µl per sample (i.e. 600 µl for 6 samples) of **Depletion Buffer** and agitate the tube well to resuspend beads.

e. Place on magnetic rack (1 min), aspirate and discard supernatant.

f. Resuspend beads in **80 µl** per sample (i.e. **480 µl** for 6 samples) of **Depletion Buffer**.

d. Aspirate and discard all supernatant.

e. Add 100 µl per sample (i.e. 600 µl for 6 samples) of **Bead Resuspension Buffer** and agitate the tube well to resuspend beads. Place on magnetic rack (1 min), aspirate and discard supernatant.

f. Repeat step 3e.

g. Add 100 µl per sample of **Bead Wash Buffer** to resuspend beads. Place on magnetic rack (1 min), aspirate and discard supernatant.

h. Resuspend beads in **160 µl** per sample (i.e. **960 µl** 6 samples) of **Depletion Buffer**.

i. For each sample, prepare two tubes, each containing 80 µl of beads from step 3h to use in two depletion steps. Leave the beads at room temperature until use.

If using Hydrophilic Streptavidin Magnetic Beads, NEB #S1421S:

a. Resuspend the beads by carefully vortexing tube at medium speed.

b. Transfer **300 µl** of bead suspension (4 mg/ml) per sample into a fresh tube.
*To prepare multiple samples, aliquot bead suspension for up to **6** samples (i.e. 1800 µl) in a single 2 ml tube.*

c. Place tube on magnetic rack (1 min), aspirate and discard supernatant.

d. Add 100 µl per sample (i.e. 600 µl for 6 samples) of **Depletion Buffer** and agitate the tube well to resuspend beads.

e. Place on magnetic rack (1 min), aspirate and discard supernatant.

f. Resuspend beads in **160 µl** per sample (i.e. **960 µl** for 6 samples) of **Depletion Buffer**.

g. For each sample, prepare two tubes, each containing 80 µl of beads from step 3f to use in two depletion steps. Leave the beads at room temperature until use.

4. Ribosomal RNA depletion

<u>One-step depletion</u>

a. Briefly centrifuge the tube containing ~20 µl hybridized riboPOOL and total RNA (from step 2) to spin down droplets.

b. Combine 80 µl of prepared beads (from step 3f) with ~20 µl of the hybridized riboPOOL-RNA solution. Agitate the tube to resuspend well.

<u>Two-step depletion</u>

a. Briefly centrifuge the tube containing ~20 µl hybridized riboPOOL and total RNA (from step 2) to spin down droplets.

b. Combine 80 µl of prepared beads (from step 3g) with ~20 µl of the hybridized riboPOOL-RNA solution. Agitate the tube to resuspend well.

c. Incubate at **37°C for 15 min**, followed by a **50°C incubation for 5 min**.

d. Briefly spin down droplets.

e. Place on magnetic rack for 2 min. Carefully transfer the supernatant into a new tube.

f. Place the new tube with the supernatant on the magnetic rack for 1 min to get rid of trace amounts of beads. (optional)

g. Carefully transfer the supernatant to a new tube (optional).

Steps f and g are recommended to remove any trace amount of beads but can be left out if desired.

c. Incubate at **37°C for 15 min**, followed by a **50°C incubation for 5 min**.

d. Immediately before use, take second tube containing 80 µl of beads from step 3g for a second depletion. Place on magnetic rack (1 min), aspirate and discard supernatant.

e. Briefly spin down droplets from first depletion reaction (4c). Place on magnetic rack for 2 min. Carefully transfer the supernatant to new tube containing the remaining beads (4d) for the second depletion.

f. Incubate once more at **37°C for 15 min**, followed by a **50°C incubation for 5 min**.

g. Briefly spin down droplets. Place on magnet for 2 min. Carefully transfer the supernatant to a new tube.

h. Place the new tube with the supernatant on the magnetic rack for 1 min to get rid of trace amounts of beads. (optional)

i. Carefully transfer the supernatant to a new tube (optional).

Steps h and i are recommended to remove any trace amount of beads but can be left out if desired.

At this point, RNA can be stored at -20°C overnight or -80°C for up to a month.

RNA Clean-up:

RNA samples that have been subject to rRNA depletion **must** be purified before sequencing library preparation to remove salts and buffer concentrates. The following clean-up methods have been successfully applied with riboPOOLs. As some clean-up methods incorporate size selection, please pay careful attention to product specifications and accompanying protocols:

- Ethanol/Isopropanol Precipitation
  Recovers small and large RNAs e.g. tRNAs, miRNAs, mRNA and large non-coding RNA.

- Silica column-based RNA clean-up, recommended kits:
  - Clean & Concentrator kit from Zymo Research (#R1013)
    Can be used to recover both small (17-200 nt) and large RNA (> 200 nt) in a single fraction or separate fractions.
  - Nucleospin RNA Clean-up XS from Macharey Nagel (#740903)
    Recovers only large RNA (> 200 nt).

- SPRI bead-based RNA clean-up, recommended kits:
  - Agencourt RNAClean XP from Beckman Coulter (#A63987, #A66514)

- End of protocol -