

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:
 - siBeads from siTOOLS Biotech (streptavidin-coated magnetic beads, 25 mg/ml)
 - Dynabeads MyOne Streptavidin C1 from Thermo Fisher (#65001, 10 mg/ml)
 - Hydrophilic Streptavidin Magnetic Beads from New England Biolabs, NEB (#S1421S, 4 mg/ml)
- 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)
- DNase treatment (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.

Additional notes:

- riboPOOLs work best for high quality RNA samples though can also be used for degraded/ribosome profiling samples.
- RNA input amount may range from 10 ng to 5 µg (ideal range is 100 ng – 1 µg)
- RNA input should be DNA-free.
- Due to abundance of rRNA, expect to lose ~95% of initial input. *Expected yield for 1 µg of input RNA is < 30-50 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 according to below table (e.g. 15 µl for 12 reaction).

Reaction size	H ₂ O to add (µl)
12 reaction	15
24 reaction	30
96 reaction	110

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

We recommend to perform ribosomal RNA depletion with riboPOOLs in one step. See [riboPOOL Application Note](#) for results.

rRNA depletion – bead preparation

If using siBeads by siTOOLS Biotech:

- a. Resuspend the siBeads by carefully vortexing tube at medium speed.
- b. Transfer 90 µl of bead suspension per sample into a fresh tube.
For batch washing of beads for multiple samples, aliquot bead suspension for up to 6 (i.e. 540 µl) or 12

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- samples (i.e. 1080 µl) in a single tube.
- Place tube on magnetic rack and wait for 1 min. Beads may stick to sides of tube making solution appear brown. Aspirated solution however, should be clear.
 - Aspirate and discard all supernatant.
 - Add 80 µl per sample (i.e. 480 µl for 6 samples, 960 µl for 12 samples) of Depletion Buffer (DB) and agitate the tube well to resuspend beads.
 - Repeat steps c to e.

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

- Resuspend the beads by carefully vortexing tube at medium speed.
- 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.
- 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.
- Aspirate and discard all supernatant.
- 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.
- Repeat step 3e.
- Add 100 µl per sample of Bead Wash Buffer to resuspend beads. Place on magnetic rack (1 min), aspirate and discard supernatant.
- 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:

- Resuspend the beads by carefully vortexing tube at medium speed.
- 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.
- Place tube on magnetic rack (1 min), aspirate and discard supernatant.
- Add 100 µl per sample (i.e. 600 µl for 6 samples) of Depletion Buffer and agitate the tube well to resuspend beads.
- Place on magnetic rack (1 min), aspirate and discard supernatant.
- Resuspend beads in 80 µl per sample (i.e. 480 µl for 6 samples) of Depletion Buffer.

4. Ribosomal RNA depletion

- Briefly centrifuge the tube containing 20 µl hybridized riboPOOL and total RNA (from step 2) to spin down droplets.
- Combine 80 µl of prepared beads (from step 3f) with 20 µl of the hybridized riboPOOL-RNA solution. Agitate the tube to resuspend well.
- Incubate at 37°C for 15 min, followed by a 50°C incubation for 5 min.
- Briefly spin down droplets.
- Place on magnetic rack for 2 min. Carefully transfer the supernatant into a new tube.
- Place the new tube with the supernatant on the magnetic rack for 1 min to get rid of trace amounts of beads. (optional)
- 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.

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.

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- 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)

Optional DNase treatment

Our experience and customer feedback supports no riboPOOL contamination after rRNA depletion, which makes a DNase treatment unnecessary. If for another reason DNA treatment is desired, please include a DNase step after RNA clean up, as salt concentration after depletion is too high for efficient DNase treatment. Please include another RNA clean up step after DNase treatment.

- End of protocol -