Protocol for ribosomal RNA depletion with riboPOOLs

Materials provided:
- riboPOOL (freeze-dried)
- Nuclease-free water

Product description:
riboPOOLs allow the efficient removal of abundant ribosomal RNAs (rRNAs) from total RNA samples isolated from cells or tissues. riboPOOLs are available for multiple prokaryotic and eukaryotic species including Human, Mouse, Rat, Drosophila, Arabidopsis Thaliana and a range of bacteria and archaea. riboPOOLs consists of complex mixtures of single-stranded 3'-biotinylated oligonucleotide-probes designed to specifically hybridize with all rRNAs of the target species. Ribosomal RNAs bound to riboPOOL-probes are efficiently removed from the samples by streptavidin-conjugated magnetic beads. riboPOOLs work independent of polyadenylated RNA and can be applied for any downstream application e.g. Next-Generation Sequencing (NGS) library preparation.

Additional materials and equipment required for this rRNA depletion protocol (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)
- 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)

Buffers (not provided):
All buffers should be made in DEPC-treated or nuclease-free water.

Tip: Prepare 150 µl per sample (i.e. 3 ml for 20 reactions) of Hybridization Buffer, and dilute 143 µl per sample of Hybridization Buffer with an equal volume of nuclease-free water to obtain the Depletion Buffer.

Buffer Name	**Components**	**Minimum volume required per sample (µl)**	**Volume for 20 reactions (µl)**
Hybridization Buffer	10 mM Tris-HCl (pH 7.5) 1 mM EDTA 2 M NaCl	5.5	110
Depletion Buffer *(1X Hybridization buffer)*	5 mM Tris-HCl (pH 7.5) 0.5 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 samples.
- RNA input amount may range from 100 ng to 5 µg and RNA should be free of DNA.
- During protocol, avoid leaving tubes with RNA at room temperature over an extended period of time.
- Take necessary precautions to avoid RNase contamination.
- After rRNA depletion, expect to lose ~80-90% of initial RNA amount due to high abundance of rRNA.
  Expected yield for 1 µg of input RNA is < 80 ng.

siTOOLs Biotech GmbH • Lochhamer Strasse 29A, 82152 Planegg / Martinsried, Germany
info@sitools.de • +49 (0) 89 12501 4800

Protocol for ribosomal RNA depletion with riboPOOLs

Protocol:

1. ***Resuspension of riboPOOL***
   a. 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 and mix:
      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.
   b. Incubate at 68°C for 10 min to denature RNA.
   c. Allow to **cool slowly** from 68°C to 37°C for optimal hybridization.
      It is important that cooling is performed slowly. 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***

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, aspirate supernatant.
   d. 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, aspirate supernatant. **Repeat** this step **once**.
   e. Add 100 µl per sample of **Bead Wash Buffer** to resuspend beads. Place on magnetic rack, aspirate supernatant.
   f. Resuspend beads in 160 µl per sample (i.e. 960 µl for 6 samples) of **Depletion Buffer**.
   g. For each sample, aliquot 80 µl of beads from step 3f into a fresh tube. In a second labelled fresh tube, aliquot another 80 µl of beads from step 3f to use in a second depletion reaction. 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, aspirate 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, aspirate supernatant.
   f. Resuspend beads in 160 µl per sample (i.e. 960 µl for 6 samples) of **Depletion Buffer**.
   g. For each sample, aliquot 80 µl of beads from step 3f into a fresh tube. In a second labelled fresh tube, aliquot another 80 µl of beads from step 3f to use in a second depletion reaction. Leave the beads at room temperature until use.

siTOOLs Biotech GmbH • Lochhamer Strasse 29A, 82152 Planegg / Martinsried, Germany
info@sitools.de • +49 (0) 89 12501 4800

4. ***Ribosomal RNA depletion***

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

   b. Pipette the hybridized riboPOOL/RNA solution into a tube containing 80 µl of beads in **Depletion Buffer** (from step 3g). Agitate the tube to resuspend well.

   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, aspirate 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. Place the new tube with the supernatant on the magnetic rack for 1 min to get rid of potential trace amounts of beads from the last step.

   h. Very carefully transfer the supernatant to a new tube (avoid having any beads in your final sample).

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 can be performed according to previously established or kit manufacturer's instructions:

- 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)

siTOOLs Biotech GmbH • Lochhamer Strasse 29A, 82152 Planegg / Martinsried, Germany
info@sitools.de • +49 (0) 89 12501 4800