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 consist of complex mixtures of single-stranded 3'- biotinylated oligonucleotide probes designed to specifically hybridize with target RNAs. Ribosomal RNAs bound to riboPOOL-probes are efficiently removed from the samples by streptavidin-conjugated magnetic beads. riboPOOLs work independently of polyadenylated RNA and can be applied for any downstream application e.g. Next-Generation Sequencing (NGS) library preparation, microarray or PCR.

Additional reference:
Kim, I. V, Ross, E. J., Dietrich, S., Döring, K., Alvarado, A. S., and Kuhn, C. D. (2019) Efficient depletion of ribosomal RNA for RNA sequencing in planarians. *bioRxiv*. 10.1101/670604

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.

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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. 1200 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 **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.

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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)

- End of Protocol-

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