Protocol

Use excel calculator to determine volumes for this protocol.

Prepare Beads

1. Calculate total amount of streptavidin beads required for reactions and add to 1.5 mL tube on magnetic rack and allow to settle.
2. Wash beads once with the bead input volume of 1x SSC.
3. Return to magnetic rack and allow beads to settle.
4. Resuspend in resuspension volume of 1x SSC; typically 7.5 uL per reaction with some additional to account for pipetting error.
5. Add Superase-In to resuspended beads.
6. Take beads off rack and leave at room temperature.

Anneal oligos to rRNA

1. If you haven't already, mix 100 uM biotinylated oligos at an eqimolar concentration (see below).
2. Prepare removal master mix of SSC, EDTA, oligos and T.1E according to calculator.
3. Add removal master mix and T.1E to each RNA sample to achieve the same concentration of RNA per reaction in a final volume of 7.5 uL. This can be conducted in striptubes or a 96-well plate.
4. Anneal oligos to rRNA by incubating 5 minutes at 70C → infinite at 25C using a slow temperature shift (0.1C per second).

Deplete rRNA

1. While the oligos are annealing, mix beads to ensure solution is homogeneous and distribute 8.5 uL (additional uL is due to addition of Superase-In) into each well of a 96-well plate.
2. After annealing reactions reach 25C, add each 7.5 uL reaction to plate containing resuspended beads, pipetting up and down 25 times per well. For just a few reactions, I do this with a single channel, but for many reactions I use a multichannel - both work.
3. Incubate bead/annealing mixture at room temperature for 5 minutes.
4. Move plate with bead/annealing mixture to pre-heated thermocycler at 50C and incubate for 5 minutes. I add a qPCR plastic sheet to ensure there's no evaporation.
5. After incubation, move plate *directly* onto magnetic rack and pull supernatent into a new plate or striptubes. I find the plastic sheets are easier to remove while the plate is on the thermocycler (the plate is more stable and less likely to jostle the reactions). To ensure I don't disturb the pellet, I don't pull all of the supernatent - usually I pull about 10-12.5 uL for a 16 uL reaction.
6. Since SSC buffer is relatively low salt and the final concentration of EDTA is <1 mM, I have had success using the supernatent directly in reverse transcription or fragmentation reactions. If this is not desired, a magnetic bead-based purification is outlined below (ethanol or column-based purifications also work). The purifications as described are not 96-well plate compatible though with volume modifications (maintaining the 1:4 ratio of reaction to binding buffer) they can be.

mRNA Purification (optional)

1. For each reaction, aliquot 50 uL of AMPure XP beads into a single 1.5 mL tube and allow to settle on a magnetic rack (this will take 10-15 minutes due to the large amount of PEG in the buffer).
2. Carefully pull off AMPure XP supernatent leaving the bead pellet.
3. Resuspend the beads in 25 uL T.1E per depletion reaction, plus some extra to account for pipetting error.
4. Add 25 uL of resuspended beads to each depletion reaction supernatent and bring to a final volume of 40 uL of reaction / bead mixture using additional T.1E.
5. Add 160 uL (1:4, final volume 200 uL) of GuHCl binding buffer to each reaction, vortex and leave at room temperature for 5 minutes.
6. Place on magnetic rack, allow to settle, and remove supernatent.
7. Without removing the tubes from the rack, wash the pellet twice with 80% ethanol, allowing ethanol to sit on beads ~30s before removing and discarding all without disturbing the bead pellet (note ethanol should be prepared fresh or kept in an airtight container, see note for binding buffer below).
8. After beads are washed (to speed drying) spin tubes very briefly (~1 second) on a benchtop mini-fuge to move all remaining ethanol to the bottom of the tube and return to magenetic rack. Use a 10 uL pipette tip to remove all remaining ethanol without disturbing the pellet. Allow to dry for < 5 minutes; pellet should not begin to crack but should have minimal liquid droplets.
9. Resuspend pellets in desired volume of T.1E. If pellets are hard to resuspend or stick to the tube, they have been over-dried which can decrease yield. Allow to incubate 5 minutes at room temperature off of the rack.
10. Place on magnetic rack and keep supernatent and discard beads - this is your mRNA.

Note: this purification protocol also generally works to replace ethanol precipitations in many contexts and keeps even very small nucleic acid species.

Reagents and Recipes

- **T.1E:**
  - 10 mM Tris pH 8, 0.1 mM EDTA
- **GuHCl binding buffer:**
  - 1 M Gu-HCl (e.g. 2.34 g in 25 mL; Sigma: G3272)
  - 0.05% Tween 20 (e.g. 125 uL 10% tween-20 in 25 mL)
  - BTV 100% EtOH (not aqueous, all liquid apart from Tween-20 solution is ethanol)
  - Solution should be parafilmed or kept in a completely air-tight container as ethanol is volatile and hydroscopic.
- **Depletion oligos:**
  - All oligos are mixed at equimolar concentration starting from 100 uM stocks. For example, for a depletion reaction with 50 oligos, each oligo would be at 2 uM final concentration. If depletion is poor for a particular region or rRNA subunit (typically the 5S), these oligos may be used at 2x concentration to improve depletion.
  - 5'-biotinylated oligos from IDT
  - Ordering in 96-well plate format reduces cost / oligo
- **NEB Streptavidin beads** (NEB: S1420S)
- **20x SSC** (Thermo/invitrogen: 15557044)
- **Superase-In** (Thermo/invitrogen: AM2696)
- **500 mM EDTA** (Thermo/invitrogen: AM9260G)
- **AMPure XP magnetic beads**: (Beckman: A63880)