**File S1. RNase H depletion protocol:** The detailed protocol used to perform the RNase H depletion.

# Ordering

* Anti-sense DNA oligos (Eurofins)
  + Synthesis scale: 10 nmol
  + Purification: Salt Free
  + Quality control: MALDI-TOF
  + Delivery: TRIS Buffer @ 100 µM
* Hybridization Buffer
  + 100 mL NaCl (5 M), RNase-free (Thermo Fisher# AM9760G)
  + 1 L UltraPure 1 M Tris-HCI Buffer, pH 7.5 (Thermo Fisher# 15567027)
* RNase H [NEB M0297S = 50 µL]
* DNase I (RNase-free) [NEB M0303S = 500 µL]

# Preparation

1. Pool Oligos
   1. Pool all oligos (total volume of each) and aliquot
2. Prepare 5x Hybridization Buffer (1000 mM NaCl, 500 mM Tris-HCl, pH 7.5)

|  |  |
| --- | --- |
| 1 M Tris-HCI Buffer, pH 7.5 | 500 µL |
| 5 M NaCl | 200 µL |
| H2O | 300 µL |
| Total | 1 mL |

# Protocol

## DNA Probe Hybridization

1. Combine the following and mix well

|  |  |
| --- | --- |
|  | **Volume** |
| 5x Hybridization Buffer | 1 µL |
| 100 uM Pooled Antisense rRNA Oligos | 0.65 µL |
| Total RNA (~ 1 ug) | 3.35 µL |
| **Total** | **5 µL** |

1. Hybridize using the following program in a thermocycler with heated lid set to 105C

|  |  |
| --- | --- |
|  |  |
| 95°C | 2 minutes |
| Ramp from 95°C to 22°C | -0.1 °C/s |
| 22°C | 5 minutes |

1. Transfer immediately to ice

## RNase H Digestion

1. Prepare the RNase H Mix on ice. If a master mix is prepared, it must be used immediately.

|  |  |
| --- | --- |
|  |  |
| RNase H (5U/µL) | 2 µL |
| 10x RNase H Reaction Buffer | 1 µL |
| H2O | 2 µL |
| **Total** | **5 µL** |

1. Add 5 µL of RNase H Mix to each hybridized sample and mix well
2. Incubate at 37°C for 30 minutes
3. Spin down, transfer immediately to ice, and then proceed with DNase I digestion

## DNase I Digestion

1. Prepare the DNase I Mix on ice. If a master mix is prepared, it must be used immediately.

|  |  |
| --- | --- |
|  |  |
| 10x DNase I Reaction Buffer | 10 µL |
| DNase I (2U/µL) | 4 µL |
| H2O | 76 µL |
| **Total** | **90 µL** |

1. Add 90 µL of DNase I Mix to each RNase H digested sample
2. Incubate at 37°C for 30 minutes
3. Spin down, transfer immediately to ice, and then proceed with cleanup

## Clean Up: Zymo RNA Clean & Concentrator-5 kit.

All centrifugation steps should be performed at 10,000 – 16,000 x g.

1. Add 200 µL (2 volumes) **RNA Binding Buffer** to each sample and mix (transfer to microcentrifuge tube if necessary)
2. Add 300 µL (equal volume) of ethanol (95-100%) and mix
3. Transfer the sample to the **Zymo-SpinTM IC Column** in a **Collection Tube** and centrifuge for 30 seconds. Discard the flow-through.
4. Add 400 μL **RNA Prep Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
5. Add 700 μL **RNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
6. Add 400 μL **RNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through and centrifuge for an additional 2 minutes to remove any residual wash buffer. Transfer the column carefully into an RNase- free tube (not provided).
7. Add 12 μL **DNase/RNase-Free Water** directly to the column matrix and centrifuge for 30 seconds.
8. Use 5 µL of the eluted RNA for library prep using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® (E7765), section 4: “Protocol for use with Purified mRNA or rRNA Depleted RNA”

# References

[1] Selective Depletion of rRNA Enables Whole Transcriptome Profiling of Archival Fixed Tissue

<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0042882#s4>

[2] Comparative analysis of RNA sequencing methods for degraded or low-input samples

<https://www.nature.com/articles/nmeth.2483#methods>