RNase H Depletion of ribosomal RNA

created 4-December-2014, Peter Wang
modified 10-June-2015, Peter Wang = v 1.1; modified 18-Nov-2015, Peter Wang = v1.2.

BACKGROUND:

This is based on the protocol of Adiconis *et al.*, which itself is an adaptation of the original method by Morlan *et al.*

• Adiconis X, Borges-Rivera D, Satija R, DeLuca DS, Busby MA, Berlin AM, Sivachenko A, Thompson DA, Wysoker A, Fennell T, Gnirke A, Pochet N, Regev A, Levin JZ. Comparative analysis of RNA sequencing methods for degraded or low-input samples. Nat Methods. 2013 Jul;10(7):623-9. doi: 10.1038/nmeth.2483. Epub 2013 May 19. Erratum in: Nat Methods. 2014 Feb;11(2):210. PubMed PMID: 23685885; PubMed Central PMCID: PMC3821180.

• Morlan JD, Qu K, Sinicropi DV. Selective depletion of rRNA enables whole transcriptome profiling of archival fixed tissue. PLoS One. 2012;7(8):e42882. doi: 10.1371/journal.pone.0042882. Epub 2012 Aug 10. PubMed PMID: 22900061; PubMed Central PMCID: PMC3416766.

Adiconis designed 50mer DNA oligos that tile the entire sequence of human ribosomal RNAs (28S, 18S, 5.8S, 5S, and the mitochondrial 18S and 16S). These are hybridized to the RNA sample and then RNase H is added, which will destroy all RNA that is present as RNA-DNA hybrids. The DNA oligos are then removed with DNase I.

Note that a commercial implementation of this method is available from New England Biolabs (NEB) as "NEBNext rRNA Depletion Kit (Human/Mouse/Rat)", catalog #E6310.

A thermostable RNase H ("Hybridase", from Epicentre) is used (at 45°C) in both papers. However, it is likely that *E. coli* RNase H could be used (at 37°C), as that is what is used in the NEB kit, but might require use of shorter oligos. Note that Hybridase is a significant part of the expense of this protocol.

Purification of the RNA after treatment can be done in a number of ways. Adiconis *et al.* uses RNAClean SPRI beads, I use a silica column (Zymo Research). The use of the Zymo "In Tube DNase Treatment"streamlines the DNase treatment a bit.

REAGENTS:

- thermal cycler, capable of doing a slow temperature ramp.
- heat block or other thermocycler that can be set to 45°C.

- DNA oligo pool for targeted depletion, at 100 μM
  -- ordered from PAN Facility using the Excel spreadsheet provided in Adiconis *et al.* Supplement
  -- note that several oligos contain I (inosine) in place of G, to break up long runs of G.
  -- oligos were unpurified. Each oligo was resuspended to 100 μM with water, and combined equi-molar.

- RNase Inhibitor, Murine (New England Biolabs, catalog # M0314)
  or any other similar, e.g., RNaseOUT (Life Technologies, catalog # 10777-019)
- Hybridase (Epicentre, catalog # H39100 or H39500)
- TURBO DNase (Ambion / Life Technologies, catalog # AM2238)
- RNA Clean & Concentrator-5 kit for clean-up (Zymo Research, catalog # R1015)

1x RNaseH Buffer = 100 mM Tris pH 7.4, 200 mM NaCl, 20 mM $MgCl_2$
(above is given for reference only; only need to make 5x buffer, below)

- **5x RNaseH(-)Mg Buffer** = 500 mM Tris pH 7.4, 1000 mM NaCl

- *for 1000 µl:* 300 µl water + 200 µl 5M NaCl + 500 µl 1M Tris pH 7.4
- 1 mM EDTA
- 1M $MgCl_2$

PROTOCOL:

input RNA amount: I have only done this protocol for inputs of 5 µg total RNA because I have required larger amounts of depleted RNA. More standard is to treat 1 µg total RNA, so I have also included amounts for that. For RNA amounts lower than 1 µg, I would use the same volumes as for 1 µg.

depletion oligo amount: should be equal mass as the input RNA.

- 1000 ng oligo / (50 nt × 325 g/nt) = 61.54 pmoles

expected recovery: 5-10% of the input RNA.

Note: the RNA must be in a relatively small volume (3.1 µl for 1 µg input). If your RNA is too dilute, I would suggest concentrating it by ethanol precipitation with linear polyacrylamide as carrier.

Prior to starting hybridization (which doesn't take very long), you should set a heat-block or other thermal cycler to 45°C, and also make up the RNase H mix without enzymes and start it pre-warming (see below).

I add EDTA to 50 µM, to chelate trace divalent metal (say in the water) to protect the RNA during the 95°C step.
Obviously it is best to make master-mixes for all samples, to minimize pipeting of tiny volumes.

hybridization mix	**5 µg input**	**1 µg input**
input RNA + water	15.67	3.134
1 mM EDTA	1.25	0.25
5x RNaseH(-)Mg buffer	5	1
rRNA oligo pool (100 µM)	3.08	0.616
	25 µl	5 µl

For thermal cycler program, the intent is to match Adiconis *et al.* which specifies to ramp temperature from 95°C to 45°C at 0.1°/sec.
However, on our GeneAmp 9700 and probably some other thermal cyclers, it is not possible to program this directly. The program below approximates it.
GeneAmp PROGRAM:

95°C 2';
99 × [95°(-0.1°/cycle) 1"; 95°(-0.1°/cycle)];
99 × [75.2°(-0.1°/cycle) 1"; 75.2°(-0.1°/cycle)];
52 × [55.4°(-0.1°/cycle) 1"; 55.4°(-0.1°/cycle)];
45°C hold.

For RNase H reaction, final Mg^{2+} is 20 mM; since there is no Mg^{2+} in the hybridization mix, the RNaseH mix needs to be 2x Mg^{2+}, that is, 40 mM.

RNase H mix	**5 µg input**	**1 µg input**
water	8.5	1.7
5x RNaseH(-)Mg buffer	5	1
1M $MgCl_2$	1	0.2
RNase Inhibitor, Murine (40 U/µl)	0.5	0.1
Hybridase (5 U/µl)	10	2
	25 µl	5 µl

Pre-warm solution *without* RNase Inhibitor and Hybridase, at 45°C.

Add RNase Inhibitor and Hybridase just before use.

When hybridization program has completed, add 25 µl (or 5 µl) pre-warmed RNaseH solution to each hybridization mix (without removing hybridization tube from thermal cycler).
Incubate RNase H reaction at 45°C for 30 min.

Make up TURBO DNase solution.
Pre-warm the water and 10x TURBO buffer to 37°C, adding enzyme just before use.

TURBO DNase mix	**5 µg input**	**1 µg input**
water	118	59
10x TURBO DNase buffer	14	7
TURBO DNase (2 U/µl, Ambion)	8	4
	140	70 µl

At end of RNase H digestion, add the pre-warmed TURBO DNase solution.
Incubate DNase reaction at 37°C for 30 min.

Purify using Zymo RNA Clean & Concentrator-5 (RCC-5) kit.
Stop reaction by adding 2 volumes (380 µl or 160 µl) of Zymo RNA Binding Buffer, mix. Transfer to a new tube.
Add equal volume (570 µl or 240 µl) of EtOH, mix.
Load onto Zymo RCC-5 column, microfuge 12K×g for 30 sec.
(For 5 µg scale, will need to load in more than one pass.)
optional: Re-load flowthrough onto column, spin 30 sec.
Add 400 µl RNA Prep Buffer, microfuge 12K×g for 1'.
Wash with 700 µl and 400 µl RNA Wash Buffer, spin 30 sec each time.
Microfuge column dry, 12K×g for 2'.
The minimum elution volume is 6 µl. If your library protocol accepts larger volumes, use more.
(I eluted column twice, with 2 × 6 µl water.)
Add ≥6 µl water to each column, incubate 1', microfuge 12K×g for 30 sec.

Measure output RNA concentration; best to use a sensitive fluorescent method, such as Qubit assay (Life Technologies).

Expected Results:
The apparent amount of RNA after depletion varies from 5-20% of the input amount. There is no clear relationship of this to the effectivenss of depletion in the RNA-Seq data.
We have seen about 1-2% (or less) ribosomal RNA reads in our RNA-Seq data, so excellent depletion.

MODIFICATION NOTES:

10-June-2015: now the TURBO DNase treatment is done before loading onto Zymo column. Previously, RNase H reaction was loaded onto column, washed once, then eluted with DNase solution and incubated; then re-loaded onto column, more tedious for 1 μg scale but perhaps better for 5 μg scale (see note below).
18-November-2015: Added "expected results" note at end. Removed comment about having only done at 5 μg RNA input, have since done for 1 μg RNA inputs and worked fine. Also removed the following note, as I have now done it exactly as written.
note: recipe here for 5 μg input is just a suggestion; I have not actually tested it. What I have actually done for 5 μg scale is loaded RNase H reaction onto Zymo column and used a modification of their "in-tube" protocol (see their RCC-25 manual): wash once, then elute with 100 μl DNase solution (I used 5 μl TURBO DNase in total 100 μl of 1x buffer) into a clean collection tube; wrap column in place with Parafilm, incubate the whole setup at 37°C for 30 min. Then the treated solution is reloaded onto the same column following the "in-tube" protocol.