rRNA subtraction protocol for metatranscriptomics

Recommended materials/kits	Price	Vendor	Stock Number
MEGAscript™ transcription kit	$249	Ambion	AM1334
MEGAclear™ kit	$89	Ambion	AM1908
RNeasy MinElute cleanup kit	$259 (50 rxn)	Qiagen	74204
Biotin-11-CTP (10 mM)	$158	Ambion	04739205001
Biotin-16-UTP (10 mM)	$158	Invitrogen	11388908910
SUPERase•In™ RNase Inhibitor	$97 (2500 U)	NEB	AM2694
Streptavidin-coated magnetic beads	$210 (5 ml)	NEB	S1420S
DynaMag™ – Spin magnet	$310 (6 sample)	Invitrogen	123-20D
20X Sodium chloride-citrate (SSC) buffer (RNase free)	$32	Ambion	AM9770
Nuclease-free Water (non-DEPC)	$62 (1 L)	Ambion	AM9932
Microcent. tubes, RNase-free (1.5 ml)	$43 (250)	Ambion	AM12450
PCR tubes, RNase-free (0.2 ml)	$120 (1000)	Ambion	AM12225
Herculase® II Fusion polymerase	$228 (200 rxn)	Stratagene	600677
RNaseZap® (250 ml)	$53	Ambion	AM9780
Quant-iT™ RiboGreen® RNA Assay	$378	Invitrogen	R11490
Formamide (100%)			
General PCR reagents			
Ethanol ($100%), ACS grade			

General notes:

- Prior to all lab work, wipe down bench surfaces and lab equipment with RNase-Zap (Ambion) to deactivate RNases.
- Replace gloves frequently during the procedure.
- Confirm the availability of all downstream reagents before proceeding to the next step.

rRNA subtraction

A. PCR amplification of rRNA genes

This step creates sample-specific amplicon pools. These will be used as template for *in vitro* transcription (using T7 RNA polymerase) to produce anti-sense rRNA probes complimentary to rRNA in the total RNA extract.

1. Order primers*.

Bacteria

16S:

Eub16S_27F *AGAGTTTGATCCTGGCTCAG*

Eub16S_1492R_T7 *GCCAGTGAATTGTAATACGACTCACTATAGGGACGGCTACCTTGTTACGACTT*

23S:

Eub23S_189F *GAASTGAAACATCTHAGTA*

Eub23S_2490R_T7 *GCCAGTGAATTGTAATACGACTCACTATAGGGCGACATCGAGGTGCCAAAC*

Archaea

16S:

Arch16S_21F *TCCGGTTGATCCYGCCGG*

Arch16S_1492_T7 GCCAGTGAATTGTAATACGACTCACTATAGGGGGYYACCTTGTTACGACTT

23S:

Arch23S_F *ASAGGGTGAHARYCCCGTA*

Arch23S_R *GCCAGTGAATTGTAATACGACTCACTATAGGG*CTGTCTCRCGACGGTCTRAACCCA

Eukaryote

18S:

Euk18S_1F *ACCTGGTTGATCCTGCCAG*

Euk18S_1520R_T7 *AATTATAATACGACTCACTATAG*ATTCYGCAGGTTCACCTAC

28S:

Euk28S_26F *ACCCGCYGAAYTTAAGCATA*

Euk28S_3126R_T7 *AATTATAATACGACTCACTATAGATTCTGRYTTAGAGGCGTTCAG*

* T7 promoter underlined above. See Delong *et al*. (1999) and Stewart *et al*. (2010) for details

on primer design. The 5' bases upstream of the T7 promoter facilitate RNA polymerase binding. Transcription efficiency increases if the first bases downstream of the promoter are GG in the transcribed sequences (CC in the template strand).

2. PCR amplify rRNA.

Protocol for 50 µl reaction* (using Herculase® II Fusion polymerase, Stratagene 600677):

Reagents		**Thermal cycler protocol***	
Template DNA	100 ng	**Bacterial 23S**	**All other reactions**
Herculase 5X buffer	10 µl	95ºC 2 min	95ºC 2 min
dNTP (10 mM)	1.25 µl	95ºC 20 sec	95ºC 20 sec
F primer (10 µM)	1.25 µl	**39ºC 20 sec**	**55ºC 20 sec**
R primer (10 µM)	1.25 µl	72ºC 2 min	72ºC 2 min
Herculase pol	1 µl	72ºC 3 min	72ºC 3 min
H_2O	To 50 µl	*** 35 cycles**	

* Modify protocol accordingly if using a different polymerase. We recommend performing multiple reactions per sample (typically four 50 µl reactions for 16S, four 50 µl reactions for 23S, etc), and pooling the products afterwards. High PCR yields (e.g., ~250-500 ng/µl) are necessary for the next step (*in vitro* transcription).

3. Purify PCR products (we recommend using the QIAquick PCR purification kit (Qiagen) with elution in **50 ul elution buffer**) and quantify DNA concentration using the Nanodrop (the Nanodrop is typically accurate enough here, as the PCR products should be at high concentration (>100 ng/µl)).

B. *In vitro* transcription (w/ T7 RNA polymerase) of biotin-labeled anti-sense RNA probes

---- based on MEGAscript™ High Yield Transcription kit (Ambion)

1. Prepare separate reactions for all probes. For a standard 20 µl reaction*:

PCR amplicons (from step A, ideally 250-500 ng)	1 µl
ATP (75 mM)	2 µl
GTP (75 mM)	2 µl
CTP (75 mM)	1.5 µl
UTP (75 mM)	1.5 µl
Biotin-11-CTP (10 mM, Roche 04739205001)	3.75 µl
Biotin-16-UTP (10 mM, Roche 11388908910)	3.75 µl
10X buffer	2 µl
SUPERase•In™ RNase inhibitor	0.5 µl
T7 RNA polymerase	2 µl

2. At room temperature (**not on ice**, as spermidine in the reaction buffer can cause DNA precipitation), mix reagents in the order listed. Incubate at 37ºC for 4-6 hrs, or up to overnight depending on yield.

3. After incubation, add 1 µl DNase I (included in the MEGAscript kit) to remove the DNA template. Incubate at 37ºC for an additional 30 min.

4. Purify synthesized RNA using the MEGAclear™ kit, with elution in 50 µl elution solution. *The MEGAclear kit is designed for recovery of high amounts of product and is therefore ideal for IVT reactions (recommended here over the RNeasy kit).*

5. Quantify RNA concentration using either RiboGreen or Nanodrop protocols. **Store probes at -80°C**.

***** The IVT reaction is linear and therefore dependent on starting DNA concentration. A 4-6 hr reaction with 250-500 ng of starting template typically yields high probe concentrations (e.g., 1500-2000 ng/µl). If yield is low, the reaction volume can be increased to 40 µl. Assuming ~50% GC, ~1 in 8 nucleotides should be biotin-labeled in the resulting aRNA probes.

C. rRNA subtraction with biotinylated aRNA

This step binds biotinylated aRNA probes (step B) to rRNA in the total RNA sample. The labeled ds rRNA is then removed via hybridization to streptavidin-coated magnetic beads (NEB S1420S), followed by magnetic separation (via a separation rack; e.g., DynaMag^{TM}-Spin Magnet). Depending on the rRNA concentration in the sample, the ratio of probe to template RNA may need to optimized. To start, we recommend a probe-to-RNA ratio of 2:1, and a reaction volume of **50 µl in 1X sodium chloride-citrate (SSC) and 20% formamide**. We have found that > 20% formamide inhibits probe-bead binding, and < 20% allows non-specific binding (non-target RNA to beads). The procedure obviously involves a significant reduction in RNA concentration. Depending on downstream applications (e.g., RNA amplification), we recommend using **as much starting RNA template as possible** (ideally 250-500 ng).

1. Bead washing (**do this before or during the hybridization step).**
    a. Transfer beads (100 µl per sample) to a 1.5 ml tube - **beads for all samples can be washed together in the same tube.** Testing indicates that for total RNA amounts (template + probe) < 2 µg per sample, 100 µl beads should effectively remove all probe (**for higher amounts, increase bead volume linearly; an excess of beads is highly favored over inefficient probe removal**)
    b. Wash 1: bind beads to magnetic separation rack (takes ~2 min), pipet off and discard supernatant, re-suspend beads in equal volume of 0.1N NaOH (*deactivates bead-associated RNases*), and flick vigorously to re-suspend, re-bind to magnet and pipet off supernatant
    c. Repeat wash twice using 1X SSC buffer. On the 3rd wash, aliquot out 100 µl beads per sample - leave beads in buffer on ice until hybridization is complete.

2. Hybridization.

a. For a 50 ul reaction*, prepare in a PCR tube:

Template, total RNA (ideally 250-500 ng)	X µl
aRNA 16S probe (500-1000 ng)**	X µl
aRNA 23S probe (500-1000 ng)**	X µl
SUPERase•In RNase inhibitor	1 µl
20X Sodium chloride-citrate (SSC) buffer (RNase free)	2.5 µl
Formamide (100%)***	10 µl
Nuclease-free water (if necessary)	To 50 µl

* Template and probe volumes will be scaled depending on concentrations.

** To avoid pipetting small volumes, make a diluted probe master-mix prior to starting

***To prevent formamide degradation, store small aliquots (1 ml) at -20°C

b. In a thermal cycler, incubate under the following conditions:

5 min at 70ºC

rampdown to 25°C using 5C increments for 1 min each.

c. Remove the reaction and let sit at RT for 2-5 min.

3. Bead binding.

a. While the hybridization reaction is at RT, capture the pre-aliquoted beads (100 ul per sample – step 1C above) on the magnetic rack, pipet off the supernatant, and remove the beads from the rack.

b. Dilute up (using 1X SSC and 20% formamide) the hybridization reaction to match the initial bead volume (100 ul).

c. Add the hybridization reaction (now 100 ul) to the dried beads.

d. **Incubate at RT for 10 min**, with occasional flicking to mix.

4. Bead removal.

a. Quick spin the tubes. Capture the beads on the magnetic rack (2-3 min), transfer the non-rRNA-containing supernatant to a 1.5 ml tube. Re-suspend remaining beads with 100 µl 1X SSC, capture beads as above, transfer supernatant to the same 1.5 ml tube (200 µl total volume).

(**For reactions using > 100 µl beads**: run the hybridization reaction at 50 µl, then dilute the sample (with 1X SSC + 20% formamide) up to match the initial volume of the dried beads.)

5. Purify subtracted RNA (200 µl) to remove formamide.
    a. Use the RNeasy MinElute kit. A second wash with RPE buffer is recommended to improve performance in downstream *in vitro* transcription. Elute in 15 µl water.
6. Run 1 µl of purified RNA (diluted 1:10 and 1:100) on the Bioanalyzer to confirm rRNA subtraction and probe removal (*not absolutely necessary, but recommended*).
7. Store RNA at -80°C for downstream applications.

DeLong EF, Taylor LT, Marsh TL, Preston CM. (1999). Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. *Appl Environ Microbiol* **65**: 5554-5563.

Stewart FJ, Ottesen EA, DeLong EF. (2010). Development and quantitative analyses of a universal rRNA-subtraction protocol for microbial metatranscriptomics. *In review.*