A finalized protocol for depletion of rRNAs and human mRNAs has been established and is ready to be shared with the HMP and dental research communities. The procedure used involves the following basic steps:

-Step 1- Ambion Microbenrich Kit extraction of the RNA sample. This step is intended to remove mammalian mRNAs (poly-adenylated message) and also 18S and 28S rRNAs.

-Step 2- RNA amplification with the Ovation RNA-Seq cDNA Synthesis Kit (optional). For many microbiome applications microbial RNA quantities will be limiting and isothermal unbiased cDNA amplification will be a useful step.

-Step 3- DNA Subtractive hybridization using Biotinylated PCR products of the rRNA genes and Streptavidin Capture. We have noted that rDNA removal with commercial kits is effective but that results from other laboratories show a significant number of reads remain that map to rDNA sequence despite what appeared to be effective removal of these sequences using the Ambion product in Step 1. We reasoned that the explanation relates to the fact that many rDNAs may be partial length or fragmented during processing making their removal with site-specific oligonucleotides impossible to achieve. For this reason we further target rDNA removal using microbial and mammalian rDNA amplicons (16S, 23S, 18S and 28S) derived from the samples under study that are subsequently biotinylated and removed from samples using streptavidin beads. The current SOP is provided below.

RNA SEQ ENRICHMENT PROTOCOL

Reagents:

MicrobEnrich kit (Ambion, AM1901)
RNeasy MinElute columns (Qiagen, 74204)
Ovation RNA Seq kit (Nugen, 7100)
MinElute column (Qiagen, 28004)
Finnzymes' Phusion® Hot Start High-Fidelity DNA Polymerase (NEB, F-549L)
Primers listed below
Label IT® µArray® Biotin Labeling Kit (Mirus, MIR8050)
Streptavidin HP SpinTrap columns (GE Healthcare, 28-9031-30)
0.5M EDTA, pH 8 (Ambion, 9260G)
EPPS (Sigma, E1894-25G)
Sodium Chloride (Sigma, 7647-14-5)
1M Tris, pH 7.0 (Ambion, 9850G)
Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, UFC801024)
Mineral Oil
2 mL Collection tubes (Qiagen, 19201)
0.2 mL tube (Biorad, TFI-0201)
0.5, and 1.5 mL tubes
1.5 – 2% agarose gel
Ice
DI water

Solutions to make

1M EPPS, pH 8.0

12.6 g	EPPS
50 mls	DI water
50 mL	

pH solution to 8.0

** store at 4°C*

2.5 M NaCl

7.3 g	NaCl
50 mL	DI water
50 mL	

** store at room temperature*

TBS Buffer

(50 mM Tris, 150 mM NaCl, pH 7.5)

5 mL	1M Tris, pH 7.0
6 mL	2.5 M NaCl
89 mL	DI water
100 mL	

pH solution to 7.5

** store at room temperature*

Subtraction Hybridization Buffer

(25 mM EPPS, 2.5 mM EDTA)

2.5 µl	1 M EPPS
0.5 µl	0.5M EDTA
97 µl	DI water
100 µl	

** store at 4°C*

Preparing cDNA

1. Run 5 µg of RNA through Ambion's MicrobEnrich kit using Ambion provided protocol to remove human contamination.
2. Purify samples using RNeasy MinElutes and elute in 14µL. (Other protocols can be used for purification, but elute in a small volume so the concentration is high enough for the RNA Seq kit.)
3. Run 10 ng of each sample through Nugen's RNA Seq kit using Nugen provided protocol.
4. Purify cDNA using MinElute columns and elute in 40 µl.

Preparing Ribosomal Driver

1. Prepare ribosomal PCR product by using the following primers and parameters:

Primers:

Primer name	5´- Sequence -3´	bp	anneal temp	ext time	Comments
16S_8F	AGAGTTTGATCCTGGCTCAG	1533	65°C	1 min	
16S_1541R	AAGGAGGTGATCCAGCCGCA				
18S_90F	ATGGCTCATTAAATCAGTTATGGTTC	1770	64°C	1.5 min	
18S_1860R	CTTTGTTACGACTTTTACTTCCTCTAG				Gel purify for template
23S_10F	YGGTGGATGCCTTGGC	2746	63°C	1.5 min	
23S_2756R	YRCTTAGATGCTTTCAGCRBTTATC				
28S.1_20F	GTGGCAACCCGCTGAA	350	60°C	30 sec	Gel purify for template
28S.1_370R	CAAAGTTCTTTTCAACTTTCCCTTAC				
28S.2_285F	GGGTTGCTTGGGAATGCA	1275 bp	60.8	1 min	Gel purify for template
28S.2_1560R	GACCTCCACCAGAGTTTCCT				
28S.3_1500F	GAGCGCACGTGTTAGGAC	915 bp	66°C	1 min	Gel purify for template
28S.3_2415R	CCCATGTTCAACTGCTGTTCA				
28S.4_3955F	GAGGTGTAGAATAAGTGGGAGG	1068 bp	60.8	1 min	Gel purify for template
28S.4_5023R	ACCCRGAAGCAGGTCG				

*18 and 28S primers produce multiple PCR bands. Set up a small PCR and gel purify the correct bp fragment using the MinElute kit. Use the gel purified product (1 µl at 0.1 ng/µl) as the template for the larger reaction. You'll likely still see multiple bands, but they will all be from the correct part of the rDNA.

Master Mix:

20 µl	5X HF Buffer
2 µl	10 mM dNTP mix
1 µl	Hot Start Phusion II
3 µl	10 uM primer
50 ng	gDNA
X µl	DI Water
100 µL	

Cycling Conditions

98°C	30 sec	
98°C	10 sec	
Annealing temp	30 sec	40 cycles
72°C	Extension time	
72°C	10 min	
4°C	Hold	

2. Using fragment lengths provided in the chart for each PCR product, combine equal picomol amounts of each of the 28S PCR fragments to make a 28S PCR mix.
3. Using fragment lengths provided in chart for each PCR product, combine equal picomol amounts of 16S, 18S, 23S and 28S PCR mix to make rRNA PCR mix.
4. Sonicate rRNA PCR mix 5 times at power 0.5 for 1 min. These conditions should be adequate to reach the appropriate size requirement for the driver DNA (250-500bp), though the size should be checked on a gel.

Biotinylating Ribosomal Driver

1. Reconstitute Label IT® µArray® Biotin Reagent with 200 µl of reconstitution buffer for MIR 8050 50 rxn kit. If the MIR 8010 10 rxn kit is used, reconstitute with 40 µl of reconstitution buffer.
2. Biotinylate the rRNA PCR mix, which will be used as the driver in the reaction. Each sample undergoing RNA Seq requires 20µg of rRNA PCR mix. Set up biotinylation in as follows. Reaction can be scaled up as needed.

1X	
5 µg	Purified, sonicated rRNA PCR DNA
10 µL	10X Labeling Buffer M
20 µL	Label IT® µArray® Biotin Reagent
X µL	DI water
100 uL	

3. Place sample(s) in 37°C incubator for 5 hours.
4. Following incubation, add 10 uL of D1 reagent. Mix well and incubate for 5 minutes at room temperature.
5. Add 10 µL of N1 reagent. Mix well and incubate for 5 minutes on ice. Samples can be purified immediately or stored at -20°C until ready to purify.

6. Purify samples on Qiaquick column using the Qiagen protocol with the following modifications.
    a. Ensure that the pH indicator is added to the PB buffer and that the pH is correct before applying sample to the column.
    b. Allow sample to incubate on the column for one minute before spinning down. Once spun through, reapply the sample and incubate an additional minute before spinning.
    c. Elute twice with 50µl of DI water each. Incubate sample for 2 minutes at room temperature before spinning down.
7. Samples can be purified immediately or stored at -20°C until ready.

Setting up rRNA Subtractive Hybridization

1. Mix 2 µg of the cDNA generated from the MicrobEnrich/RNA Seq kit protocols above as the tester DNA with 10 µg of biotinylated rRNA PCR mix as the driver in a 0.2 mL PCR tube.
2. Completely dry sample in speed vac.
3. Resuspend dried tester/driver mix in 4 µl of subtraction hybridization buffer. Pipette sample up and down several times, scraping any material off the sides of the walls, to ensure sample is thoroughly resuspended.
4. Denature sample at 98°C for 5 minutes.
5. Spin down sample and add 1 µl of 2.5 M NaCl.
6. Mix sample and spin down.
7. Cover sample with 10 µl of mineral oil.
8. Incubate at 65°C for 48 hours.

Prepare Streptavidin HP SpinTrap column for extraction (after 48 hr incubation)

1. Break off the bottom cap from the spin column. Save the bottom cap.
2. Remove the storage solution by centrifugation for 1 min at 200 × g.
3. Add 400 µl of TBS buffer and centrifuge for 1 min at 200 × g to equilibrate the medium.
4. Repeat step 3 a total of three times.
5. Place cap back on the bottom of the column.

Prepare sample for extraction

1. Following 48 hour incubation, remove samples from thermocycler and spin down.
2. Add 200 µl of TBS buffer underneath the layer of mineral oil.
3. Spin down tube to ensure separation of mineral oil and sample.
4. Remove sample, being careful not to pull up any of the mineral oil.

    Optional: Pipet droplets of sample on a piece of parafilm. Mineral oil tends to stick to the parafilm and lag behind as droplets are rolled. Roll droplets until no mineral oil is observed. **Caution**: Pay attention. Droplet rolls fast!

Extract Biotinylated Sample.

1. Immediately after the equilibration, add entire subtracted sample (200 µl) to the equilibrated column.

2. Fully suspend the medium by manual inversion for approximately 5 minutes.
3. Place column on a lab quake and incubate at room temperature for one hour.
4. Following incubation, remove bottom cap and centrifuge for 1 min at 200 × *g* to remove unbound DNA. COLLECT FLOW THROUGH as it contains desired DNA.
5. Add 400 µl of TBS buffer and centrifuge for 1 min at 200 × *g*.
6. Perform step 5 a total of 5 times, collecting flow through at each wash.
7. Place cap back on the bottom of the column.
8. Add 200 µl of TBS buffer and mix by inversion. Remove cap.
9. Centrifuge for 1 min at 1000 × *g*. Collect flow through.
10. Perform step 8-9 a total of 3 times, collecting flow through at all times.
11. Flow through can be stored at -20°C until ready to use.

Purifying/Concentrating Sample.

1. Add entire sample volume into the Amicon Ultra filter device.
2. Place capped filter device into swing bucket.
3. Spin the device at 4,000 × g (maximum) for approximately 20 minutes.
4. Discard flow through.
5. Add 4 mL of DI water. Spin at 4,000 x g for 30 minutes.
6. Recover the concentrated solute by inserting a pipettor into the bottom of the filter device and withdrawing the sample using a side-to-side sweeping motion to ensure total recovery. Place sample in clean 1.5 mL tube.
7. Spin sample an additional minute at 4,000 x g to bring down any remaining sample.
8. Recover any additional sample.
9. Purified sample can be stored at -20ºC until ready to use.

Second Round of Subtraction.

1. Mix purified 1X subtracted sample with 10 µg of biotinylated rRNA PCR mix in a .2 mL PCR tube.
2. Completely dry sample in speed vac.
3. Resuspend dried tester/driver mix in 4 µl of subtraction hybridization buffer. Pipet sample up and down several times, scraping any material off the sides of the walls, to ensure sample is thoroughly resuspended.
4. Denature sample at 98°C for 5 minutes.
5. Spin down sample and add 1 µl of 2.5 M NaCl.
6. Mix sample and spin down.
7. Cover sample with 10 µl of mineral oil.
8. Incubate at 65°C for 48 hours.

Extraction, Purification and Concentration of 2X Subtracted Sample.

1. Follow protocols above for extracting samples with Streptavidin HP SpinTrap columns and purifying with Amicon Ultra Filter device.
2. Repeat extraction and purification a total of two times before proceeding to second strand synthesis.

Second Strand Synthesis Protocol

1. Set up second strand cDNA reaction as follows:

1X	
112 µl	cDNA (increase volume with DI water if necessary)
8 µl	3ug/ul Random Hexamers
120 µl	

2. Incubate for 10 minutes at 100°C.
3. Add additional reagents as follows:

1X	
15 µl	NEB Buffer 2
10 µl	10mM dNTP mix
0.6 µl	DNA Polymerase I (10,000U/ml)
0.2 µl	E.coli DNA Ligase (10,000 U/ml)
4 µl	E.coli DNA Ligase Buffer

4. Incubate 15°C for 4.5 hours.
5. Samples can be stored at -20 until ready for purification.

Purify samples using MinElute columns.