Metatranscriptomics sample preparation protocol w/ScriptSeq

Ed DeLong

Abstract

This protocol is an update of the Metatranscriptomics protocol from DeLong lab 2012 which incorporates ScriptSeq v2 RNA library preparation.

Part A: RNA extraction, DNase treatment, purification, and quantification.
Part B: rRNA subtraction
Part C: cDNA synthesis and Illumina library prep

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Guidelines

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.

Part A: RNA exraction, DNase treatment, purification, and quantification

-- for filters in 300 µl RNAlater

A1. RNA extraction
--- based on *mir*Vana™ miRNA Isolation kit (Ambion)

Preparation:

- Add 21 ml of ACS grade 100% ethanol to Wash Solution 1, and mix well.
- Add 40 ml of ACS grade 100% ethanol to Wash Solution 2/3, and mix well.
- Make sure the Acid-Phenol:Chloroform is not more than 6-months old.

A2. DNase Treatment (~100 µl rxn)
---based on Ambion TURBO DNA-free™ kit

Preparation: set heat block or hyb oven to 37°C

A3. Concentrating and purifying DNase treated RNA

---based on RNeasy MinElute Cleanup Kit (Qiagen)

Preparation: add 4 volumes of ethanol (100%) to buffer RPE.

A4. RNA quantification

Depending on your application, the recommended option for RNA quantification is:

1. Bioanalyzer (Agilent) using the RNA 6000 Pico total RNA kit: quantitative range = 0.05-5.0 ng/µl (dilute samples 1:10 and 1:100 prior to analysis), allows visualization of RNA size distribution, less accurate, ~1 hr processing time.

Part B: rRNA subtraction

B1. PCR amplification of rRNA genes

This step creates sample-specific amplicon pools using DNA template extracted from samples collected in parallel with those used to generate the total RNA extract. It is important to use DNA template that roughly mirrors the taxonomic composition of the microbial community in the samples to be subtracted. The sample-specific amplicon pools 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.

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_T7 GCCAGTGAATTGTAATACGACTCACTATAGGGCTGTCTCRCGACGGTCTRAACCCA

Eukaryotes

18S:

Euk18S_1F ACCTGGTTGATCCTGCCAG
Euk18S_1520R_T7 AATTATAATACGACTCACTATAGATTCYGCAGGTTCACCTAC

28S:
Euk28S_26F ACCCGCYGAAYTTAAGCATA
Euk28S_3126R_T7 AATTATAATACGACTCACTATAGATTCTGRYTTAGAGGCGTTCAG

See DeLong et al. 1999 (AEM 65:5554-5563) for design of T7-appended primers and Hunt et al. 2006 (AEM 72:2221-2225) for bacterial 23S primers. Archaeal primers are based on conserved stretches in an alignment of taxa representing major Crenarchaeal and Euryarchaeal divisions. Eukaryotic primers are based on conserved stretches in representative marine taxa. 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).

Protocol for 50 µl reaction* (using Herculase® II Fusion polymerase, Agilent 600675):

Template DNA	100 ng	Bact 23S (35 cycles)		All other primers (35 cycles)	
Herculase 5X buffer	10µl	95°C	2 min	95°C	2 min
dNTP (10mM)	1.25µl	95°C	20 sec	95°C	20 sec
F primer (10µM)	1.25µl	39°C	20 sec	55°C	20 sec
R primer (10µM)	1.25µl	72°C	2 min	72°C	2 min
Herculase pol	1µl	72°C	3 min	72°C	3 min
H_2O	To 50µl				

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

B2: In vitro transcription (w/ T7 RNA polymerase) of biotin-labeled antisense RNA probes
---based on MEGAscript™ High Yield Transcription kit (Ambion)

For a standard 20 µl reaction*:

PCR amplicons (step B1, suggest 250-500 ng)*	1µl
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•InTM RNase inhibitor	0.5 µl
T7 RNA polymerase	2 µl

*Use Speedvac to concentrate PCR amplicons down to 250 ng/µl

* 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.

B3. rRNA subtraction with biotinylated aRNA

This step binds biotinylated aRNA probes (step B2) 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™-Spin Magnet, Invitrogen 123-20D). 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. For bacterioplankton samples, we've 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) however we can usually generate enough subtracted RNA for ScriptSeq using as little as 100 ng starting RNA template.

Part C: cDNA synthesis and Illumina library prep
---based on EpiCenter ScriptSeq v2 RNA-Seq library preparation kit

Notes:
- Remove all components except enzymes and Finishing solution, allow to thaw at RT and store on ice. Centrifuge briefly before use.
- A thermal cycler with heated lid is recommended for fragmentation and PCR
- For cDNA synthesis steps, leave lid of PCR machine open, use Styrofoam lid instead
- Otherwise, follow standard ScriptSeq protocol, reproduced below

C1. Fragment RNA and anneal cDNA synthesis primer

C2. Synthesize cDNA
Note: Use sytrofoam lid on thermocycler, not heated lid for C2-C3

Thermocycler setting for part C2:

25°C for 5 minutes
42°C for 20 minutes
37°C Pause
37°C for 10 minutes
95°C for 3 minutes
25°C Pause

C3. Terminal-tag the cDNA
Note: Use sytrofoam lid on thermocycler, not heated lid for C2-C3. Thoroughly mix the terminal-tagging premix before use.

Termocycler settings for Part C3:

25°C for 15 minutes
95°C for 3 minutes
4°C hold or ice

C4. Purify the cDNA

C5. Amplify the library and add an index
Notes: Typically, 12-15 cycles of PCR are performed, more can be done to increase library yields if needed. For 500 pg-5ng input, do 15 PCR cycles. To add an index, replace the Reverse PCR primer included with this kit with one of the ScriptSeq Index PCR primers, available from EpiCenter.

C6. Purify the RNA-Seq library

At this point, the samples are ready for pooling (check forcompatible indices) and sequencing according to the latest MiSeq sequencing protocol. For v2 sequencing kits we recommend a final pooled library concentration of 6 pM to get a 800K cluster density.

Before start

* 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.

Protocol

A1. RNA extraction

Step 1.
Thaw 2-ml screw-cap tube **on ice** (should take a while). If using sterivex filters, follow notes in annotations.

NOTES
Paul Den Uyl 24 Aug 2015
Preparation:
- Add 21 ml of ACS grade 100% ethanol to Wash Solution 1, and mix well.
- Add 40 ml of ACS grade 100% ethanol to Wash Solution 2/3, and mix well.
- Make sure the Acid-Phenol:Chloroform is not more than 6-month old.

A1. RNA extraction

Step 2.
Lyse the cells on the filters: Remove the 300 µl RNAlater carefully and discard.

A1. RNA extraction

Step 3.
Add 750 µl (1.5mL)* Lysis/Binding buffer, vortex vigorously to completely lyse the cells.

NOTES

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*If using sterivex filters: add 1.5mL

A1. RNA extraction

Step 4.

Add 75 µl (150 µl)* miRNA Homogenate Additive. Vortex to mix.

NOTES

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*If using sterivex filters: Add 150 µl

Homogenate Additive: *2M sodium acetate, PH 4; the sodium acetate buffer helps maintain the pH of the denatured cell lysate during acid extraction and provides the salt necessary for RNA precipitation later.*

A1. RNA extraction

Step 5.

Leave the mixture on ice for 10 min.

DURATION

00:10:00

A1. RNA extraction

Step 6.

Transfer the 825 µl lysate to a new Non-stick RNase-free tube or a 2 mL tube with o-ring (might need to spin the tubes to get as much lysate out as possible).

NOTES

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If using sterivex filters, split lysate equally into two tubes.

A1. RNA extraction

Step 7.

Add 750 µl Acid-Phenol:Chloroform, vortex for 30-60 sec to mix.

DURATION

00:01:00

A1. RNA extraction

Step 8.

Centrifuge for 5 min at maximum 10,000 X g at RT.

DURATION

00:05:00

NOTES

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After centrifugation, the interphase should be compact, if not, repeat centrifugation.

A1. RNA extraction

Step 9.

Carefully remove the top aqueous phase w/o disturbing the lower phase, transfer it to a fresh tube.

NOTES

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The volume of the aqueous phase should be similar to initial volume of Lysis/Binding buffer.

A1. RNA extraction

Step 10.
Pre-heat Elution Solution (or Nuclease-free water) to 95ºC.

A1. RNA extraction

Step 11.
Add 1.25 volumes of RT 100% ethanol to the aqueous phase, mix well, load to a Filter Cartridge in the collection tube (provided).

A1. RNA extraction

Step 12.
Centrifuge for 15 sec at 10,000 X g, discard the flow-through.

DURATION
00:00:15

A1. RNA extraction

Step 13.
Apply 700 µl miRNA Wash Solution 1 to the Filter Cartridge and centrifuge for 10 sec at 10,000 X g, discard the flow-through.

DURATION
00:00:10

A1. RNA extraction

Step 14.
Apply 500 µl Wash Solution 2/3, spin 15 sec at 10,000 X g, and discard the flow-through.

DURATION
00:00:15

A1. RNA extraction

Step 15.
Repeat step 14.

A1. RNA extraction

Step 16.
Put the Filter Cartridge back in the tube, centrifuge for 1.5 min at 10,000 X g to remove residual fluid from the filter.

DURATION
00:01:30

A1. RNA extraction

Step 17.
Transfer the Filter Cartridge into a fresh collection tube, apply 50 µl pre-heated (95 ºC) Elution Solution (0.1 mM EDTA) to the center of the filter.

A1. RNA extraction

Step 18.
Wait 1 minute.

DURATION
00:01:00

A1. RNA extraction

Step 19.
Spin for 30 sec at 10,000 X g.

DURATION
00:00:30

A1. RNA extraction

Step 20.
Repeat steps 17-19 with another 50 µl pre-heated (95ºC) Elution Solution.

A2. DNase Treatment

Step 21.
Add 1/10th volume of DNase I buffer (10 µl) and 2 µl DNase I (2U/µl), gently mix and incubate at 37 ºC for 30 min.

DURATION
00:30:00

NOTES
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Preparation: set heat block or hyb oven to 37C before starting this section.

A2. DNase Treatment

Step 22.
If RNeasy MinElute is to be used immediately after this, there is no need to use the DNase inactivation reagent – go directly to the RNeasy MinElute Procedure.

A2. DNase Treatment

Step 23.
Add 0.1 volume (10 µl) of DNase inactivation reagent, mix well, incubate at RT for 2 min, flicking the tube occasionally.

DURATION
00:02:00

A2. DNase Treatment

Step 24.
Centrifuge for 1.5 min at 10,000 X g, transfer RNA to a fresh new 0.5 ml non-stick Rnase-free tube, store in –70 ºC.

DURATION
00:01:30

A3. Concentrating and purifying DNase treated RNA

Step 25.
Pre-heat elution buffer or water to 40°C.

NOTES
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Preparation: add 4 volumes of ethanol (100%) to buffer RPE before starting this section.

A3. Concentrating and purifying DNase treated RNA

Step 26.
Combine two 100 µl DNase-treated RNA samples in a 2ml Nonsticky RNase-free tube (200 µl total).

NOTES
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Adjust starting volumes accordingly depending on sample - see kit instructions.

A3. Concentrating and purifying DNase treated RNA

Step 27.
Add 700 µl RLT buffer, and mix with by pipeting.

NOTES
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Adjust starting volumes accordingly depending on sample - see kit instructions.

A3. Concentrating and purifying DNase treated RNA

Step 28.

Add 500 µl 100% ethanol, mix thoroughly by pipetting. **DO NOT CENTRIFUGE.**

A3. Concentrating and purifying DNase treated RNA

Step 29.

Immediately load 700 µl onto an RNeasy MinElute Column in a 2 ml tube, close the tube gently, centrifuge for 15 s at ≥ 8,000 X g. Discard flow through.

DURATION

00:00:15

A3. Concentrating and purifying DNase treated RNA

Step 30.

Repeat step 29 to process the remaining sample volume through the column.

A3. Concentrating and purifying DNase treated RNA

Step 31.

Transfer the spin column to a new 2 ml collection tube.

A3. Concentrating and purifying DNase treated RNA

Step 32.

Pipet 500 µl **buffer RPE** onto the column, close tube gently.

A3. Concentrating and purifying DNase treated RNA

Step 33.

Centrifuge for 15 s at ≥ 8,000 X g to wash.

DURATION

00:00:15

A3. Concentrating and purifying DNase treated RNA

Step 34.

Discard the flow-through. Reuse the collection tube.

A3. Concentrating and purifying DNase treated RNA

Step 35.

Add 500 µl of 80% ethanol to the column, close tube gently.

A3. Concentrating and purifying DNase treated RNA

Step 36.

Centrifuge for 2 min at ≥ 8,000 X g, discard flow-through.

DURATION

00:02:00

A3. Concentrating and purifying DNase treated RNA

Step 37.

Transfer the column to a new 2 ml collection tube.

A3. Concentrating and purifying DNase treated RNA

Step 38.

OPEN the cap of the tube, and centrifuge at 12,000 X g for 5 min, discard flow-through.

DURATION

00:05:00

A3. Concentrating and purifying DNase treated RNA

Step 39.

Elute with 25 µl pre-heated nuclease-free water or RNA storage buffer.

A3. Concentrating and purifying DNase treated RNA

Step 40.
Transfer the spin column to a new 1.5 ml tube, pipet water or buffer directly onto the center of the silica-gel membrane, wait for 2 min at RT, close gently.

DURATION
00:02:00

A3. Concentrating and purifying DNase treated RNA

Step 41.
Centrifuge for 1 min at 12,000 X g to elute.

DURATION
00:01:00

A3. Concentrating and purifying DNase treated RNA

Step 42.
Repeat elution using 15 µl of water or buffer.

A3. Concentrating and purifying DNase treated RNA

Step 43.
Store elutions at -80°C or continue with quantification.

A4. RNA quantification

Step 44.
Depending on your application, the recommended option for RNA quantification is:

Bioanalyzer (Agilent) using the RNA 6000 Pico total RNA kit: quantitative range = 0.05-5.0 ng / µl (dilute samples 1:10 and 1:100 prior to analysis), allows visualization of RNA size distribution, less accurate, 1 hr processing time.

B1. PCR amplification of rRNA

Step 45.
Order primers (found in guidelines).

NOTES
Paul Den Uyl 26 Aug 2015
This section creates sample-specific amplicon pools using DNA template extracted from samples collected in parallel with those used to generate the total RNA extract. It is important to use DNA template that roughly mirrors the taxonomic composition of the microbial community in the samples to be subtracted. The sample-specific amplicon pools 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.

B1. PCR amplification of rRNA

Step 46.
PCR amplify rRNA.

Template DNA	100 ng	Bact 23S (35 cycles)		All other primers (35 cycles)	
Herculase 5X buffer	10µl	95°C	2 min	95°C	2 min
dNTP (10mM)	1.25µl	95°C	20 sec	95°C	20 sec
F primer (10µM)	1.25µl	39°C	20 sec	55°C	20 sec
R primer (10µM)	1.25µl	72°C	2 min	72°C	2 min
Herculase pol	1µl	72°C	3 min	72°C	3 min
H2O	To 50µl				

NOTES

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

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This is for 50 µl reaction* (using Herculase® II Fusion polymerase, Agilent 600675):

B1. PCR amplification of rRNA

Step 47.

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

NOTES

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**The Herculase buffer changes the pH of the PB buffer in this kit. You will need to add 10 µl of sodium acetate to each PB+PCR product mix to change pH indicator back to yellow.

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 48.

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

PCR amplicons (step B1, suggest 250-500 ng)*	1µl
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

NOTES

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*Use Speedvac to concentrate PCR amplicons down to 250 ng/µl

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* 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.

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 49.

At room temperature (not on ice, as spermidine in the reaction buffer may cause DNA precipitation), mix reagents in the order listed.

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 50.

Incubate at 37ºC for 4-6 hrs.

NOTES

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Recommended: use a thermocycler, or up to overnight depending on yield.

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 51.

After incubation, add 1 µl DNase I (included in the MEGAscript kit) to remove the DNA template.

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 52.

Incubate at 37ºC for an additional 30 min.

DURATION

00:30:00

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 53.

Purify synthesized RNA using the MEGAclear™ kit, with elution in 50 µl elution solution.

NOTES

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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).

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 54.

Quantify RNA concentration using either RiboGreen or Nanodrop (Nanodrop should work fine as RNA samples should be at high concentration).

B2. In vitro transcription (w/ T7 RNA polymerase) of biotin-labled antisense RNA probes

Step 55.

Store probes at -80°C.

B3. rRNA subtraction with biotinylated aRNA

Step 56.

Bead washing (do this before or during the hybridization step). For each sample, transfer 400 µl of beads* to a 1.5 ml tube.

NOTES

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*Vortex beads before use and before measuring at each step

B3. rRNA subtraction with biotinylated aRNA

Step 57.

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 quick vortex to re-suspend, spin down, re-bind to magnet and pipet off supernatant. Do this step quickly.

B3. rRNA subtraction with biotinylated aRNA

Step 58.

Wash #2: Repeat wash in step57 using 1X SSC buffer. On the 3rd wash, leave beads in buffer on ice until hybridization is complete.

B3. rRNA subtraction with biotinylated aRNA

Step 59.
Wash #3: Repeat wash in step57 using 1X SSC buffer and leave beads in buffer on ice until hybridization is complete.

B3. rRNA subtraction with biotinylated aRNA

Step 60.
Hybridization. For a 50 µl reaction*, prepare in a PCR tube:

Template, total RNA (ideally 250-500 ng in <36.5 ul)	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

NOTES

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

** To avoid pipetting small volumes, make a diluted probe master-mix (100 ng/µl) prior to starting.

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

B3. rRNA subtraction with biotinylated aRNA

Step 61.
In a thermal cycler, incubate under the following conditions:

5 min at 70ºC
Rampdown to 25°C using 5°C increments for 1 min each.

B3. rRNA subtraction with biotinylated aRNA

Step 62.
Remove the reaction and let sit at RT for 2-5 min.

DURATION

00:05:00

B3. rRNA subtraction with biotinylated aRNA

Step 63.
Bead binding. While the hybridization reaction is at RT, capture the pre-aliquoted beads (400 µl per sample) on the magnetic rack, pipet off the supernatant, and remove the beads from the rack.

B3. rRNA subtraction with biotinylated aRNA

Step 64.
Dilute the hybridization reaction in PCR tubes to 100 µl using 20% formamide in 1X SSC.

B3. rRNA subtraction with biotinylated aRNA

Step 65.
Add the hybridization reaction (now 100 µl) to the dried beads.

B3. rRNA subtraction with biotinylated aRNA

Step 66.
Incubate at RT for 10 min, with occasional flicking to mix.

DURATION

00:10:00

B3. rRNA subtraction with biotinylated aRNA

Step 67.

Bead removal. Quick spin the tubes. Capture the beads on the magnetic rack (2-3 min).

B3. rRNA subtraction with biotinylated aRNA

Step 68.

Transfer the non-rRNA-containing supernatant to a 1.5 ml tube using a P200 pipettor.

B3. rRNA subtraction with biotinylated aRNA

Step 69.

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).

B3. rRNA subtraction with biotinylated aRNA

Step 70.

Purify subtracted RNA (200 µl) to remove formamide. Use the RNeasy MinElute kit. Elute in 15 µl and 5 µl pre-heated to 50 °C (combine the elutions).

B3. rRNA subtraction with biotinylated aRNA

Step 71.

Run 1 µl of purified RNA (diluted 1:10 and 1:100) on the Bioanalyzer to confirm rRNA subtraction and probe removal.

NOTES

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Not absolutely necessary, but recommended.

B3. rRNA subtraction with biotinylated aRNA

Step 72.

Store RNA at -80°C for downstream applications.

C1. Fragment RNA and anneal cDNA synthesis primer

Step 73.

If needed, speedvac the subtracted RNA to maximum volume of 9 µl. Use all of the template material remaining after the rRNA-subtraction step.

NOTES

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- Remove all components except enzymes and Finishing solution, allow to thaw at RT and store on ice. Centrifuge briefly before use.

- A thermal cycler with heated lid is recommended for fragmentation and PCR

- For cDNA synthesis steps, leave lid of PCR machine open, use Styrofoam lid instead

- Otherwise, follow standard ScriptSeq protocol, reproduced below

C1. Fragment RNA and anneal cDNA synthesis primer

Step 74.

In a 0.2 mL PCR tube assemble the following reaction:

Nuclease-free water	x µl
rRNA-subtracted RNA	y µl

RNA fragmentation solution	1.0 µl
cDNA synthesis primer	2.0 µl
Total reaction volume	12 µl

C1. Fragment RNA and anneal cDNA synthesis primer

Step 75.

Fragment RNA by incubation at 85 ºC for 5 minutes in thermocycler with heated lid.

DURATION

00:05:00

C1. Fragment RNA and anneal cDNA synthesis primer

Step 76.

Stop fragmentation reaction by placing the tube on ice.

C2. Synthesize cDNA

Step 77.

On ice preare the cDNA synthesis master mix, volumes below are per rxn:

cDNA synthesis premix	3 µl
100 mM DTT	0.5 µl
StarScript Reverse Transcriptase	0.5 µl
Total volume	4 µl

NOTES

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Thermocycler setting found in guidelines.

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Use sytrofoam lid on thermocycler, not heated lid for C2-C3

C2. Synthesize cDNA

Step 78.

Gently but thoroughly mix the cDNA synthesis master mix by pipetting.

C2. Synthesize cDNA

Step 79.

Add 4 µl of the cDNA synthesis master mix to each reaction on ice from Part C1 and mix by pipetting.

C2. Synthesize cDNA

Step 80.

Incubate at 25 °C for 5 minutes followed by 42 °C for 20 minutes.

C2. Synthesize cDNA

Step 81.

Cool the reactions to 37 °C and pause the thermocycler.

C2. Synthesize cDNA

Step 82.

Remove one reaction at a time from the thermocycler, add 1.0 µl of Finishing solution and mix gently by pipetting. Return each reaction to the thermocycler before proceeding to the next.

C2. Synthesize cDNA

Step 83.

Incubate at 37 °C for 10 minutes.

DURATION

00:10:00

C2. Synthesize cDNA

Step 84.

Incubate each reaction at 95°C for 3 minutes, then cool reactions to 25 °C and pause the thermocycler.

DURATION

00:03:00

C2. Synthesize cDNA

Step 85.

During the 95 °C incubation prepare the terminal tagging master mix described in C3.

C3. Terminal-tag the cDNA

Step 86.

On ice, prepare the terminal tagging premix: For each reaction, combine on ice:

Terminal tagging premix	7.5 µl
DNA polymerase	0.5 µl
Total volume	8 µl

NOTES

Paul Den Uyl 27 Jul 2015

Thermocycler setting found in guidelines.

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Thoroughly mix the terminal-tagging premix before use.

C3. Terminal-tag the cDNA

Step 87.

Remove one reaction at a time from the thermocycler (25 °C paused) and add 8 µl of the terminal tagging master mix.

C3. Terminal-tag the cDNA

Step 88.

Gently mix by pipetting. Return each reaction to the thermocycler before proceeding to the next.

C3. Terminal-tag the cDNA

Step 89.

Incubate each reaction at 25 °C for 15 minutes.

DURATION

00:15:00

C3. Terminal-tag the cDNA

Step 90.

Incubate each reaction at 95 °C for 3 minutes. Then cool to 4 °C on ice or in thermocycler.

DURATION

00:03:00

C4. Purify the cDNA

Step 91.

Use the Agencourt AmpureXP system to purify the cDNA with 1.8X purification.

C4. Purify the cDNA

Step 92.

Warm Ampure beads to room temperature for 30 minutes.

DURATION
00:30:00

C4. Purify the cDNA

Step 93.
Prepare 400 µl fresh 80% ethanol at room temperature for each sample.

C4. Purify the cDNA

Step 94.
Add 45 µl of beads to each microfuge tube containing the di-tagged cDNA from part 3C.

C4. Purify the cDNA

Step 95.
Mix thoroughly by pipetting 10 times.

C4. Purify the cDNA

Step 96.
Transfer volume to 1.5 mL tube.

C4. Purify the cDNA

Step 97.
Incubate at room temp for 15 minutes.

DURATION
00:15:00

C4. Purify the cDNA

Step 98.
Place tubes in magnetic stand at room temp for at least 5 minutes until liquid clear.

DURATION
00:05:00

C4. Purify the cDNA

Step 99.
Remove and discard supernatant using a pipet, some liquid may remain in tube.

C4. Purify the cDNA

Step 100.
Ethanol Wash #1: With tubes on stand, add 200 µl 80% ethanol to each tube without disturbing the beads.

C4. Purify the cDNA

Step 101.
Ethanol Wash #1: Incubate for 30 seconds, then remove and discard supernatant.

DURATION
00:00:30

C4. Purify the cDNA

Step 102.
Ethanol Wash #2: With tubes on stand, add 200 µl 80% ethanol to each tube without disturbing the beads.

C4. Purify the cDNA

Step 103.
Ethanol Wash #2: Incubate for 30 seconds, then remove and discard supernatant.

DURATION
00:00:30

C4. Purify the cDNA

Step 104.
Allow tubes to air-dry on magnetic stand for 15 minutes at room temp.

DURATION
00:15:00

C4. Purify the cDNA

Step 105.
Add 24.5 µl of nuclease-free water to each tube and remove from magnetic stand.

C4. Purify the cDNA

Step 106.
Thoroughly resuspend beads by pipetting 10 times.

C4. Purify the cDNA

Step 107.
Incubate tube at room temperature for 2 minutes.

DURATION
00:02:00

C4. Purify the cDNA

Step 108.
Place the tube on magnetic stand at room temp for at least 5 minutes, until liquid clears.

DURATION
00:05:00

C4. Purify the cDNA

Step 109.
Transfer 22.5 µl supernatant, which contains di-tagged cDNA, from each tube to a new 0.2 mL PCR tube, place on ice.

C5. Amplify the library and add an index

Step 110.
In a 0.2 mL PCR tube containing 22.5 µl of di-tagged cDNA from C4, add on ice:

FailSafe PCR Premix E	25 µl
Forward PCR primer	1 µl
Reverse or ScriptSeq index primer	1 µl
FailSafe PCR Enzyme (1.25 U)	0.5 µl
Total Volume	50 µl

NOTES

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Typically, 12-15 cycles of PCR are performed, more can be done to increase library yields if needed. For 500 pg-5ng input, do 15 PCR cycles. To add an index, replace the Reverse PCR primer included with this kit with one of the ScriptSeq Index PCR primers, available from EpiCenter.

C5. Amplify the library and add an index

Step 111.
Perform PCR:

Denature ds DNA at 95 °C for 1 minute

Followed by 12-15 cycles of:
95 °C for 30 seconds
55 °C for 30 seconds
68 °C for 3 minutes
Finish with 68 °C for 7 minutes
Bring thermocycler temperature down to 4 °C

C5. Amplify the library and add an index

Step 112.
After PCR complete, proceed immediately to purification step C6.

C5. Purify the RNA-Seq library

Step 113.
Use the Ampure XP system to purify the PCR reaction to remove the primer-dimers that can occur during PCR.

C5. Purify the RNA-Seq library

Step 114.
Warm Ampure beads to room temperature for 30 minutes.

DURATION
00:30:00

C5. Purify the RNA-Seq library

Step 115.
Prepare 400 µl fresh 80% ethanol at room temperature for each sample.

C5. Purify the RNA-Seq library

Step 116.
Add 50 µl of beads to each tube containing amplified library from part C5.

C5. Purify the RNA-Seq library

Step 117.
Mix thoroughly by pipetting 10 times.

C5. Purify the RNA-Seq library

Step 118.
Transfer each 100 µl volume to 1.5 mL tube.

C5. Purify the RNA-Seq library

Step 119.
Incubate at room temp for 15 minutes.

DURATION
00:15:00

C5. Purify the RNA-Seq library

Step 120.
Place tubes in magnetic stand at room temp for at least 5 minutes until liquid clear.

DURATION
00:05:00

C5. Purify the RNA-Seq library

Step 121.
Remove and discard supernatant using a pipet, some liquid may remain in tube.

C5. Purify the RNA-Seq library

Step 122.

Ethanol Wash #1: With tubes on stand, add 200 µl 80% ethanol to each tube without disturbing the beads.

C5. Purify the RNA-Seq library

Step 123.

Ethanol Wash #1: Incubate for 30 seconds, then remove and discard supernatant.

DURATION

00:00:30

C5. Purify the RNA-Seq library

Step 124.

Ethanol Wash #2: With tubes on stand, add 200 µl 80% ethanol to each tube without disturbing the beads.

C5. Purify the RNA-Seq library

Step 125.

Ethanol Wash #2: Incubate for 30 seconds, then remove and discard supernatant.

DURATION

00:00:30

C5. Purify the RNA-Seq library

Step 126.

Allow tubes to air-dry on magnetic stand for 15 minutes at room temp.

DURATION

00:15:00

C5. Purify the RNA-Seq library

Step 127.

Add 20 µl of nuclease-free water to each tube and remove from magnetic stand.

C5. Purify the RNA-Seq library

Step 128.

Thoroughly resuspend beads by pipetting 10 times.

C5. Purify the RNA-Seq library

Step 129.

Incubate tube at room temperature for 2 minutes.

DURATION

00:02:00

C5. Purify the RNA-Seq library

Step 130.

Place the tube on magnetic stand at room temp for at least 5 minutes, until liquid clears.

DURATION

00:05:00

C5. Purify the RNA-Seq library

Step 131.

Transfer the supernatant, which contains the RNA-Seq library, from each tube to a new tube.

C5. Purify the RNA-Seq library

Step 132.

Make 1:10 and 1:100 dilutions for Bioanalyzer analysis of average library size.

C5. Purify the RNA-Seq library

Step 133.

Perform Picogreen to get accurate quantification of library concentration.

C5. Purify the RNA-Seq library

Step 134.

At this point, the samples are ready for pooling (check for compatible indices) and sequencing according to the latest MiSeq/NextSeq sequencing protocol. For v2 sequencing kits with the MiSeq and NextSeq, we recommend a final pooled library concentration of 6 pM and 1.0 pM respectively.