Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: SM-A4SE5 Small intestine terminal ileum

RNA ID: 376T

Library ID : 296640

Composite Library ID: 296642

Protocol ID: Illumina Truseq Small RNA, Ribo V2

Cold Spring Harbor Laboratory
Genome Center
500 Sunnyside Blvd
Woodbury, NY 11797

LAB MEMBERS

Wet lab: Carrie A. Davis, Jorg Drenkow, Lei Hoon See.
Computational Lab: Alex Dobin, Chris Zaleski.

PI: Tom Gingeras

Sample: Tissues were provided by G-Tex

Kits: RNeasy Mini kit (QIAGEN cat#: 74106)
miRNeasy Mini kit (QIAGEN cat#:217004)
RNeasy MinElute cleanup kit (QIAGEN cat#:74204)
MirVana miRNA Isolation Kit Cat(Ambion cat#: AM1561)

RNA extraction from Human Tissue
Guidelines for Disruption and Homogenization of Tissues Using the TissueLyser II
The TissueLyser II and TissueLyser Adapter Set 2 x 24 allow high throughput, rapid, and effective disruption of 48 biological samples in 2–4 minutes. Homogenization and disruption with the TissueLyser II gives results comparable to using rotor–stator homogenization. The following guidelines can be used for disruption and homogenization of tissues using the TissueLyser II. Be sure to work quickly in order to prevent RNA degradation.

Either one of the three RNA extraction methods was used depending on the tissue type. Please see below for the appropriate method for each tissue type.

RNeasy Fibrous Tissue Mini Kit (Qiagen)
Reagents :
Things to do before starting
• β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.
•Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96 – 100%) as indicated on the bottle to obtain a working solution.
•Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNasefree water provided in the RNase-Free DNase Set box. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex. For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure
1. Heat a water bath or heating block to 55°C for proteinase K digestion.
2On dry ice, excise a piece of tissue < 30mg, keeping the tissue frozen at all times. Weigh the tissue.
3. Add 300µl Buffer RLT with β-ME to the tissue
4. Then add 1.5ul reagent DX (Qiagen) to reduce foaming of sample.
5. Add 1/3 volume of Zirconia beads (1mm diameter, Biospec Products) to the tube and close the tube tightly. Use parafilm to wrap around the lid of the tube to prevent leakage.
6. Position the tubes in the outermost rack positions on the TissueLyser. Set the Tissue

Lyser to 30Hz and beat for 5 min. TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.
7. Carefully pipet the lysates into new microcentrifuge tubes (not supplied). Do not reuse the Zirconia beads.
8. Add 590 µl RNase-free water to the lysate. Then add 10 µl proteinase K solution, and mix thoroughly by pipetting.
9. Incubate at 55°C for 10 min.
10.Centrifuge at 20–25°C for 3 min at 10,000 x g. A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.
11. Pipet the supernatant into a new 1.5 ml or 2 ml microcentrifuge tube (not supplied).
Avoid transferring any of the pellet. If this is unavoidable, a small amount of pelleted debris may be carried over without affecting the RNeasy procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.
12. Add 0.5 volumes of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge. Precipitates may be visible after addition of ethanol. This does not affect the procedure.
13. Transfer 700 µl of the sample, including any precipitate that may have formed, to a RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge at 20–25°C for 15 s at 8000 x g (10,000 rpm). Discard the flowthrough.* Reuse the collection tube in step 14.
14. Repeat step 13 using the remainder of the sample. Discard the flow-through.*
15. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at 8000 x g (10,000 rpm) to wash the membrane. Discard the flow-through.*Reuse the collection tube in step 15.
Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at _8000 x g, and discard the flow-through* (but not the collection tube). Proceed to step 16.
* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.
16. Add 10µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.
17. Add the DNase I incubation mix (80µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.
Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.
18. Add 350µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) at 20–25°C. Discard the flow-through.*Reuse the collection tube in step 18.
19. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at 8000 x g (10,000 rpm) to wash the membrane. Discard the flow-through. Reuse the collection tube in step 19.
Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

20. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 2 min at 8000 x g (10,000 rpm) to wash the membrane.
The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
21. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 20.
* Flow-through contains Buffer RLT and is therefore not compatible with bleach.
22. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add30–50 µl RNase-free water directly to the RNeasy spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at 8000 x g (10,000 rpm) at 20–25°C.
23. Repeat step 22 using another 30–50µl RNase-free water, or using the eluate from step 19 (if high RNA concentration is required). Reuse the collection tube from step 22. If the expected RNA yield is >30µg, there is no need to repeat step 22. If using the eluate from step 22, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.
24. Proceed with Ethanol precipitation.

RNeasy Lipid Tissue Mini Kit (Qiagen)

Notes before starting
Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

1. Disrupt and homogenize ≤100 mg fatty tissue (≤50 mg other tissue) in 1 ml QIAzol Lysis Reagent and 5ul reagent DX (Qiagen) using the TissueRuptor®, TissueLyser LT, or TissueLyser II (see Table 1).
2. Incubate the homogenate at room temperature (15–25°C) for 5 min.
3. Add 200µl chloroform, and shake vigorously for 15 s.
4. Incubate sample at room temperature for 2–3 min.
5. Centrifuge at 12,000 x g for 15 min at 4°C.
6. Transfer upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 1 volume of 70% ethanol, and vortex. Do not centrifuge. Proceed at once to step 7.
7. Transfer up to 700 µl of the sample to RNeasy Mini spin column in 2 ml collection tube (supplied). Close the lid, centrifuge at room temperature for 15 s at ≥8000 x g, and discard flow-through.
8. Using the same collection tube, repeat step 7 using the remainder of the sample. Discard the flow-through.
Optional DNase digest: Follow steps in “Optional on-column DNase digestion with the RNase-Free DNase Set” in Appendix C of the RNeasy Lipid Tissue Handbook.
9. Add 700 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g, and discard flow-through. (Skip this step if performing optional DNase digestion.)
10. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g, and discard flow-through.

11. Add 500 µl Buffer RPE to RNeasy column. Close lid and centrifuge for 2 min at ≥8000x g.
Optional: To further dry membrane, place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.)
12. Place RNeasy column in a new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid, and centrifuge for 1 min at ≥8000 x g.

miRNeasy Mini Kit

1. On dry ice, excise a piece of tissue 50 –80 mg, keeping the tissue frozen at all times.
Add to a collection tube and weigh the tissue.
2. Pipet 700µl QIAzol Lysis Reagent and 3.5ul reagent DX (Qiagen, to reduce foaming of sample) into the tube.
3. Add 1/3 volume of Zirconia beads (1mm diameter, Biospec Products) to the tube and close the tube tightly. Use parafilm to wrap around the lid of the tube to prevent leakage.
4. Position the tubes in the outermost rack positions on the Tissue Lyser. Set the TissueLyser to 30Hz and beat for 5 min. TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.
5. Carefully transfer the homogenate to a new microcentrifuge tube (not supplied) by pipetting. Do not reuse the stainless steel bead.
6. Place the tube containing the homogenate on the benchtop at room temperature (15– 25°C) for 5 min. This step promotes dissociation of nucleoprotein complexes.
5. Add 140µl chloroform to the tube containing the homogenate and cap it securely.
Shake the tube vigorously for 15 s.Thorough mixing is important for subsequent phase separation.
6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.
7. Centrifuge for 15 min at 12,000 x g at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.
After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 350 µl.
Note: If you want to purify a separate miRNA-enriched fraction, follow the steps in Appendix A (page 31) after performing this step.
8. Transfer the upper aqueous phase to a new collection tube (supplied). Add 1.5 volumes (usually 525 µl) of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9. A precipitate may form after addition of ethanol, but this will not affect the RNeasy procedure.
9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.*
Reuse the collection tube in step 10.
10. Repeat step 9 using the remainder of the sample. Discard the flow-through.*
Reuse the collection tube in step 11.
Optional: If performing optional on-column DNase digestion (see “Important points before starting”), follow the steps in Appendix B (page 34) after performing this step.

11. Add 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow-through.†
Skip this step if performing the optional on-column DNase digestion (page 34). Reuse the collection tube in step 12.
12. Pipet 500 µl Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow-through.
Reuse the collection tube in step 13.
13. Add another 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the RNeasy Mini spin column membrane.
The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
Note: Following centrifugation, remove the RNeasy Mini spin column from the collection tube carefully so the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
14. Optional: Place the RNeasy Mini spin column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE or if residual flow-through remains on the outside of the RNeasy Mini spin column after step 13.
15. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube (supplied). Pipet 30–50µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
16. If the expected RNA yield is >30µg, repeat step 15 with a second volume of 30–50µl RNase-free water. Elute into the same collection tube. To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 15). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

Ethanol Precipitation (same for Total, Small and Large RNA)

1. Add 2.5 volumes of 100% ethanol and 1/10 volumes of NaOAc PH 5.5 to the eluted RNA.
2. Freeze in -80°C for 2hrs.
3. Centrifuge for 30 min at max speed at 4°C.
4. Pipette and discard the supernatant making sure not to touch the pellet of RNA.
5. Wash with 500µL of 70% ethanol and centrifuge at max speed for 5 min.
6. Pipette and discard the supernatant.
7. Open the cap and speed vacuum at low heat for 3-5 min making sure that the pellet is dry.
8. Resuspend the pellet with RNase-free water.

Long and Small RNA Separation

MirVana kit: Ambion MirVana miRNA Isolation Kit Cat # AM1561

1. Add 5 volumes of Lysis/Binding solution to the total RNA.

2. Vortex or pipet to completely lyse cells and create a homogenous lysate.

3. Add 1/10 volume of miRNA Homogenate Additive to the cell lysate and mix well by vortexing or inverting tube several times.

4. Leave the mixture on ice for 10 minutes.

5. Add 1/3 volume of 100% ethanol to the RNA mixture from before.

6. Mix thoroughly by vortexing or inverting tube several times.

7. For each sample, place a Filter Cartridge into one of the collection tubes supplied.

8. Pipet up to 700 uL of the lysate/ethanol mixture (from the previous step) onto the filter cartridge.

9. Centrifuge for approximately 15 seconds at 5,000 x g to pass the mixture through the filter (vacuum can also be used). Apply the mixture in successive applications to the same filter and repeat centrifugation until all the mixture is passed through. **(Do not discard the filtrate- this contains the small RNA, while the filter contains the Long RNA. Save the filter cartridge and wash the Long RNA using RNA cleanup section.)**

10. Collect the filtrate (pool successive passes through the filter in a new tube) and record the volume.

11. Add 2/3 volume room temperature 100% ethanol to filtrate (flow-through) and mix thoroughly.

12. Pass 700 uL of each sample through a second filter cartridge and centrifuge for 15 seconds at 5,000 x g.

13. Discard the flow through.

14. Continue passing sample until the entire sample is through the filter.

15. Proceed to **RNA Cleanup**.

RNA Cleanup

1. Apply 700 uL miRNA Wash Solution 1 (make sure ethanol was added) to the filter cartridge and centrifuge for 5-10 seconds (can also use vacuum).

2. Discard the flow through.

3. Apply 500 uL Wash Solution 2/3 (make sure ethanol is added) to filter cartridge and centrifuge for 5-10 seconds (can also use vacuum).

4. Discard the flow through.

5. Repeat steps 3 and 4.

6. Spin the filter cartridge for 1 minute at 10,000g to remove residual fluid from the filter.

7. Transfer the filter cartridge into a new collection tube and apply 100 uL of pre-heated (95°C) nuclease free water to the filter.

8. Spin for 20-30 seconds at 10,000 x g to elute RNA.

9. Proceed to Ethanol Precipitation.

DNase I treatment (same for Small and Large RNA)

Reagents	*100 µL Sample (100 µg RNA max)*	*50 µL Sample (50 µg RNA max)*
Total RNA (100 µg max)	78 µL	39 µL
10X One-phor-all Buffer	10 µL	5 µL
10 U/µL DNase/RNase Free	8 µL	4 µL
20 U/µL RNasin/anti-RNase	4 µL	2 µL
Total Volume	*100 µL*	*50 µL*

1. Add all reagents to resuspended RNA and pipette to mix well.
2. Place in a 37°C waterbath for 30 min.
3. Proceed to RNA Cleanup, which is different for Small and Large RNA.

Small RNA Cleanup

1. Make sure there is a max of 45 µg RNA in the 100 µL RNA sample.
2. Add 350 µL Buffer RLT to the 100 µL sample of RNA. Vortex to mix well.
3. Add 675 µL of 100% ethanol to the reaction and mix by inverting.
4. Transfer 700 µL of sample into an RNeasy MinElute column in a 2 mL collection tube. Close the lid gently and centrifuge for 30 s at 8000 x g (≥10,000 rpm). Discard the flow through. Repeat this step with the remaining sample.
5. Add 700 µL Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow through.
6. Pipet 500 µL Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
7. Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the spin column membrane. Discard the flow-through and the collection tube.
8. Place the RNeasy MinElute spin column into a new 2 mL collection tube, making sure that the column does not come in contact with the flow through. Open the lid and centrifuge for 5 min at ≥8000 x g (≥10,000 rpm).
9. Place the RNeasy MinElute spin column into a 1.5 mL collection tube and pipet 20 µL RNase free water onto the spin column membrane. Close the lid gently and wait 1 min. Then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).

10. Repeat step 9 with a second volume of 20 µL RNase free water.
11. Proceed to ethanol precipitation.

RIBOMINUS TREATMENT:

Kits : RiboMinus™ Eukaryote System v2 (Life Technologies cat# A15026)

Before you begin:
• Pre-heat 2X Hybridization Buffer in a 50°C heat block or bath, to bring salts into solution.
• Set heat blocks to 37°C and 70°C. Arrange heat blocks in close proximity to each other for optimal transfer and slow cooling in step 3.
1. Add the following components to a sterile, RNase-free 1.5-mL microcentrifuge tube in the order listed:

Component	**Volume**
2X Hybridization Buffer	50 µL
RiboMinus™ Eukaryote Probe Mix v2	4 µL
Total RNA, 1–5 µg	X µL
Nuclease-free Water	to 100 µL

2. Mix by gentle vortexing, and incubate the tube with the RNA/probe mix sample (100 µL) at 70°C for 10 minutes to denature the RNA.
3. Immediately transfer the tube to a 37°C heat block, and allow the RNA/probe mix sample to cool to 37°C over a period of 20 minutes.
IMPORTANT! Do not allow the sample to cool quickly by excessive time at room temperature during transfer to 37°C or by placing the tubes on ice. Slow cooling promotes sequence-specific hybridization.
While the sample is cooling, prepare the RiboMinus™ Magnetic Beads as described in the following section.

Prepare RiboMinus™ Magnetic Beads

1. Resuspend the RiboMinus™ Magnetic Beads in its bottle (blue cap) by thorough vortexing.
2. For each RNA sample, prepare 200 µL of 1X Hybridization buffer by diluting 2X Hybridization Buffer with an equal volume of Nuclease free Water.
3. For each sample, pipet 500 µL of bead suspension into a sterile, RNase-free, 1.5-mL microcentrifuge tube.
4. Place each tube with the bead suspension on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
5. Remove the tubes from the magnetic stand, and wash the beads with 500 µL Nuclease free Water by dispensing the water down the side of the tube where the beads are collected.
6. Place each tube on a magnetic stand for 1 minute or until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.
7. Repeat steps 5–6 once.

8. Resuspend the beads in 200 µL of prepared 1X Hybridization Buffer. Place the prepared beads in a 37°C heat block for at least 5 minutes, or longer until the 20-minute incubation of the RNA/probe mix at 37°C is complete.

Capture and remove rRNAprobe complexes

1. After the 20-minute incubation of the RNA/probe mix at 37°C is complete, briefly centrifuge the RNA/probe mix to collect the mixture at the bottom of the tube.
2. Transfer the RNA/probe mix (100 µL) to the prepared RiboMinus™ Magnetic Beads (200 µL). Mix well by pipetting up and down or by low speed vortexing.
3. Place the tube in a 37°C heat block or bath, and incubate for 5 minutes.
4. Briefly centrifuge the tube and place it on a magnetic stand for 1 minute or until the solution clears.
5. Transfer the supernatant (~300 µL) containing the
rRNA-depleted RNA to a new tube.
IMPORTANT! Do not discard the supernatant—this contains the rRNA-depleted RNA.
6. Proceed to Ethanol Precipitation.

RNA 5'Pyrophosphohydrolase (RppH, NEB M0356S)

1. Denature RNA at 65^{o}C for 5 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
    a. RNA 85 µL
    b. 10X Thermopol reaction buffer 10 µL
    c. Anti-RNase (Ambion 20U/uL) 1 µL
    d. RppH (5 U/µL) 4 µL
3. Incubate at 37^{o}C for 1hr.
4. Proceed to **Small RNA Cleanup**.
5. Proceed to ethanol precipitation.
6. After drying the pellet, resuspend in 5 µL H_2O.

LIBRARY PROTOCOL:

The Illumina Truseq small RNA sample preparation protocol is used to make the small RNA library. Two bioreplicates were made starting with 1µg RNA each for the Ribominus step and using 2 different primer indices in the PCR. Both libraries were pooled at the end and sequenced on a single lane.

***3' and 5'* Ligation**

This process describes the sequential ligation of the RNA 3' and RNA 5' RNA adapters to the sample. Start the protocol with at least 100ng total small RNA.

Illumina-supplied consumables:
- Ligation buffer (HML)
- 10 mM ATP
- RNA 3' adapter (RA3)
- RNA 5' adapter (RA5)
- RNase inhibitor
- Stop solution (STP)
- T4 RNA ligase
- Ultrapure water

User-supplied consumables:
- T4 RNA ligase 2, truncated

Ligate 3' adapter

1. Set up the ligation reaction in a sterile PCR tube on ice:

RNA 3' adapter diluted 1/2 (RA3)	1 µL
Small RNA in dH_2O	5 µL

2. Gently pipette up and down 6-8 times to mix thoroughly, then spin down briefly.
3. Incubate at 70^{o}C for 2 min and then immediately place on ice.
4. Preheat the thermal cycler to 28^{o}C.
5. Prepare the following mix in a separate PCR tube on ice :

Ligation buffer (HML)	2 µL
RNase inhibitor	1 µL
T4 RNA ligase 2, truncated	1 µL

6. Gently pipette up and down 6-8 times to mix thoroughly, then spin down briefly.
7. Add 4 µL of the mix to the reaction tube from step 1 and gently pipette the entire volume up and down 6-8 times to mix thoroughly. The total volume should be 10 µL.
8. Incubate the tube at 28^{o}C for 1 hr.
9. With the reaction remaining on the thermal cycler, add 1 µL Stop solution (STP) and gently pipette the entire volume up and down 6-8 times to mix thoroughly. Continue to incubate the reaction at 28^{o}C for 15 min, and then place the tube on ice.

Ligate 5' adapter

1. Preheat the thermal cycler to 70^{o}C.
2. Aliquot 1.1 X N µL of the RNA 5' adapter (RA5, diluted 1/2) into a separate PCR tube, with N equal to the number of samples being processed for the current experiment.
3. Incubate the adapter at 70^{o}C for 2 min and then immediately place on ice.

4. Pre-heat the thermal cycler to 28°C.
5. Add 1.1 X N µL of 10mM ATP to the RA5 tube. Gently pipette up and down 6-8 times to mix thoroughly.
6. Add 1.1 X N µL of T4 RNA ligase to the RA5 tube. Gently pipette up and down 6-8 times to mix thoroughly.
7. Add 3 µL of the mix to the reaction from step 9 of Ligate 3' adapter. Gently pipette up and down 6-8 times to mix thoroughly. The total reaction volume should now be 14 µL.
8. Incubate the reaction tube at 28°C for 1 hr and then place the tube on ice.

Reverse transcribe and amplify

Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 3' and 5' adapters. This process selectively enriches those fragments that have adapter molecules on both ends. PCR is performed with 2 primers that anneal to the ends of the adapters.

Illumina-supplied consumables:
- 25 mM dNTP mix, dilute to 12.5 mM with water
- PCR mix (PML)
- RNA PCR primer (RP1)
- RNA PCR primer index 1- 48 (RPI1-RPI48)
- RNA RT primer (RTP)
- RNase inhibitor
- Ultrapure water

User-supplied consumables:
- 5' and 3' adapter-ligated RNA (6 µL)
- 5X first strand buffer
- 100mM DTT
- Superscript III reverse transcriptase

Reverse transcription

1. Combine the following in a separate PCR tube :

5' and 3' adapter-ligated RNA	6 µL
RNA RT primer diluted 1/2 (RTP)	1 µL

2. Gently pipette up and down 6-8 times to mix thoroughly, then spin down briefly.
3. Incubate the tube at 70°C for 2 min and then immediately place on ice.
4. Preheat the thermal cycler to 50°C.
5. Prepare the following mix in a separate tube on ice:

5X first strand buffer	2 µL
12.5 mM dNTP mix	0.5 µL

100mM DTT	1 µL
RNase inhibitor	1 µL
Superscript III reverse transcriptase	1 µL

6. Gently pipette up and down 6-8 times to mix thoroughly, then spin down briefly.
7. Add 5.5 µL of the mix to the reaction tube from step 3. Gently pipette up and down 6-8 times to mix thoroughly, then spin down briefly. The total volume should now be 12.5 µL.
8. Incubate the tube at 50^{o}C for 1 hr and then place the tube on ice.

PCR amplification

1. Set up the reaction by adding :

Ultrapure water	10.5 µL
PCR mix (PML)	25 µL
RNA PCR primer diluted ½ (RP1)	1 µL
RNA PCR primer index (RPI1- 8)*	1 µL

2. Gently pipette up and down 6-8 times to mix thoroughly, spin down briefly and then place the tube on ice.
3. Add 37.5 µL of PCR master mix to the reaction tube from step 8.
4. Gently pipette up and down 6-8 times to mix thoroughly, spin down briefly and then place the tube on ice. The total volume should now be 50 µL.
5. Amplify the reaction in the thermal cycler using the following PCR cycling conditions:

1. 98^{o}C 30s.
2. 98^{o}C 10 s.
3. 60 ^{o}C 30 s.
4. 72 ^{o}C 15 s.
5. Go back to step 2 and repeat 16 more times.
6. 72 ^{o}C 10min
7. 4 ^{o}C forever

*1 index primer was used for each sample. Indices 1-8 from the Truseq kit were used.

AMPure XP PCR Cleanup Protocol

(Agencourt AMPure XP beads Cat. # A63881)

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled. Add 1.2X volume of Agencourt AMPure XP to sample.

2. Mix reagent and PCR reaction thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.

3. Place the reaction tubes onto a magnetic stand for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.

4. Aspirate the cleared solution and discard.

5. Dispense 200 µL of 70% ethanol in each reaction and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.

6. Let the beads air dry for 5 minutes off of the magnet.

7. Off of the magnet add 50 µL of elution buffer (Qiagen Elution Buffer was used) and pipette mix 10 times.

8. Place the tubes back on the magnet for 1 minute to separate beads from the solution.

9. Transfer the eluant to a new tube.

10. Measure sample concentration on Thermo Scientific NanoDrop and run final libraries on Agilent Bioanalyzer to visualize final library.

Starting small RNA	After Ribominus	Final library