Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: Biochain 118S
Sample ID : SID38462
Library ID: LID57189
Protocol ID: Illumina Truseq Small RNA

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RNA Isolation

Human tissue total RNA was received from BioChain and run out on the bioanalyzer to assess the quality. Then long and small RNA were separated using this MirVana kit procedure (Ambion MirVana miRNA Isolation Kit Cat # AM1561).

1. Add 1.2 mL Lysis/Binding solution to 100-10^7 cells containing around 200 uL of RNA later (add more lysis/binding solution for greater volumes of RNA later).

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 an equal volume of Acid-Phenol:Chloroform to the volume of the lysate before the addition of miRNA Homogenate Additive.

6. Vortex for 30-60 seconds to mix.

7. Centrifuge for 5 minutes at maximum speed (10,000 x g) at room temperature to separate the aqueous and organic phases. After centrifugation the interphase should be compact; if not repeat centrifugation.

8. Carefully remove the aqueous (upper) phase without disturbing the lower phase and transfer it to a fresh tube. Note the volume of the aqueous phase removed.

Separating Large RNA (start here if total RNA has already been extracted)

1. Mix total RNA with 5 volumes Lysis/Binding Buffer.

2. Add 1/10 volume of miRNA Homogenate Additive to the RNA mixture from the previous step, and mix well by vortexing or inverting the tube several times. Leave the mixture on ice for 10 min.

3. Add 1/3 volume of 100% ethanol to the RNA mixture from the previous step. Mix thoroughly by inverting the tube several times.

4. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet 700 uL of the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter. Centrifuge at 5,000 x g for 30 seconds to pass the mixture through the filter. Collect the filtrate. (If the RNA mixture is >700 µL, transfer *the flow-through* to a fresh tube, and repeat until all of the RNA mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrate.) (Proceed to Separating Small RNA section to use the filtrate containing the small RNA.)

5. Proceed to RNA Cleanup.

Separating Small RNA

1. Add 2/3 volume room temperature 100% ethanol to filtrate (i.e.flow-through) acquired in step 4 of long RNA procedure.

2. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet 700 uL of the filtrate/ethanol mixture (from the previous step) onto a second Filter Cartridge. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter. Centrifuge for ~1 min at 5,000 x g to pass the mixture through the filter. Discard the flow-through, and repeat until all of the filtrate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.

3. 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 at 5,000 x g (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 at 5,000 x g (can also use vacuum).

4. Discard the flow through.

5. Repeat steps 3 and 4.

6. Spin the filter cartridge for 1 minute at 5,000 x g 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. Ethanol precipitate RNA.

Ethanol Precipitation

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 at least 30 min.

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 500 uL 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 5 min making sure that the pellet is dry.

8. Resuspend the pellet with RNase-free water.

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:

Hybridization Step
Instructions are provided below to perform hybridization for 1–10 µg of your total RNA sample with the RiboMinus™ Eukaryote Probe. To process >10 µg total RNA sample, divide your sample into two samples, each containing <10 µg total RNA.

1. Set a water bath or heat block to 70–75°C.
2. To a sterile, RNase-free 1.5 mL microcentrifuge tube, add the following:
Total RNA (1 µg): <10 µL
RiboMinus™ Probe (15 pmol/L): 8 µL
Hybridization Buffer: 100 µL

3. Incubate the tube at 70–75°C for 5 minutes to denature RNA.
4. Allow the sample to cool to 37°C slowly over a period of 30 min by placing the tube in a 37°C water bath (a heat block works as well). To promote sequence-specific hybridization, it is important to allow slow cooling. **Do not** cool samples quickly by placing tubes in cold water.
5. While the sample is cooling down, proceed to **Preparing Beads**.

*****An earlier version of this protocol says to use RNA in less than 20 µL, add 10 µL of probe and 300 µL hybridization buffer, this larger volume means you need to precipitate the ribominused RNA in a 2 mL tube at the end. Either way works. It doesn't change anything else expect the supernatant volumes and the precipitation tube size.

Preparing Beads

1. Resuspend RiboMinus™ Magnetic Beads in its bottle by thorough vortexing.
2. Pipet 750 µL of the bead suspension into a sterile, RNase-free, 1.5 mL microcentrifuge tube.
3. Place the tube with the bead suspension on a magnetic separator for 1 min. The beads settle to the tube side that faces the magnet. Gently aspirate and discard the supernatant.
4. Add 750 µL sterile, DEPC Water to the beads and resuspend beads by pipetting
5. Place tube on a magnetic separator for 1 min. Aspirate and discard the supernatant.
6. Repeat Steps 4–5 once.
7. Resuspend beads in 750 µL Hybridization Buffer and transfer 250 µL beads to a new tube and maintain the tube at 37ºC for use at a later step.
8. Place the tube with 500 µL beads on a magnetic separator for 1 min. Aspirate and discard the supernatant.
9. Resuspend beads in 200 µL Hybridization Buffer and keep the beads at 37°C until use.

Removing rRNA
1. After the incubation at 37°C for 30 min of the hybridized sample (above), briefly centrifuge the tube to collect the sample to the bottom of the tube.
2. Transfer the sample (~120 µL - this will be ~330 µL with the older protocol) to the prepared RiboMinus™ Magnetic beads from Step 9 (**Preparing Beads**, above). Mix well by pipetting up and down
3. Incubate the tube at 37°C for 15 min. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
4. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. **Do not discard the supernatant. The supernatant contains RiboMinus™ RNA.**
5. Place the tube with 250 µL beads from Step 7 (**Preparing Beads**, above) on a magnetic separator for 1 min. Aspirate and discard the supernatant.
6. To this tube of beads, add ~320 µL (~500 µL with older protocol) supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or low speed vortexing.
7. Incubate the tube at 37°C for 15 min. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
8. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. **Do not discard the supernatant as the supernatant contains RiboMinus**™ RNA.
9. Transfer the supernatant (~ 320 µL - ~500 µL with older protocol) containing **RiboMinus™ RNA** to a small filter column and spin at max speed for 2 min to remove any remaining magnetic particles.
10. Transfer flow through (ribominus RNA) to a new tube (1.5 mL for small volume, 2 mL for large volume)
11. Ethanol precipitate as before but add 1 µL glycoblue to facilitate the precipitation.
12. After drying the pellet, resuspend in 43.5 µL H_2O. Keep 1 µL for running a small RNA Bioanalyzer chip.

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 (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°C for 2 min and then immediately place on ice.
4. Preheat the thermal cycler to 28°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°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°C for 15 min, and then place the tube on ice.

Ligate 5' adapter

1. Preheat the thermal cycler to 70°C.
2. Aliquot 1.1 X N µL of the RNA 5' adapter (RA5) 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°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 (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°C for 1 hr and then place the tube on ice.

PCR amplification

Sequences of primers:

RPIC	CAAGCAGAAGACGGCATACGAGATCACACAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
RPIA	CAAGCAGAAGACGGCATACGAGATACACACGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
RPIG	CAAGCAGAAGACGGCATACGAGATGTGTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
RPIT	CAAGCAGAAGACGGCATACGAGATTGTGTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

1. Set up the reaction by adding :

Ultrapure water	8.5 µL
PCR mix (PML)	25 µL
RNA PCR primer (RP1)	2 µL
RNA PCR primer index* (12.5 µM)	2 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°C	30s.
2. 98°C	10 s.

3. 60 °C 30 s.
4. 72 °C 15 s.
5. Go back to step 2 and repeat 14 more times.
6. 72 °C 10min
7. 4 °C forever

* In this experiment, the RNA PCR primer index used were custom designed RPIA/RPIC in one bioreplicate and RPIG/RPIT in the other bioreplicate that is to be multiplexed together.

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 1.5X 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 ThermoScientific NanoDrop and run final libraries on Agilent Bioanalyzer to visualize final library.

Staring small RNA	After PCR	Final library