Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: GM12878 190S

RNA ID: 190S

Sample ID: SID38799

Protocol ID: Illumina TruSeq Small RNA Library Protocol

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RNA Isolation

GM12878 cell pellets (190WC had 4.8 x 10^7 cells and 191WC had 4.5 x 10^7 cells) were received from Brent Graveley at UCHC Genetics and Develpmental Biology and small and large RNA was extracted using the Qiagen miRNeasy Mini Kit.

Kits: miRNeasy Mini kit (QIAGEN cat #:217004)
RNeasy MinElute cleanup kit (QIAGEN cat #:74204)

1. Add 1 mL of QIAzol Lysis Reagent for every 10^7 cells. Add QIAzol the pellet and vortex to mix.
2. If number of cells is $\leq 3x10^{6,}$ vortex for 1 min to homogenize the cells.
If number of cells is $>3x10^{6,}$ homogenize by placing 700 µL of sample into a QIAshredder homogenizer and centrifuge for 2.5 min at maximum speed.
3. Place the tube(s) containing the homogenate on the benchtop at room temperature (15-25°C) for 5 min.
4. Add 20% volume of chloroform to the homogenate and cap it securely. Shake the tube vigorously for 15 s.
5. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 min.
6. Centrifuge the homogenate for 15 min at 12,000 x g at 4°C.
7. Transfer the upper, colorless, aqueous phase containing the RNA to a new collection tube.
8. Add an equal volume of 70% ethanol and mix thoroughly by vortexing. Do not centrifuge.
9. Pipet 700 µL of the sample including any precipitate that may have formed into an RNeasy Mini spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Repeat this step until the whole sample has been pipetted into the spin column and discard the flow-through each time.
10. Pipet the flow-through (which contains miRNA) into a 2 mL RNase free tube. The spin column contains the large RNA.

Small RNA Purification

S1. Add 450 µL of 100% ethanol (0.65 volumes) to the flow-through from step 15 and mix thoroughly by vortexing. Do not centrifuge.
S2. Pipet 700 µL of the sample into an RNeasy MinElute spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Discard the flow through. Repeat this step until the whole sample has been pipetted into the spin column and discard the flow-through each time.
S3. 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.
S4. 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.
S5. 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.
S6. 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).

S7. 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) to elute the small RNA fraction. Repeat with a second volume of 20 µL RNase free water.
S8. Proceed to ethanol precipitation.

Ethanol Precipitation (same for 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 at least 30 min.
3. Centrifuge for 35 min at max speed at 4°C.
4. Pipette and discard the supernatant making sure not to touch the RNA pellet.
5. Wash with 1 mL 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.

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 21.25 µL H_2O. Keep 1 µL for running a small RNA Bioanalyzer chip.

Tobacco Acid Pyrophosphatase Reaction (TAP, Epicentre T19250)

1. Denature RNA at 65°C for 5 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
    a. RNA 21.25 µL
    b. 10X TAP reaction buffer 2.5 µL
    c. Anti-RNase (Ambion 20U/uL) 1 µL
    d. TAP (10 U/µL) 0.25 µL
3. Incubate at 37°C for 1hr.
4. Proceed to small RNA column cleanup, then ethanol precipitation.
5. After drying the pellet, resuspend in 5 µL H_2O.

Small RNA Column Cleanup

1. Bring sample up to 100 µL. (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.

Ilumina TruSeq Library Protocol

The Illumina Truseq small RNA sample preparation protocol is used to make the small RNA library. Two bioreplicates were made starting with 1 µg 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 100 ng 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.

RT and PCR
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

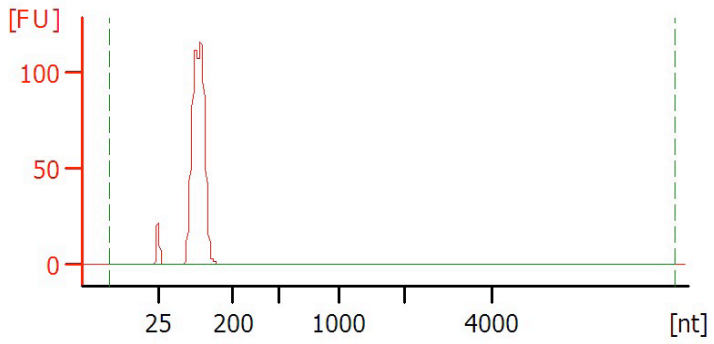
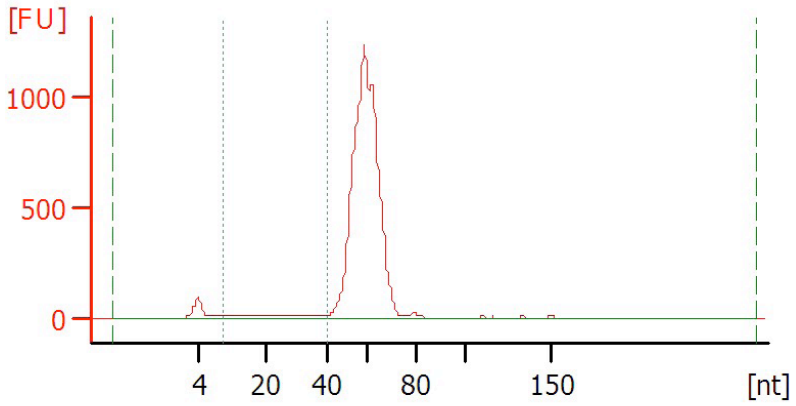
* In this experiment, the RNA PCR primer index used were custom designed RPIA/RPIC in one bioreplicate and RPIG/RPIT in the other bioreplicate that is to be multiplexed together.

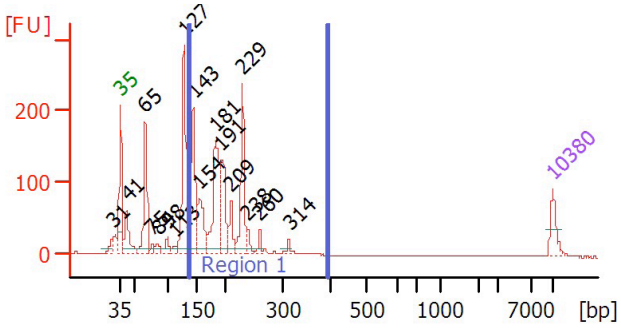
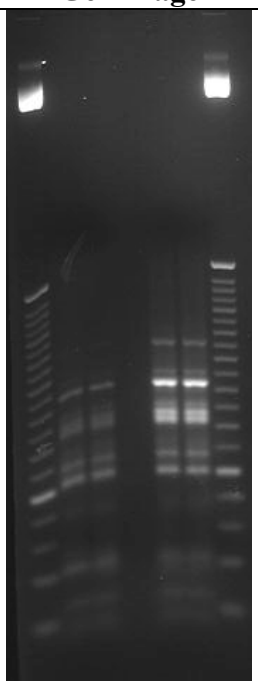
Gel extraction

1. Run the PCR product in a 2% agarose gel.
2. Excise the DNA from >125bp to 400bp.
3. Weigh the gel slice and add 3 volumes of QG buffer.
4. Incubate at 50°C for 30 min or until the gel slice has completely dissolved.
5. After the gel slice has dissolved completely, check that the color of the mixture is yellow. **Note** : If the color of the mixture is orange or violet, add 10 µL of 3M NaOAc pH 5.0 and mix. The color of the mixture will turn to yellow.
6. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.
7. Place a Minelute column in a provided 2 mL collection tube.
8. To bind DNA, apply the sample to the Minelute column and spin for 1 min. The maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, simply load and spin again.
9. Discard the flow-through and place the Minelute column back in the same collection tube.
10. Add 500 µL QG buffer to the spin column and spin for 1 min.
11. Discard the flow-through and place the Minelute column back in the same collection tube.
12. To wash, add 750 µL PE buffer to the Minelute column and spin for 1 min.
13. Discard the flow-through and spin the column for an additional 1 min at >10,000g.
14. Place the Minelute column into a clean 1.5 mL tube.
15. To elute DNA, add 10 µL EB buffer to the center of the membrane, let the column stand for 1 min. and spin for 1 min.
16. Repeat the elution to get a higher yield of DNA.
17. Proceed to ethanol precipitation.

Quantification

1. After drying the pellet, resuspend in 20 μL H_2O.
2. Measure the concentration on the Nanodrop.
3. Run 1 uL on the High sensitivity DNA Bioanalyzer chip.

Bioanalyzer: Small RNA	Bioanalyzer: Post Ribominus
	

Bioanalyzer: Pre-gel	Gel Image
	 Sample on 1 and 2 wells

Bioanalyzer: Final Library