# *Gingeras Lab RNA-Seq Library Production Document*

## ENCODE Transcriptome

### Sample Description: GM12878 191L

### RNA ID: 191L

### Library ID: SID38813

### Protocol ID:

Cold Spring Harbor Laboratory
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**LAB MEMBERS**

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# RNA Isolation

Cell pellets 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)

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.

**Large RNA Purification**

L1. Pipet 700 µL Buffer RWT into the RNeasy Mini spin column. Close the lid gently and centrifuge for 30 at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow through.
L2. Add 500 µL Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow through.
L3. Add another 500 µL Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow through and the collection tube.
L4. Place the RNeasy Mini spin column in a new 2 mL collection tube. Open the lid and centrifuge at full speed for 1 min.
L5. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube. Pipet 30-50 µL RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently. Wait one minute and then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the total RNA (containing large RNA).
L6. If the expected RNA yield is >30 µg, repeat step L5 with an additional volume of 30-50 µL RNase-free water. Elute into the same collection tube.
L7. Proceed to ethanol precipitation.

**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.

**Large RNA Cleanup**

1. Add 350 µL Buffer RLT to the 100 µL (100 µg) sample of RNA. Vortex to mix well.
2. Add 250 µL of 100% ethanol to the reaction and mix by inverting.
3. Transfer the 700 µL of sample to an RNeasy mini spin column placed 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.
4. Add 700 µL Buffer RW1 to the RNeasy mini spin column. Close the lid gently and centrifuge for 30 s at 8000 x g (≥10,000 rpm). Discard the flow through.
5. Repeat Step 4.
6. Add 500 µL Buffer RPE to the RNeasy mini spin column. Close the lid gently and centrifuge for 30 s at 8000 x g (≥10,000 rpm). Discard the flow through.
7. Repeat Step 6.
8. Transfer the RNeasy spin column to a new collection tube. Centrifuge for 2 min at 10,000 x g to dry the RNeasy membrane.
9. Place the RNeasy spin column into a new 1.5 mL collection tube and discard the old tube. Add 30-50 µL of RNase-free water directly on the spin column membrane. Close the lid gently and let stand for 1 min. Centrifuge for 1 min at 10,000 x g to elute the RNA.
10. Add another 30-50 µL of RNase-free water onto the membrane using the same centrifuge tube. Wait 1 min and then centrifuge for 1 min at 10,000 x g to elute the RNA.
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
ERCC Spike In Mix diluted 1/20: 1 uL (Ambion Cat. #4456740)

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.

## Multiplexing Long RNA Library Protocol: T-U

**1st strand cDNA Synthesis**
6.75 ul RNA sample
2ul 50ng/ul random primers (Invitrogen Cat. # 48190-011)
2.5 50uM oligo-DT primer (Invitrogen Cat. # 18418-020)
1.25ul RNAse inhibitor (Ambion Cat. # AM2692)

12.5 ul total

Use AD-1st program on cycler (98° for 2 min; 70° for 5 min; 0.1°/s ramp to 15°; 15° for 30 min ; 0.1°/s ramp to 25°; 25° for 10 min ; 0.1°/s ramp to 42°; 42° for 45 min; 0.1°/s ramp to 50°; 50° for 15 min ; 75° for 15 min; 4° forever)

**As soon as** 15 degrees is reached (after ~16 min), pause program and add:

5 ul 5X First Strand Buffer (Invitrogen Cat. # sold with SS III)
1.25 ul 0.1 M $MgCl_2$ (Ambion Cat. # AM9530G)
1.25 ul 10 mM dNTPs (Invitrogen Cat. # 18427-013)
2.5 ul 0.1M DTT (Invitrogen Cat. # sold with SS III)

*Add 10 uL of mix to each sample*
22.5 ul total reaction so far

Dilute 1 mg/ml stock of Actinomycin D to 120 ng/ul by mixing:
1.5 (or 3) ul 1 mg/ul AD + 11 (or 22) ul 10 mM Tris pH 7.6 (Sigma Cat. # T2444-1L)

After 30 minutes at 15 degrees, pause program and add:

1.25 ul Actinomycin-D (120 ng/ul) (Invitrogen Cat. # A7592)
1.25 ul Superscript III (Invitrogen Cat. # 18080-044)

*Add 2.5 ul of mix to each sample*

25 ul final volume for 1st strand reaction

Rest of reaction takes about 1 hour 40 minutes
Then, 4 degree hold

Bring reaction volume to 100ul (add 75ul Rnase free H20)
Add 5 volumes Buffer PB (500 ul), mix and apply to Minelute spin column
Follow Qiagen Minelute cleanup protocol (Qiagen Cat. # 28006)
Elute 2 x 15 ul EB

**2nd Strand cDNA Synthesis**
1 ul 5X 1st Strand Buffer (Invitrogen Cat. # sold with SS III)
15 ul 5X 2nd Strand Buffer (Invitrogen Cat. # 10812-014)
0.5 ul 0.1 M MgCl2 (Ambion Cat. # AM9530G)
1 ul DTT (Invitrogen Cat. # sold with SS III)
2 ul dU/dNTPs (Roche dUTP Cat. # 13796926 dNTPs Cat. # 11969064001)
0.5 ul E. coli DNA ligase (Invitrogen Cat. # 18052-019)
2 ul E. coli DNA polymerase I (Invitrogen Cat. # 18010-025)
0.5 ul RNAse H (Invitrogen Cat. # 18021-071)

Mix:
30 ul first strand reaction
22.5 ul second strand mix
22.5 ul RNAse free water

75ul final reaction volume

Use program 2nd on thermocycler: 2 hours at 16°; 4° forever

Bring volume up to 100 ul by adding 25 ul water
Add 500 ul of Buffer PB
Minelute cleanup, as before
Elute 2 x 26 ul of EB

Run a high sensitivity DNA chip on the bioanalyzer to determine fragmentation time.
If peak is around 1000 bp then sonicate for 60 s, if it is smaller sonicate for less time.

**Fragment cDNA**

Using Covaris sonicator:
Fill appropriate chambers with autoclaved DI water
Degas sonicator for 30 minutes prior to use

Transfer cDNA sample to the sonicator tube (Covaris Cat. # 520045)
Place on machine and run program "degas100ulsnapcap60s" (60s sonication)

Run a high sensitivity DNA chip to check fragment size

**End-Repair cDNA**
50 ul sample
Add 25 ul H20 to each sample

10 ul T4 DNA ligase buffer with 10mM ATP (New England Biolabs Cat. # B0202S)
4 ul dNTP mix 10mM (Invitrogen Cat. # 18427-013)
5 ul T4 DNA polymerase 3U/ul (New England Biolabs Cat. # M0203L)
1 ul Klenow DNA polymerase 5U/ul (New England Biolabs Cat. # M0210S)
5 ul T4 PNK 10U/ul (New England Biolabs Cat. # M0201L)

*Add 25 ul of mix to each sample*
100ul final volume

Place at room temperature for 30 min.

Add 500 ul of Buffer PB and minelute cleanup, elute 2 x 16ul

**Addition of single <A> Base**
32 ul eluted cDNA
5 ul NEBuffer2 (New England Biolabs Cat. # B7002S)
10 ul dATP (1mM) (Roche 11934511001)
3 ul Klenow fragment 3' to 5' exo- 5U/ul (New England Biolabs Cat. # M0212S)

*Add 18 ul of mix to each sample*
50 ul final volume

37 degrees, 30 min. (heat block)

Add 50 ul water, 500 ul Buffer PB, Minelute cleanup, elute 1 x 19ul

**Adapter Ligation**
19 ul eluted cDNA
25 ul 2x Rapid Ligation Buffer (Enzymatics Cat. # B101L)
1 ul Index Paired End adapter oligo mix (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
5 ul T4 DNA ligase 1U/ul (Enzymatics Cat. # L603-HC-L)

*Add 31 ul of mix to each sample*
50 ul final volume

Room temp, 15 min.

Add 50 ul water, 500 ul Buffer PB, Minelute cleanup, elute 1 x 15ul

**UNG Treatment**
15 ul eluted cDNA
1.7 ul 0.5 M KCl (Ambion Cat. # AM9640G)
1 ul UNG (Uracil N-Glycosylase) (Roche Cat. # N808-0096)

37 degrees, 15 min
95 degrees, 10 min
Hold on ice

Add 10ul loading buffer
Run out samples on a 2% agarose gel.
Cut out 200 bp band, and another band just slightly larger (freeze larger slice at -20).
Then use the Qiaquick gel extraction kit (Qiagen Cat. #28706) and elute 2 x 15ul.

**PCR Amplification**
15 ul eluted cDNA from gel-extraction (can use more or less)
2 ul PCR Primer In PE 1.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
2 ul PCR Primer In PE 2.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
2 ul of mix to 2 index primers *(PE 2 Index 1 and PE 2 Index 2 each diluted to 25uM)* (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
50 ul 2X HF Phusion Mix (New England Biolabs Cat. # M0531L)
29 ul water or up to 100 ul with water (depends on amount of DNA used)

98° 1 min then 16 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use AMPure XP Cleanup for PCR purification.

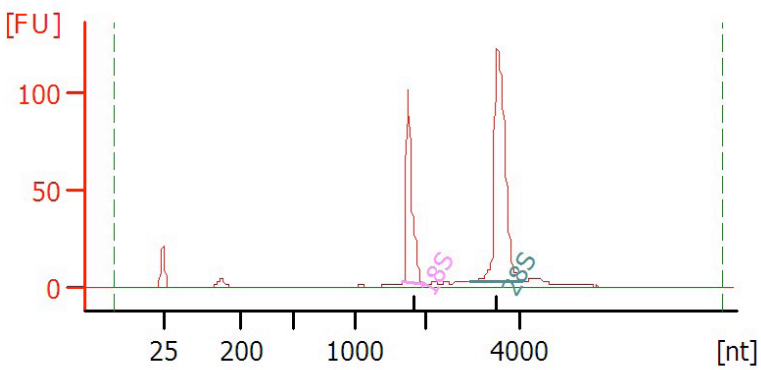
## AMPure XP PCR Cleanup Protocol

(Agencourt AMPure XP beads Cat. # A63881)

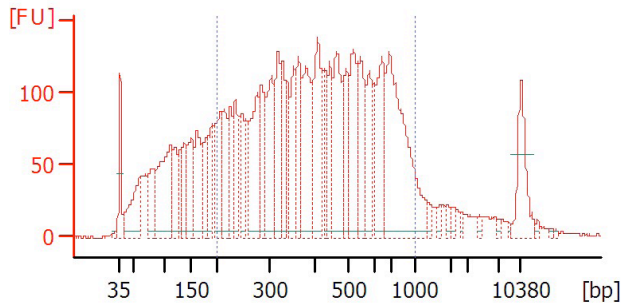
1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled. Add 80 uL Agencourt AMPure XP to sample.
2. Mix reagent and PCR reaction thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
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 30 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.

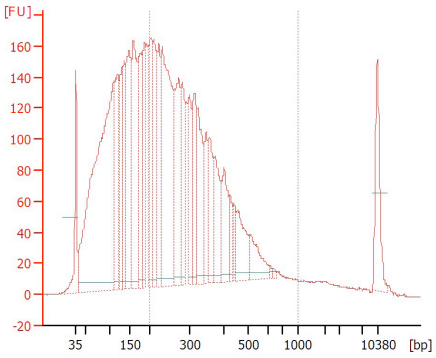
**Bioanalyzer: Long RNA after separation**



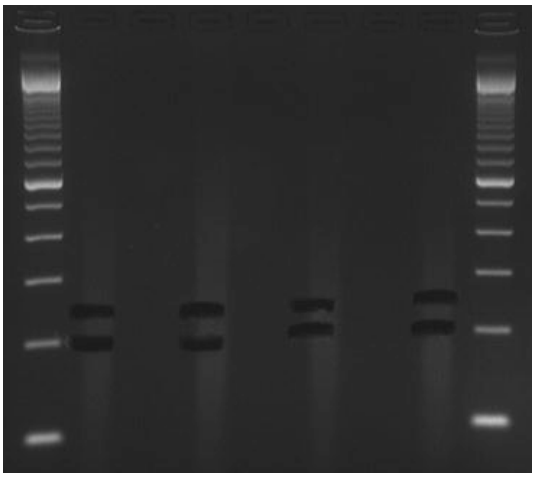

**Bioanalyzer: cDNA**




**Bioanalyzer: Post-Fragmentation**




**Gel Size Selection**



Sample is in fourth well.

**Final Library**