# *Gingeras Lab RNA-Seq Library Production Document*

## ENCODE Transcriptome

### Sample Description:
### RNA ID: 004N
### Library ID: LID20903
### Protocol ID: 004Ns

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**STRATEGY:** This document contains information about small RNA libraries generated as part of the ENCODE Consortia. It describes the Small RNA Isolation and cloning methods used to generate stranded libraries that capture the 5' ends of RNAs <200 nucleotides in length. The libraries can then be sequenced on the Illumina platform. The 3' ends are A-tailed followed by ligating on a RNA linker to the 5' ends and RT-PCR.

**CELL CULTURE**: Cells are grown according to the ENCODE growth protocol and standards. Each bioreplicate was grown and isolated independently.

**RNA ISOLATION**:

**Kits: RNeasy Mini kit (QIAGEN cat#: 74106)**
**miRNeasy Mini kit (QIAGEN cat#:217004)**
**RNeasy MinElute cleanup kit (QIAGEN cat#:74204)**

**Separate Cytosol and Nuclear From the Whole Cells**

1. Regular harvest and count cells and centrifuge for 10 min at 1900 rpm 4°C.
2. Completely aspirate supernatant.
3. Resuspend all pellets in 10-30 mL of cold PBS by pipetting up and down.
4. Centrifuge for 5 min at 2000 rpm 4°C.
5. Carefully aspirate the supernatant.

| Number of Cells | RLN Buffer (4°C) (mL) |
|---|---|
| $5X10^6$- $5X10^7$ | 0.5 |
| $5X10^7$- $1X10^8$ | 1.0 |
| $5X10^7$- $2.5X10^8$ | 2.0 |
| $2.5X10^6$- $5X10^8$ | 4.0 |

6. Add the appropriate amount of cold Buffer RLN to lyse plasma membrane. For the pelleted cells loosen them by pipetting up and down. Incubate on ice for 5 min.
7. Centrifugate lysate at 4°C for 10 min at 3200 rpm. Transfer the supernatant (the cytosol) to an RNase free centrifuge tube and keep the nuclear pellet.

For the nuclear pellet, put 1mL RLN buffer and try to resuspend the pellet. Centrifuge at 4°C for 5 min at 3200 rpm. Carefully discard the supernatant, add 800 µL RLT (+BME 1:100, 1ml RLT buffer add 100 µL BME). Homogenize the sample using a syringe and 18 gauge needle. Pass the sample through the needle until it becomes smooth and there are no thick globs. It is important that the sample is homogenized completely. Freeze the lysate in -80°C.

**To purify small RNA fraction proceed to **Small RNA Purification**.**
**To purify large RNA fraction proceed to Total RNA **(Containing Large RNA) Purification**.** (Large RNAs were sequenced using RNA-Seq as well as CAGE).

N1. Thaw the frozen tubes from above. Transfer them to a Falcon tube then put 6X volumes of QIAzol lysis buffer. Votex for 30 s.
N2. Place the tube(s) containing the homogenate on the benchtop at room temperature (15-25°C) for 5 min.
N3. Add 20% volumes of chloroform to the homogenate and cap it securely. Shake the tube vigorously for 15 s.
N4. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 min.
N5. Centrifuge the homogenate for 15 min at 12,000 x g at 4°C.
N6. Transfer the upper, colorless, aqueous phase containing the RNA to a new Falcon tube.
N7. To purify total nuclear RNA continue by adding 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge.
N8. 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.

N9. Close the lid and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Discard the flow through.
N10. Repeat step N9 using the remainder of the sample. Continue to use the same collection tube.
N11. Add 700 µL Buffer RWT to the RNeasy Mini spin column. Close the lid and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow through. (This step can be skipped if an on column DNase digestion is performed.)
N12. Pipet 500 µL Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow through.
N13. Add another 500 µL Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the RNeasy Mini spin column.
N14. Carefully place the RNeasy Mini spin column into a new 2 mL collection tube making sure the column does not touch the flow through. Discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.
N15. 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 RNA.
N16. If the expected RNA yield is >30 µg, repeat step N15 with an additional volume of 30-50 µL RNase-free water. Elute into the same collection tube. Continue onto ethanol precipitation.

**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 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 pellet of RNA.
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 Digest (same for Total, 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 Total or Large RNA.

**RNA Cleanup and Separation of Small RNA**

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 and 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). Keep the flow-through which contains the small RNA. Then follow the small RNA protocol below.

**Small RNA Separation**

S1. Add another 450 µL of 100% ethanol to the flow-through and mix by inverting.

S2. Transfer 700 µL of the 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.
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). Repeat with a second volume of 20 µL RNase free water.
S8. Proceed to ethanol precipitation.

**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 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 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 5 min making sure that the pellet is dry.
8. Resuspend the pellet with RNase-free water.

**RIBOMINUS TREATMENT**: In addition to the probes supplied with the Ribominus kit we also spike-in our own LNA probes against the 5S and 5.8S rRNA.

5S-LNAprobe-1 tt+Ccc+Agg+Cgg+Tct+Ccc+At
5S-LNAprobe-2 tc+Agg+Gtg+Gta+Tgg+Ccg+Tag
5.8S-LNAprobe-1 ct+Tca+Tcg+Acg+Cac+Gag+Cc
5.8S-LNAprobe-2 cg+Ctc+Aga+Cag+Gcg+Tagc

**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 (1–10 µg): <10 µL
RiboMinus™ Probe (15 pmol/L): 8 µL
Custom probes (5S, 5.8S 100uM each) : 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 minutes 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 minutes 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 following primers and RNA Linker are needed to perform this procedure*:

5'SBS3_Adapter (This is the RNA ligated onto the 5' end): "r" = ribose, RNA base
**5'- rArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrU**
A-Tail RT Primer (This is the primer used in the RT reaction):
**5'-TCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTVN**
PE 5' PCR (PCR Primer):
**5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC**
PE 3' PCR (PCR Primer):
**5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTC**

**Tobacco Acid Pyrophosphatase Reaction (TAP, Epicentre T19250)**

1. Denature RNA at 85°C for 2 min or 65°C for 10 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
    a. RNA 42.5 µL
    b. 10X TAP reaction buffer 5 µL
    c. Anti-RNase (Ambion 20U/ µL) 2 µL

   d. TAP (10U/ul) 0.5 µL
3. Incubate at 37 °C for 2hr.
4. Proceed to phenol chloroform extraction.

**Phenol Chloroform Extraction**

1. Add 1 volume phenol-chloroform 5:1 (pH 4.5) and vortex for 10 s.
2. Spin at 13000rpm 4°C for 20 min.
3. Transfer the upper phase to a new tube.
4. Add 1 volume chloroform and vortex for 10 s.
5. Spin at 13000rpm 4°C for 15 min.
6. Transfer the upper phase to a new tube.
7. Proceed to ethanol precipitation.
8. After drying the pellet, resuspend in 56 µL $H_2O$.

**A-tailing (PolyA kit, Ambion AM1350)**

1. Denature RNA at 85°C for 2 min or 65°C for 1 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
   a. 5X PolyA buffer 20 µL
   b. 25 mM $MnCl_2$ 10 µL
   c. BSA 1mg/ml 5 µL
   d. 100mM ATP (Roche) 2 µL
   e. Anti- RNase 2 µL
   f. E_PAP Poly A polymerase 5 µL
   g. RNA 56 µL

3. Incubate at 37°C for 30 min.
4. Proceed to phenol-chloroform extraction.
5. Proceed to ethanol precipitation.
6. After drying the pellet, resuspend in 21 µL $H_2O$. Keep 1 µL for running a small RNA Bioanalyzer chip.

**Ligation**

1. Denature RNA at 85°C for 2 min or 65°C for 10 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
   a. Anti-RNase 1 µL
   b. 10X T4 ligase buffer 3 µL
   c. BSA 0.5 µL
   d. 5'SBS 3 adapter (100 µM) 4 µL
   e. T4 RNA ligase (Ambion 5U/ µL) 1.5 µL
   f. RNA 20 µL
3. Incubate at 4 °C overnight.
4. After overnight incubation, add 1 µL T4 RNA ligase per tube and incubate at room temperature for 1 hr.
5. Proceed to phenol-chloroform extraction.
6. Proceed to ethanol precipitation.
7. After drying the pellet, resuspend in 16.5 µL $H_2O$.

**First Strand cDNA Synthesis**

1. To 16.5 µL RNA, add 2 µL A-Tail RT primer.
2. Incubate at 65 °C for 5 min. Leave on ice for 5 min.
3. Then add the following :
   a. 10mM dNTPs 1.5 µL
   b. 5X first strand cDNA buffer 6 µL
   c. 0.1M DTT 1.5 µL

d. Anti-RNase 1 µL
e. Superscript RT III 1.5 µL

**PCR**

1. Set up the reaction by adding :
   a. First strand cDNA 5 µL
   b. PE 5' PCR primer 100uM 0.5 µL
   c. PE 3' PCR primer 100uM 0.5 µL
   d. 2X Phusion mix (NEB F-531L) 50 µL
   e. $H_2O$ 44 µL

2. Program the thermal cycler as follows:

   1. 94°C 2 min.
   2. 94°C 15 s.
   3. 54 °C 30 s.
   4. 72 °C 20 s.
   5. Go back to step 2 and repeat 4 more times.
   6. 94°C 15 s.
   7. 60 °C 30 s.
   8. 72 °C 20 s.
   9. Go back to step 6 and repeat 24 more times.
   10. 4 °C forever

3. After PCR, clean up the reaction by putting through a Minelute column as follows:
   a. Add 5 volumes of PB buffer to the reaction.
   b. To bind DNA, apply the sample to a Minelute column. Spin at 13000rpm for 1 min. Discard the flow-through.
   c. Wash with 750 µL PE buffer. Spin at 13000rpm for 1 min. Discard the flow-through.
   d. Spin at 13000rpm for 1 min to dry the column.
   e. Add 10 µL EB buffer. Spin at 13000rpm for 1 min to elute DNA.
   f. Repeat the elution one more time.
   g. Use 1 µL of the elute for running on a High sensitivity DNA Bioanalyzer chip.

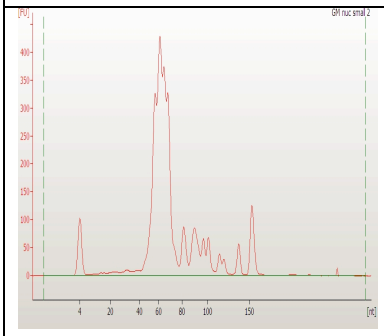
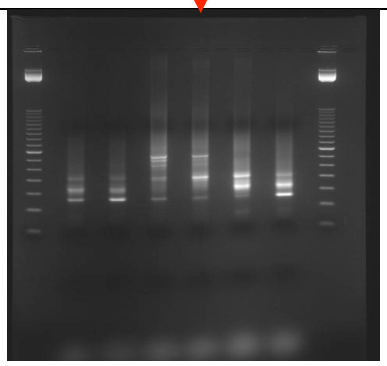
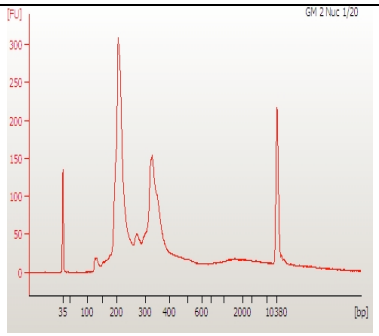
**Gel extraction**

1. Run the rest of the sample in a 2% agarose gel.
2. Excise the DNA from >128bp to 350bp.
3. Weigh the gel slice and add 3 volumes of QG buffer.
4. Incubate at 50°C for 10 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 µL on the High sensitivity DNA Bioanalyzer chip.

| Input RNA | Gel Purification | Final Library Image |
|---|---|---|
|  |  |  |