Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: Mouse liver 31B

RNA ID: 31B

Sample ID: SID38135

Cold Spring Harbor Laboratory

Genome Center

500 Sunnyside Blvd

Woodbury, NY 11797

LAB MEMBERS

Wet lab: Meagan Fastuca, Carrie A. Davis, Jorg Drenkow, Lei-Hoon See.

Computational Lab: Alex Dobin, Felix Schlesinger, Chris Zaleski, Chenghai Xue.

PI: Tom Gingeras

RNA Isolation

Mouse tissue was obtained from the CSHL animal resources. Tissue was preserved in RNAlater-ice (Life Technologies cat# AM7030M) and total RNA was isolated using the miRNeasy Mini kit.

Treatment of tissue

1. Place tissue in 10 volumes of RNAlater-ice at -20^{o}C overnight.
2. After overnight incubation, fish tissue out of RNA later-ice and blot dry on paper napkin.

RNA isolation

Kits: miRNeasy Mini kit (QIAGEN cat#:217004)

RNeasy MinElute cleanup kit (QIAGEN cat#:74204)

1. Remove the tissue sample from storage. Determine the amount of tissue.
2. Place it directly into 3ml QIAzol Lysis Reagent in a 15ml Falcon tube for disruption and homogenization.
3. Homogenize immediately using the TissueRuptor.
4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
5. Separate the homogenization to three 1.5ml eppendorf tubes (1ml/tube). Add 200µl chloroform to the tube containing the homogenate and cap it securely. Shake the tube vigorously for 15 s.
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.
8. Transfer the upper aqueous phase to a new collection tube. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.
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. 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.
10. Repeat step 9 using the remainder of the sample. Discard the flow-through.

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.
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.
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.
14. Place the RNeasy Mini spin column into a new 2 ml collection tube, and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.
15. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube. Pipet 50 µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently, wait for 1min and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.
16. Repeat step 15 with a second volume of 50 µl RNase-free water. Elute into the same collection tube.
17. Proceed to Ethanol Precipitation.

Ethanol Precipitation

1. Add 2.5 volumes of 100% ethanol and 1/10 volumes of NaOAc pH 5.5 (Ambion Cat. # AM9740) to the eluted RNA.
2. Freeze at -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 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

Reagents	*100 µL Sample*	*50 µL Sample*

	(100 µg RNA max)	*(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.

RNA Cleanup

1. Add 350 µL Buffer RLT to the 100 µL sample of RNA. Vortex to mix well.
2. Add 250 µL of 100% ethanol to the reaction and mix by inverting.
3. Transfer 700 µL of sample into an RNeasy mini 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.
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) to wash the column. Discard the flow through. Repeat.
5. Pipet 500 µL Buffer RPE into 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. Repeat.
6. Place the RNeasy mini spin column into a 1.5 mL collection tube and pipet 100 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).
7. Repeat step 9 with a second volume of 100 µL RNase free water.
8. Proceed to ethanol precipitation.

Ribo Zero Gold Protocol (r-RNA Removal)

(Epicentre Ribo-Zero Gold Kit- Human/Mouse/Rat- Cat. No. RZG1224)

A. Preparation of the Ribo-Zero Microspheres

1. Remove the Ribo-Zero rRNA Removal Kit from –70°C to –80°C storage, thaw the tubes, and place them on ice.
2. Vigorously mix the Microspheres (at room temperature) for 20 seconds by vortexing to produce a homogeneous suspension. The Microspheres are capable of withstanding vigorous vortex mixing and remain as a homogeneous suspension for several minutes.

3. For each reaction, pipette 65 µl of Microspheres into a separate 2-ml Microsphere Wash Tube. Aspirate the Microsphere suspension *slowly* to avoid air bubbles and to ensure pipetting the full required volume. Return the unused Microspheres to storage at 4°C.
Important! *Prepare the Microspheres for each RNA sample separately.* ***Do not*** *batch-wash the Microspheres for multiple samples.*
4. Centrifuge the dispensed Microspheres at 12,000 x g in a bench-top microcentrifuge for 3 minutes. *Carefully* pipette off and discard the supernatant, without disturbing the Microsphere pellet.
Caution: *The supernatant contains 0.1% sodium azide. Discard the supernatant according to local ordinances.*
5. Wash the Microspheres by adding 130 µl of Microsphere Wash Solution to each tube. Mix the tube(s) by vortexing at maximum speed to resuspend the Microspheres. Centrifuge the tube(s) at 12,000 x g for 3 minutes in a bench-top microcentrifuge. *Carefully* pipette off and discard all of the supernatant without disturbing the Microsphere pellet.
6. Add 65 µl of Microsphere Resuspension Solution to each tube and resuspend the Microspheres by vortex mixing at maximum speed until a homogeneous suspension is produced.
7. Add 1 µl of RiboGuard RNase Inhibitor to each tube of resuspended Microspheres. Mix by vortexing briefly (10 seconds) and store the tubes at room temperature for use in Part C.

B. Treatment of the Total RNA Sample with Ribo-Zero rRNA Removal Solution

1. In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine in the order given: x µl RNase-Free Water, 4 µl Ribo-Zero Reaction Buffer, 5 µg Total RNA sample (see Table 1), 10 µl Ribo-Zero Gold rRNA Removal Solution, and 1 uL Pool 13 Spike ins, for a 40 µl Total volume.
2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. During the incubation, return the remaining Ribo-Zero Gold rRNA Removal Solution and Ribo-Zero Reaction Buffer to storage at –70°C to –80°C.
3. Remove the reaction tube(s) and incubate each at room temperature for 15 minutes.

C. Microsphere Reaction and rRNA Removal

1. Briefly mix by vortexing (at medium speed for about 20 seconds) the washed, room-temperature Microspheres in the 2.0-ml Wash Tube from Part A, Step 7. If necessary, pulse-centrifuge (5 seconds) to collect the Microsphere suspension in the bottom of the tube, then resuspend by pipetting the slurry several times. It is important to have a homogeneous slurry before adding the hybridized RNA from Part B, Step 3.

2. Using a pipet, add the hybridized RNA sample from Part B, Step 3 to the resuspended Microspheres in the 2.0-ml Wash Tube and, without changing the pipet tip, *immediately* mix the contents of the tube by rapidly pipetting 10-15 times. Then, immediately mix the contents of the tube by vortexing (at medium speed) for 5 seconds and place at room temperature before proceeding to the next sample.

Important! Always *add the RNA sample to the resuspended Microspheres in the 2-ml Wash Tube and immediately and rapidly mix by pipetting the contents of the tube.* ***Never*** *add the Microspheres to the RNA sample.*

3. Incubate the tubes at room temperature for 10 minutes with vortex mixing (at medium speed) for 5 seconds every 3 to 4 minutes.

Important! Do not *use a shaker platform as this does not provide sufficient mixing.*

4. At the end of the 10-minute incubation at room temperature, mix by vortexing (at medium speed) the sample for 5 seconds and then place at 50°C for 10 minutes in a water bath, heating block, or other temperature-controlled device.

5. After 10 minutes at 50°C, immediately transfer the RNA-Microspheres suspension to a Microsphere Removal Unit (filtration unit; provided in the Ribo-Zero Core Kit) and centrifuge at 10,000 x g for 1 minute at room temperature. Save the eluate that is in the collection tube and discard the filter unit with the microspheres.

Important! *The eluate contains the rRNA-depleted sample!*

6. Ethanol precipitate and resuspend in 6.75 uL for library construction.

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

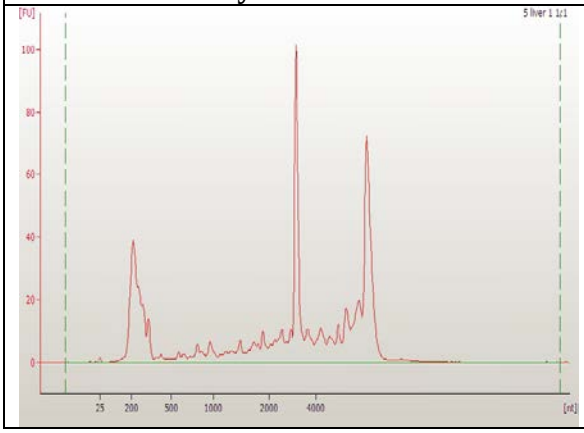
15 ul eluted cDNA from gel-extraction (can use more or less)
2 ul PCR Primer In PE 1.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
2 ul PCR Primer In PE 2.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
2 ul of mix to 2 index primers *(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)

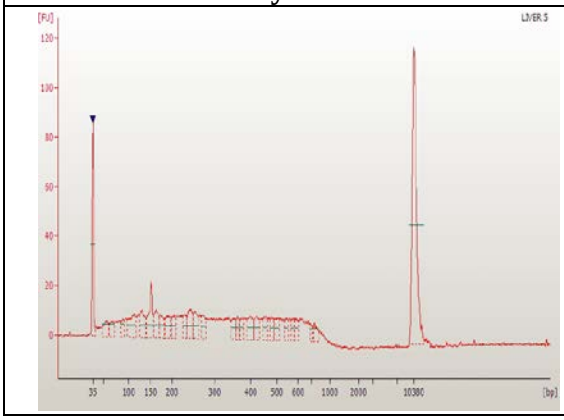
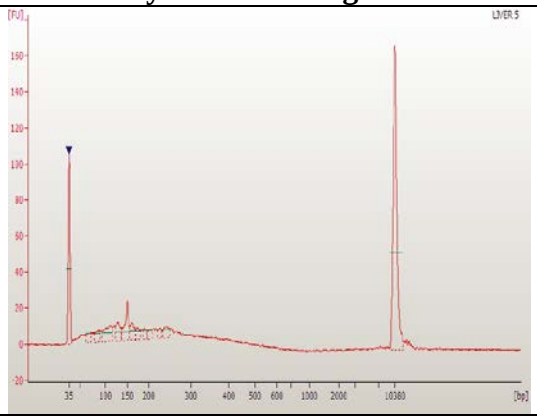
98° 1 min then 16 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use AMPure XP Cleanup for PCR purification.

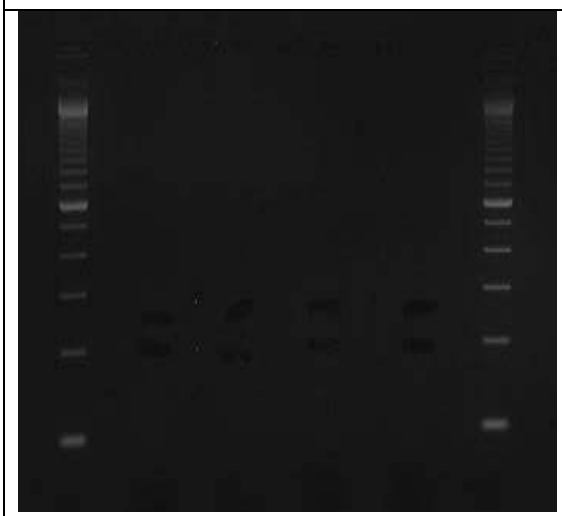
AMPure XP PCR Cleanup Protocol

(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: Total RNA	
	

Bioanalyzer: cDNA	Bioanalyzer: Post-Fragmentation
	

Gel Size Selection	Final Library
 Sample is in second well.