Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: Mouse hindbrain (HB) P0
RNA ID: 266WC
library ID: 292126
Composite library ID : 292132

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LAB MEMBERS

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RNA Isolation

Frozen pulverized mouse tissue was provided by UCSD. Use a neThen long and small RNA were extracted from the cells using the MirVana kit procedure (Ambion MirVana miRNA Isolation Kit Cat # AM1561).

1.Add 300uL Lysis/Binding solution to the cells.
2.Homogenize the cells by passing through an 18 gauge needle and syringe at least 10 times.
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.

Long and Small RNA Separation

*Preheat nuclease free water to 95°C for elution steps.
1. Add 1/3 volume of 100% ethanol to the aqueous phase recovered previously from step 8.
2. Mix thoroughly by vortexing or inverting tube several times.
3. For each sample, place a Filter Cartridge into one of the collection tubes supplied.
4. Pipet up to 700 uL of the lysate/ethanol mixture (from the previous step) onto the filter cartridge.
5. Centrifuge for approximately 15 seconds at 10,000 x g to pass the mixture through the filter (vacuum can also be used). Apply the mixture in successive applications to the same filter and repeat centrifugation until all the mixture is passed through. **(Do not discard the filtrate- this contains the small RNA, while the filter contains the Long RNA. Save the filter cartridge and wash Long RNA using RNA cleanup section.)**
6. Collect the filtrate (pool successive passes through the filter in a new tube) and record the volume.
7. Add 2/3 volume room temperature 100% ethanol to filtrate (flow-through) and mix thoroughly.
8. Pass 700 uL of each sample through a second filter cartridge and centrifuge for 15 seconds at 10,000 x g.
9. Discard the flow through.
10. Continue passing sample until the entire sample is through the filter.
11. 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 (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 (can also use vacuum).
4. Discard the flow through.
5. Repeat steps 3 and 4.
6. Spin the filter cartridge for 1 minute 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. Store eluate with RNA at -20°C or colder.

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 Long RNA. For long RNA, follow the Long RNA Purification procedure.

Long RNA Purification

L1. Pipet 700µl Buffer RWT into the RNeasy Mini spin column from step 10. Close the lid gently and centrifuge for 15 s at ≥10,000 rpm to wash the spin column membrane. Discard the flow-through.

L2. Add 500µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥10,000 rpm to wash the spin column membrane. Discard the flow-through.
L3. Pipet another 500µl Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥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. Place the RNeasy Mini spin column into a new 1.5 ml collection tube. Pipet 30–50µl RNase-free water directly onto the spin column membrane. Close the lid gently and centrifuge for 1 min at ≥10,000 rpm to elute the total RNA.
L6. If the expected RNA yield is >30 µg, repeat step L5 with a second volume of 30–50µl RNase-free water. Elute into the same collection tube.
L7. Proceed to ethanol precipitation.

Ribo-Zero Magnetic Kit
(Epicentre Cat. # MRZH116)

Beads Batch Washing Procedure

1. For each Ribo-Zero reaction, 225 µl of the Magnetic Beads is required. ***Note:*** *Mix the Magnetic Beads well by pipetting or gentle vortexing.*
2. Determine the amount of Magnetic Beads required for the total number of reactions and dispense a maximum of 1,350 µl into each 1.5-ml RNase-free microcentrifuge tube (sufficient for six reactions). Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C. ***Note:*** *When setting up more than six Ribo-Zero reactions, either multiples of 1,350-µl aliquots can be washed in a RNase-free 1.5 ml microcentrifuge tubes, or a larger volume can be washed in RNase-free 15-ml tubes (e.g., using a 15-ml magnetic stand).*
3. Place the 1.5-ml microcentrifuge tube containing the Magnetic Beads on the magnetic stand for at least 1 minute (until the solution appears clear).
4. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant. ***Caution:*** *The supernatant contains 0.1% sodium azide*
5. Remove the 1.5-ml microcentrifuge tube from the stand and add an equal volume of RNase-Free Water. Mix well by repeated pipetting or by vortexing at medium speed.
6. Repeat steps 3, 4 and 5 (i.e. wash the beads a total of 2 times with RNase-Free water).
7. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add a volume of Magnetic Bead Resuspension Solution equal to the number of reactions x 60 µl (e.g., for 6 reactions, add 6 x 60 µl = 360 µl Magnetic Bead Resuspension Solution). Mix well by repeated pipetting or by vortexing at medium speed. ***Note:*** *The volumes of the beads and Resuspension Solution are additive. Although the washed beads are resuspended in 60 µl per reaction, each reaction uses 65 µl of resuspended beads.*

8. Aliquot 65 µl of the washed Magnetic Beads into each new 1.5-ml RNase-free microcentrifuge tube (corresponding to the number of Ribo-Zero reactions).
Optional: Add 1 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.
Store the microcentrifuge tubes at room temperature until required in ***Part: Magnetic Bead Reaction and rRNA removal***.

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

1. In an RNase-free microcentrifuge tube combine:
    1 uL of ERCC Spike In Mix diluted 1/20: 1 uL (Ambion Cat. #4456740)
    4 uL Ribo-Zero Reaction Buffer
    25 uL (5 ug) Total RNA Sample
    10 uL Ribo-Zero rRNA Removal Solution

    Total Volume 40 uL

2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. Store the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer at –70°C to –80°C.
3. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

Magnetic Bead Reaction and rRNA Removal

1. Using a pipette, add the treated RNA from earlier to the 1.5-ml microcentrifuge tube containing the washed Magnetic Beads and, without changing the pipet tip, ***immediately and thoroughly mix the contents of the tube by pipetting at least 10 times. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.*** Repeat this process for each sample. ***Important!*** *Always add the treated RNA sample to the washed Magnetic Beads and immediately mix by pipetting. Never add the Magnetic Beads to the treated RNA sample. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact the efficiency of the rRNA removal.*
2. Incubate the 1.5 ml microcentrifuge tube at room temperature for 5 minutes.
3. Following incubation, mix the reactions by vortexing at medium speed for 10 seconds and then place at 50°C for 5 minutes in an appropriate water bath or heating block. Avoid any significant condensation during this incubation step.
4. After the 5-minute incubation at 50°C, remove the microcentrifuge tubes and immediately place them on a magnetic stand for at least 1 minute (until the solution appears clear).
5. Carefully remove each supernatant (85-90 µl) containing the RNA and transfer to a labeled 1.5-ml RNase-free microcentrifuge tube. ***Important!*** *The supernatant contains rRNA-depleted RNA.* ***Optional:*** *If a small amount of Magnetic Beads are still visible in the supernatant, place the collected supernatant onto the magnetic stand for 1 minute. Remove the supernatant containing the rRNA-depleted RNA and transfer to a new 1.5-ml RNase-free microcentrifuge tube.*
6. Proceed to Ethanol Precipitation.

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)

11.25 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)
1.25ul RNAse inhibitor (Ambion Cat. # AM2692)

Add 11.25 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 250 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)

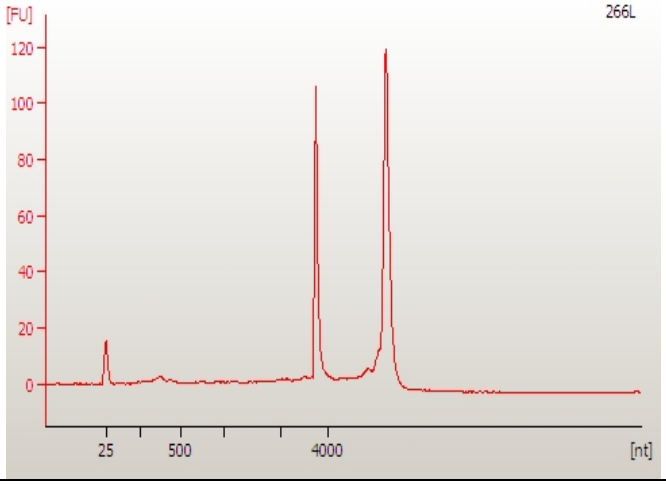
98° 1 min then 16 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use AMPure XP Cleanup for PCR purification.

AMPure XP PCR Cleanup Protocol

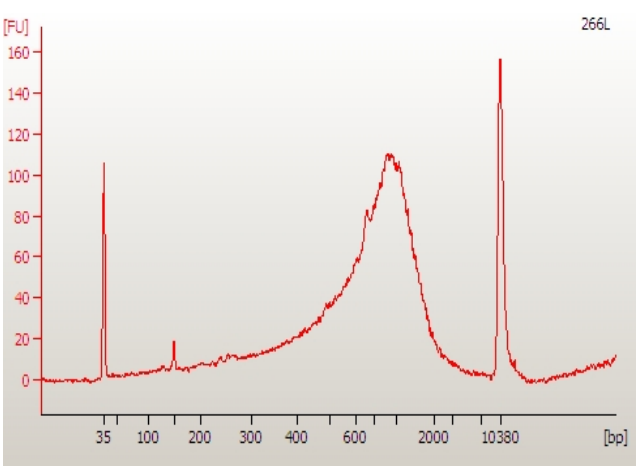
(Agencourt AMPure XP beads Cat. # A63881)

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.

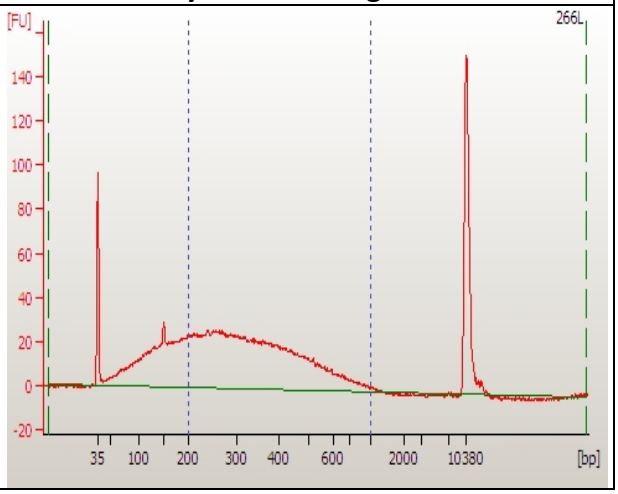
Bioanalyzer: Long RNA after separation



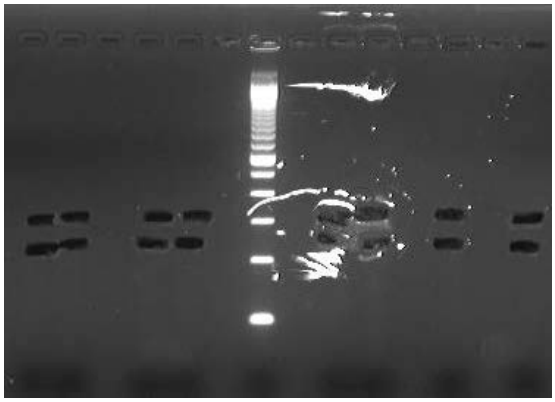
Bioanalyzer: cDNA



Bioanalyzer: Post-Fragmentation



Gel Size Selection



Sample is in fifth occupied well.

Final Library