Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: HDLEC_8022602.1P

RNA ID: 161WC

Sample ID: SID38193

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RNA Isolation

We purchased cells (~ 10^6 per vial) in RNAlater from PromoCell GmbH. Then long and small RNA were extracted from the cells using the MirVana kit procedure (Ambion MirVana miRNA Isolation Kit Cat # AM1561).

1. Add 1.2 mL Lysis/Binding solution to 100-10^7 cells containing around 200 uL of RNA later (add more lysis/binding solution for greater volumes of RNA later).
2. Vortex or pipet to completely lyse cells and create a homogenous lysate.
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.

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 Ambion ERCC RNA Spike-in mix, 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.

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 Paired End adapter oligo mix
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
30 ul eluted cDNA from gel-extraction (can use more or less)
1ul PE primer 1 (25uM)
1ul PE primer 2 (25uM)

50 ul 2X HF Phusion Mix (New England Biolabs Cat. # M0531L)
18 ul water or up to 100 ul with water (depends on amount of DNA used)

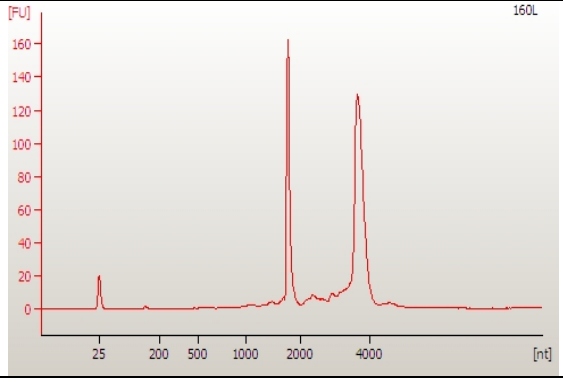
98° 1 min then 18 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use AMPure XP Cleanup for PCR purification.

AMPure XP PCR Cleanup Protocol

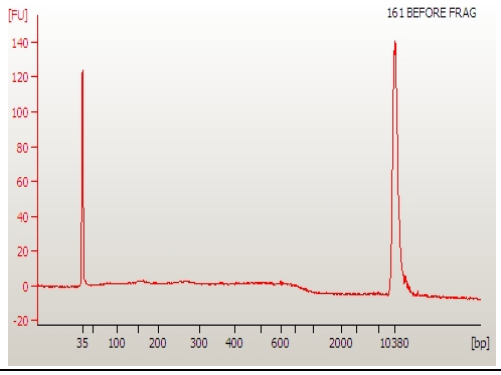
(Agencourt AMPure XP beads Cat. # A63881)

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.

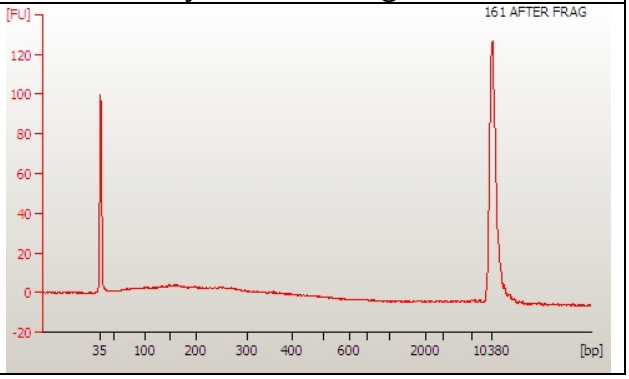
Bioanalyzer: Long RNA after separation



Bioanalyzer: cDNA



Bioanalyzer: Post-Fragmentation



Gel Size Selection

Sample is in first well.

Final Library