

Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: Artery Smooth Muscle Cells 152L

RNA ID: 152WC

Library ID: SID38234

Protocol ID:

Cold Spring Harbor Laboratory

Genome Center

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RNA Isolation

Human cell pellets in RNA Later were received from PromoCell. Then large and small RNA were separated using this MirVana kit procedure (Ambion MirVana miRNA Isolation Kit Cat # AM1561).

1. Add 1.2 mL Lysis/Binding solution to $100\text{--}10^7$ cells containing around 200 μL 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 ($10,000 \times g$) 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.

Separating Large RNA (start here if total RNA has already been extracted)

1. Mix total RNA with 5 volumes Lysis/Binding Buffer.
2. Add 1/10 volume of miRNA Homogenate Additive to the RNA mixture from the previous step, and mix well by vortexing or inverting the tube several times. Leave the mixture on ice for 10 min.
3. Add 1/3 volume of 100% ethanol to the RNA mixture from the previous step. Mix thoroughly by inverting the tube several times.
4. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet 700 μL of the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. For sample volumes greater than 700 μL , apply the mixture in successive applications to the same filter. Centrifuge at $5,000 \times g$ for 30 seconds to pass the mixture through the filter. Collect the filtrate. (If the RNA mixture is $>700 \mu\text{L}$, transfer *the flow-through* to a fresh tube, and repeat until all of the RNA mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrate.) (Proceed to **Separating Small RNA** section to use the filtrate containing the small RNA.)
5. Proceed to RNA Cleanup.

Separating Small RNA

1. Add 2/3 volume room temperature 100% ethanol to filtrate (i.e. flow-through) acquired in step 4 of long RNA procedure.
2. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet 700 μL of the filtrate/ethanol mixture (from the previous step) onto a second Filter Cartridge. For sample volumes greater than 700 μL , apply the mixture in successive applications to the same filter. Centrifuge for ~ 1 min at $5,000 \times g$ to pass the mixture through the filter. Discard the flow-through, and repeat until all of the filtrate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.

3. 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 at 5,000 x g (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 at 5,000 x g (can also use vacuum).
4. Discard the flow through.
5. Repeat steps 3 and 4.
6. Spin the filter cartridge for 1 minute at 5,000 x g 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. Ethanol precipitate RNA.

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 μ l 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 μ l 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 μ l 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 μ l RNase-Free Water, 4 μ l Ribo-Zero Reaction Buffer, 5 μ g Total RNA sample, 10 μ l Ribo-Zero Gold rRNA Removal Solution, and 1 uL ERCC Spike In Mix diluted 1/20 (Ambion Cat. #4456740), for a 40 μ l 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 MgCl₂ (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 H₂O)

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 H₂O 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 (Illumina Cat. # 1003455)
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)

1 ul PCR Primer PE 1.0 (primer from IDT)

1 ul PCR Primer PE 2.0 (primer from IDT)

50 ul 2X HF Phusion Mix (New England Biolabs Cat. # M0531L)

33 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.

