Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: CH-12 Large 32A

RNA ID: 32A

Library ID: SID38132

Protocol ID:

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LAB MEMBERS

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RNA Isolation

CH-12 cells in RNA Later were received from John Stam's lab and were isolated using this MirVana 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 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.

DNase Digest

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 (Roche 04716728001)	8 µL	4 µL
20 U/µL anti-RNase (Ambion Cat. # AM2692)	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 water bath for 30 min.
3. Proceed to RNA Cleanup.

Large RNA Cleanup

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 an 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). Discard the flow through.
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). Discard the flow through.
5. Repeat Step 4.

6. Add 500 µL Buffer RPE to 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.
7. Repeat Step 6.
8. Transfer the RNeasy spin column to a new collection tube. Centrifuge for 2 min at 10,000 x g to dry the RNeasy membrane.
9. Place the RNeasy spin column into a new 1.5 mL collection tube and discard the old tube. Add 30-50 µL of RNase-free water directly on the spin column membrane. Close the lid gently and let stand for 1 min. Centrifuge for 1 min at 10,000 x g to elute the RNA.
10. Add another 30-50 µL of RNase-free water onto the membrane using the same centrifuge tube. Wait 1 min and then centrifuge for 1 min at 10,000 x g to elute the RNA.
11. Proceed to ethanol precipitation, then run on bioanalyzer to visualize RNA quality.

Poly A+ Selection

Qiagen mRNA Isolation Protocol (using Oligotex mini kit)

- Heat Oligotex Suspension to 37°C in a water bath or heating block for 10 minutes. Mix by vortexing, and then place at room temperature.
- Heat a water bath or heating block to 70°C, and heat Buffer OEB.
- Heat Buffer OBB to 37°C in heat block to dissolve precipitate.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C (room temp).
- All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x *g*).

Procedure

1. Determine the amount of starting RNA. Do not use more than 3 mg total RNA.
2. Add the appropriate volume of Buffer OBB and Oligotex Suspension (see Table) and mix the contents thoroughly by pipetting or flicking the tube.

Buffer amounts for Oligotex mRNA Batch Protocol

Total RNA	Add RNAse free water to:	Buffer OBB (ul)	Oligotex Suspension (ul)	Prep size
≤0.25 mg	250 ul	250	20	Mini
0.25–0.50 mg	500ul	500	30	Midi
0.50–0.75 mg	500ul	500	45	Midi
0.75–1.00 mg	500ul	500	55	Midi

3. Incubate the sample for 3 min at 70°C in a water bath or heating block. (This step disrupts secondary structure of the RNA.)
4. Remove sample from the water bath/heating block, and place at 20 to 30°C for 12 min.
(This step allows hybridization between the Oligotex particle and the poly-A tail of the mRNA.)
5. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at 18,000x *g*, and carefully remove the supernatant by pipetting **(save the supernatant for A- in a separate tube)**.
(Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure.)

6. Resuspend the Oligotex:mRNA pellet in 1 ml Buffer OW2 by vortexing or pipetting. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.
7. Repeat step 6 once.
8. Add 100 µl hot (70°C) Buffer OEB. Pipet up and down 10-15 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A+ mRNA, to a small spin column, close column and set aside.
Note: Ensure that Buffer OEB does not cool significantly during handling.
9. To ensure maximal yield, add another 100 µl hot (70°C) Buffer OEB to the Oligotex pellet. Pipet up and down 10-15 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A+ mRNA, into the spin filter with the previous 100ul of eluate.
10. Spin filter column for 2 min at 18000xg to remove any remaining Oligotex suspension from the A+ RNA.
11. Ethanol precipitate and run samples on Agilent bioanalyzer to visualize RNA.

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
2 ul 50 ng/ul random primers
2.5 ul 50 uM oligo-DT primer
1.25 ul Anti-RNAase

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
1.25 ul 0.1 M $MgCl_2$
1.25 ul 10 mM dNTPs
2.5 ul 0.1M DTT

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

After 30 minutes at 15 degrees, pause program and add:

1.25 ul Actinomycin-D (120 ng/ul)
1.25 ul Superscript III

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
Elute 2 x 15 ul EB

2nd Strand cDNA Synthesis
1 ul 5X 1st Strand Buffer
15 ul 5X 2nd Strand Buffer
0.5 ul $MgCl_2$
1 ul DTT
2 ul dU/dNTPs
0.5 ul E. coli DNA ligase
2 ul E. coli DNA polymerase I
0.5 ul RNAse H

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
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 10 mM ATP ("10X ER")
4 ul dNTP mix 10mM
5 ul T4 DNA polymerase 3U/ul (NEB)
1 ul DNA polymerase I Lg Klenow Fragment 5U/ul (NEB)
5 ul T4 PNK 10U/ul (NEB)

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

10 ul dATP (1 mM)
3 ul Klenow fragment 3' to 5' exo- 5 U/ul

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

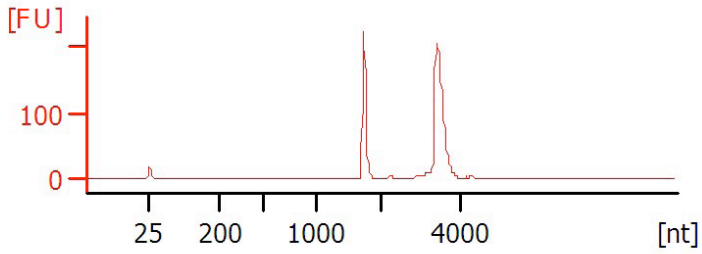
Add 10ul loading buffer
Run out samples on a 2% agarose gel.
Cut out 200 bp band, and another band just slightly larger (freeze larger slice at -20).
Then use the Qiaquick gel extraction kit (Qiagen Cat. #28706) and elute 2 x 15ul.

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)

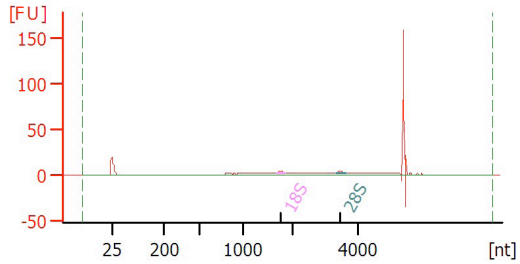
50 ul 2X HF Phusion Mix (New England Biolabs Cat. # M0531L)
29 ul water or up to 100 ul with water (depends on amount of DNA used)

98° 1 min then 16 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use QiaQuick PCR cleanup kit and elute 1x30uL of elution buffer.

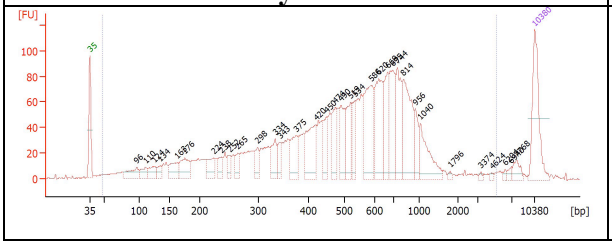
Bioanalyzer: Total RNA



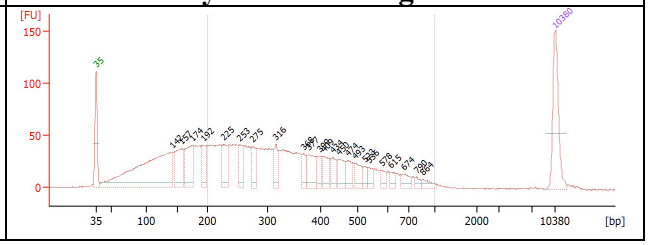
Bioanalyzer: Poly A+ Selection



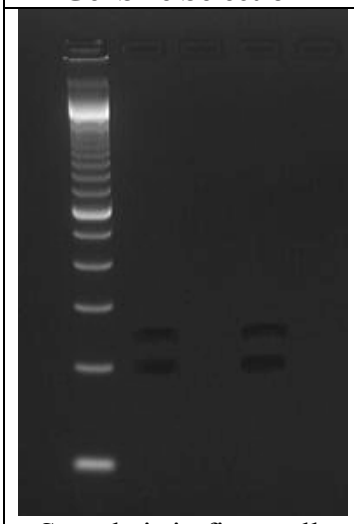
Bioanalyzer: cDNA



Bioanalyzer: Post-Fragmentation



Gel Size Selection



Sample is in first well.

Final Library