Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: CD14 Whole Cell Poly A- RNA, BioRep 1
RNA ID:
Library ID: LID44657
Protocol ID:

LAB MEMBERS

Wet lab: Carrie A. Davis, Jorg Drenkow, Meagan Fastuca, Kimberly Persaud, Lei-Hoon See, Huaien Wang.
Computational Lab: Alex Dobin, Sonali Jha, Wei Lin, Felix Schlesinger, Chris Zaleski.

PI: Tom Gingeras

CELL CULTURE: Cells were obtained from John “Stam”

POLY-A+ SELECTION:

Qiagen mRNA Isolation Protocol (using Oligotex mini kit (Qiagen Cat. # 70042))

The batch protocol has been used for the recent library production, but from other experience the spin column protocol (listed in the handbook prior to the batch protocol) gives the same results (as far as bioanalyzer image goes)

Important notes before starting
• This protocol may be necessary if you are using impure total RNA or if you are unsure about the purity of your total RNA. Many isolation procedures do not remove contaminants such as protein that can clog Oligotex spin columns. Better results are generally obtained with purer starting material.

• **Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature.**
• **Heat a water bath or heating block to 70°C, and heat Buffer OEB.**
• Review the introductory material on pages 12–19 before starting.
• If working with RNA for the first time, please read Appendix A (page 76).
• Determine the amount of total RNA in the RNA sample (see “Quantification of starting RNA”, page 18).

• Buffer OBB may (and almost always does) form a precipitate upon storage. If necessary, redissolve by warming at 37°C for approximately 10 minutes, and then place at room temperature. You can wrap the OBB bottle in parafilm and carefully, partially, submerge it in the water bath, or aliquot the needed amount in 1.5ml tubes and use the heat block

• 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. Pipet total RNA into an RNase-free 1.5 ml microcentrifuge tube, and adjust the volume with RNase-free water (if necessary) to the volume indicated in Table 5.

Note: The initial volume of the RNA solution is not important so long as the volume can be brought up to the indicated amount with RNase-free water. If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of RNase-free water by heating the tube for 3 min at 60°C followed by vortexing for 5 s and sharply flicking the tube. Repeat at least twice.

2. Add the appropriate volume of Buffer OBB and Oligotex Suspension (see Table 5). Mix the contents thoroughly by pipetting or flicking the tube.

Table 5. 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	15	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
1.0–1.5 mg	650ul	650	85	Maxi
1.5–2.0 mg	650ul	650	115	Maxi
2.0–2.5 mg	650ul	650	135	Maxi
2.5–3.0 mg	650ul	650	175	Maxi

*We generally use slightly more than the recommended amount of beads (~5ul)

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 (manual says 10, we say 12)**.**
This step allows hybridization between the oligo dT30 of the Oligotex particle and the poly-A tail of the mRNA.
5. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000 x *g*), and carefully remove the supernatant by pipetting.
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. **Note:** Save the supernatant until certain that satisfactory binding and elution of poly A+ mRNA has occurred. **We save the supernatant always, as to save the A- fraction. EtOH precipitate and resuspend in 100 µl of RNAse free water.**

6. Resuspend the Oligotex:mRNA pellet in 1 ml Buffer OW2 by vortexing or pipetting (pipetting works better, be sure to resuspend well) Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.
Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatantis left in the microcentrifuge tube. The remaining solution will not affect the procedure.
7. Repeat step 6 once.
8. Add 20–100 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.
***We always use 100ul, it gives better yields.**
Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with Oligotex and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.
9. To ensure maximal yield, add another 20–100 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. EtOH precipitate.

A^- Fraction Purification

Using the saved A- fraction from oligo-dT selection (step 5) perform two more rounds of Oligotex selection to remove any remaining A+ RNA.

Add 20µl Oligotex suspension to A^- fraction
Mix well by pipetting
Put in 70^o heatblock for 3 minutes.
Put at room temp. for 12 minutes, mix by inverting the tubes every so often
Spin at 18000xg at room temp for 2 minutes to pellet the Oligotex.
Remove and keep the supernatant.
EtOH Precipitate and resuspend in 100 µl of RNAse Free Water.

Repeat steps **6-11 of the Poly A+ Selection** to extract the A+ fraction from the Oligotex Pellet.

Repeat A^- Fraction Purification steps to perform another round of Oligotex selection.

Used final A^- Fraction for the remainder of the protocol

Ribosomal RNA Depletion

Kit: EPICENTRE rRNA Removal Kit (RZH1046)

A. Prepare the Ribo-Zero Microspheres

1. Remove the Ribo-Zero Core from 4°C storage and allow the tubes to warm to room temperature for 30 minutes. These components must be at room temperature for use in Step A3.
2. Remove the Ribo-Zero rRNA Removal Kit from -80°C storage, thaw the tubes and place them on ice.
3. Vigorously vortex the room-temperate Microspheres for 20 seconds to produce a homogenous suspension. The Microspheres are capable of withstanding vigorous vortex mixing and remain as a homogenous suspension for several minutes.
4. For each reactions, pipette 65 µL of Microspheres into a separate 2-ml Microsphere Wash Tube. Aspirate the Microspheres suspension SLOWLY to avoid air bubbles and to ensure pipetting the full required volume. Return the unused Microspheres to 4°C.
5. Centrifuge the dispensed Microspheres at 12,000 x g for 3 minutes. Remove each tube from the centrifuge, keeping it in the same orientation as was in the centrifuge, and carefully pipette off and discard the supernatant, without disturbing the pellet.
6. Wash the Microspheres by adding 130 µL of Microsphere Wash Solution to each tube. Vigorously vortex to resuspend.
7. Centrifuge the tubes at 12,000 x g for 3 minutes. Remove each tube from the centrifuge, keeping it in the same orientation as was in the centrifuge, and carefully pipette off and discard the supernatant, without disturbing the pellet.
8. Add 65 µL of Microsphere Resuspension Solution to each tube and resuspend the Microspheres by vigorous vortex at max speed until a homogenous suspension is produced.
9. Add 1 µL of Riboguard RNase Inhibitor to each tube of resuspended Microspheres. Vortex briefly and store the tubes at room temperate.

B. Treat the total RNA sample with Ribo-Zero rRNA Removal Solution

Amount of Input Total RNA	Max Volume of Total RNA That Can Be Added to Reaction	Volume of Ribo-Zero rRNA Removal Solution Used Per Reaction
1-2.5 µg	28 µL	8 µL
>2.5-5 µg	26 µL	10 µL

1. The maximum volume of the RNA sample and the volume of the Ribo-Zero rRNA Removal Solution used per reaction is dependent on the amount of total RNA in the sample. See Table Above.
2. In a RNase-free microcentrifuge tube, combine in the order given:
   21 µL RNase Free Water
   4 µL Ribo-Zero Reaction Buffer
   5 µg Total RNA Sample (see table)
   10 µL Ribo-Zero rRNA Removal Solution
   _____
   40 µL Total Volume
3. Gently mix the reaction and incubate at 68°C for 10 minutes. During the incubation return the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer to -80°C storage.
4. Remove the reaction tubes and incubate each at room temperature for 15 minutes.

C. Microsphere Reaction and rRNA Removal

1. Briefly vortex the wash, room temperature Microspheres from step A9.
2. Using a pipet, add the hybridized RNA sample from step B4 to the resuspended Microspheres. Mix the contents by pipetting 10-15 times immediately. Vortex for five seconds.
3. Incubate the tubes at room temperature for 10 minutes, with vortexing for 5 seconds every 3-4 minutes.
4. At the end of the 10 minute incubation at room temperature, vortex the sample for 5 seconds and then place at 50^0C for 10 minutes in a water bath, heating block, or other temperature-control device.
5. After 10 minutes, immediately transfer the RNA Microspheres suspension to a Microspher Removal Unit and centrifuge at 12,000 x g for 1 minute at room temperature. Save the eluate in the collection tube and discard the filter.

D. Purify the rRNA Depleted Sample

1. Adjust the volume of the sample to 180 µL using RNase Free Water
2. Proceed with ethanol precipitation procedure, **ALSO ADDING** 2 µL of Glycogen (10mg/ml) to the sample before freezing at -80^{o}C for 30 minutes.

LIBRARY PROTOCOL: Adapted from… *Transcriptome analysis by strand-specific sequencing of complementary DNA* Dmitri Parkhomchuk, Tatiana Borodina, Vyacheslav Amstislavskiy, Mariya Banaru, Linda Hallen, Sylvia Krobitsch, Hans Lehrach & Alexey Soldatov.

Use 100 ng of ribominused RNA (add spike ins, pool14 NIST beta) in 4.75ul or less of RNase free H20 for cDNA synthesis

cDNA- 1st strand: Mix

4.75 ul sample r- RNA
2ul 50ng/ul random primers (Invitrogen Cat. # 48190-011)
2.5 50uM oligo-DT primer (Invitrogen Cat. # 18418-020)
2ul NIST spike-ins
1.25ul RNAse inhibitor (Ambion Cat. # AM2692)
Up to 12.5ul with RNAse free H20 if needed

98° 2 min
70° 5 min
.1°/s ramp to 15°
15° 30 min
.1°/s ramp to 25°
25° 10 min
.1°/s ramp to 42°
42° 45 min
.1°/s ramp to 50°
50° 15 min
75° 15 min
4° hold

As soon as 15 degrees is reached (after ~15min), pause program and add:

5ul 5X First Strand Buffer (Invitrogen Cat. # sold with SS III)

1.25ul .1M MgCl2 (Ambion Cat. # AM9530G)
1.25ul 10mM dNTPs (Invitrogen Cat. # 18427-013)
2.5ul .1M DTT (Invitrogen Cat. # sold with SS III)

22.5ul (total at this point)

After 30 minutes at 15 degrees, pause program and add (**before temp. ramp!**):

1.25ul Actinomycin-D (Invitrogen Cat. # A7592) (we have a 1mg/ml stock, dilute to 120ng/ul in 10mM Tris-Cp pH 7.6 (Sigma Cat. # T2444-1L) before use)
1.25ul Superscript III (Invitrogen Cat. # 18080-044)

25ul 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 volume PB (500ul) mix and apply to Minelute spin column
Follow Qiagen Minelute cleanup protocol (Qiagen Cat. # 28006)
Elute 2 x 15ul EB

2nd Strand Synthesis

Prepare 2nd strand mix:
(22.5ul per sample)

1ul 5X 1st Strand Buffer (Invitrogen Cat. # sold with SS III)
15ul 5X 2nd Strand Buffer (Invitrogen Cat. # 10812-014)
.5ul MgCl2 (Ambion Cat. # AM9530G)
1ul DTT (Invitrogen Cat. # sold with SS III)
2ul dUNTPs (Roche dUTP Cat. # 13796926 dNTPs Cat. # 11969064001)
.5ul E. coli DNA ligase (Invitrogen Cat. # 18052-019)
2 ul E. coli DNA polymerase I (Invitrogen Cat. # 18010-025)
.5ul RNAse H (Invitrogen Cat. # 18021-071)

22.5ul

Mix:
30ul first strand reaction
22.5ul second strand mix
22.5ul RNase free H20

75ul final reaction volume

2 hours 16 degrees, 4 degrees hold in PCR machine
Bringing volume to 100ul with H20, then add 500ul PB, follow minelute cleanup protocol
Elute 2 x 26ul (fragmentation takes place in 50ul).
Bioanalyzer- high sensitivity DNA chip (to see if cDNA is full length, peak should be around 1000bp- if it is not, you need to lessen fragmentation time) (Agilent Cat. # 5067-4626)

Fragment cDNA: Covaris
If machine is not on:
Fill appropriate chambers with autoclaved DI water
Run degas program (~30 minutes)

Transfer your 50ul cDNA sample to the sonicator tube (Covaris Cat. # 520045) (using pipette)
Place on machine (snaps in) and run program degas60snapcap100ul (60s sonication)
Run Bioanalyzer- high sensitivity DNA chip to check fragment size (peak should be 200-300)
(Agilent Cat. # 5067-4626)

End-Repair cDNA

48ul sample
27ul H20
10ul T4 DNA ligase buffer with 10mM ATP ("10X ER") (New England Biolabs Cat. # B0202S)
4ul dNTP mix 10mM (Invitrogen Cat. # 18427-013)
5ul T4 DNA polymerase 3U/ul (New England Biolabs Cat. # M0203L)
1ul Klenow DNA polymerase 5U/ul (New England Biolabs Cat. # M0210S)
5ul T4 PNK 10U/ul (New England Biolabs Cat. # M0201L)

100ul final volume

Room temp. 30min.
Add 500ul PB, follow Minelute cleanup, elute 2 x 16ul

Addition of single <A> Base

32ul eluted cDNA
5ul NEBuffer2 (New England Biolabs Cat. # B7002S)
10ul dATP (1mM) (Roche 11934511001)
3ul Klenow fragment 3' to 5' exo- 5U/ul (New England Biolabs Cat. # M0212S)

50ul final volume

37 degrees, 30 min.

Bring volume to 100ul (add 50ul H20), then add 500ul PB
Follow minelute cleanup, elute 1 x 19ul

Adapter Ligation

19ul eluted cDNA
25ul DNA ligase buffer (Enzymatics Cat. # B101L)
1ul adapter oligo mix (Illumina Cat. # 1003455)
5ul DNA ligase 1U/ul (Enzymatics Cat. # L603-HC-L)

50ul final volume

Room temp, 15 min.
Bring volume to 100ul with H20 (add 50ul), then add 500ul PB
Minelute cleanup, elute 1 x 15ul

UNG Treatment

15ul eluted cDNA
1.7ul 500 mM KCl (Ambion Cat. # AM9640G)
1ul UNG (Roche Cat. # N808-0096)

37 degrees, 15 min
95 degrees, 10 min
Hold on ice

Add 10ul loading buffer (Sigma Cat. # G2526-5ML)

Run on 2% Ultra-pure agarose gel (Invitrogen Cat. 15510-019) for 2 hours, 70V (use 100bp ladder (Invitrogen Cat. # 15628-019)
Cut out 200bp band, and another band just slightly larger (freeze larger slice, -20)

If you do not see anything on the gel at this point, do not be alarmed, cut bands anyway

Use Qiaquick gel extraction kit, used 250bp band (Qiagen Cat. #28706), elute 2 x 15ul

PCR Amplification: Mix

15ul eluted cDNA from gel-extraction (freeze remaining cDNA)- If you suspect you need more or less for good amplification, use more or less
1ul PE primer 1 (IDT)
1ul PE primer 2 (IDT)
50ul HF Phusion Mix (Finnzymes Cat. # F-531L)
33ul H20 (adjust this volume according to how much cDNA was used)

100ul final PCR volume

98° 1 min

18 cycles of:
98° 10s
60° 30s
72° 30s

72° 5 min
4° hold

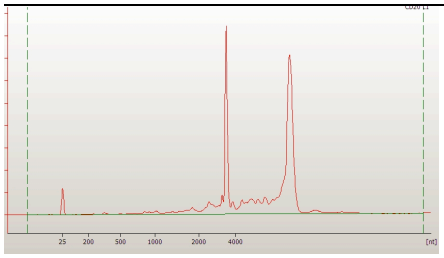
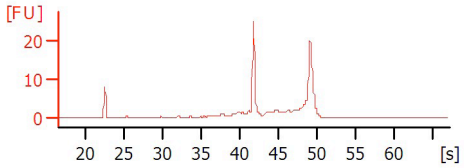
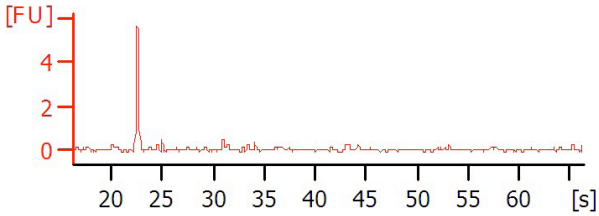
Add 500ul PB, Min-elute clean up, elute 1 x 15ul

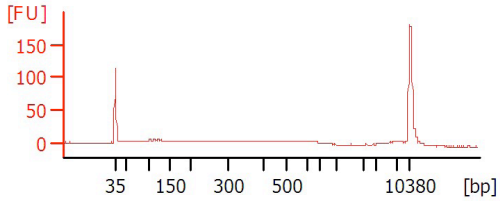
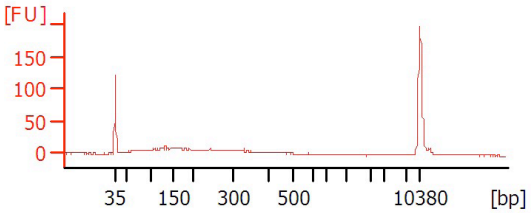
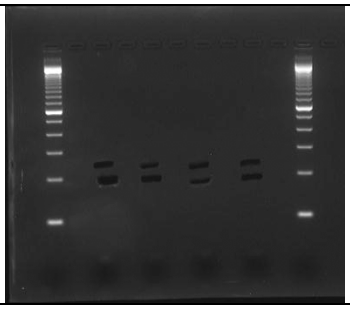
Add 10ul loading dye, run on 2% gel at 70V for 2 hours

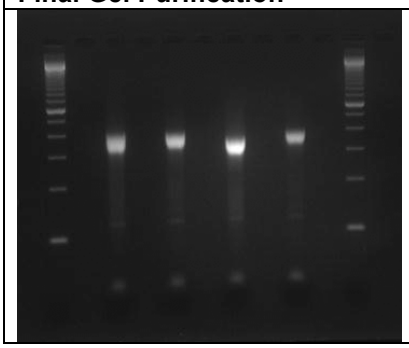
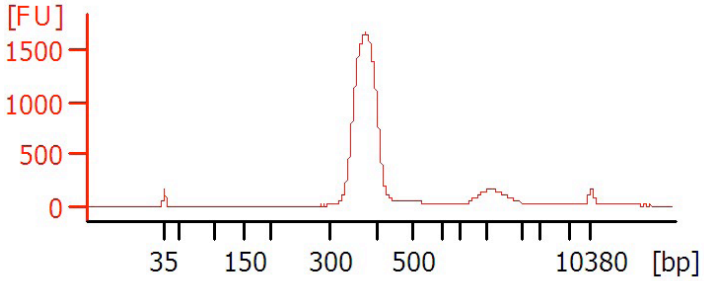
Cut distinct band (should be ~100bp larger than cDNA band)
You may have more than 1 band at this point, cut whichever band is ~100bp larger than your cDNA cut was

Gel purify as before
Elute 2 x 15ul
Precipitate
Resuspend 25ul H20

Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul) (Agilent Cat. # 5067-4626)

Bioanalyzer: Total RNA	Bioanalyzer: A- after two rounds of Oligotex Selection	BioAnalyzer: After Ribosomal Depletion
		

BioAnalyzer: cDNA	BioAnalyzer: Post-Fragmentation	Gel Size Selection
		

Final Gel Purification*	BioAnalyzer: Final Library**
	

** The major band is cut out.*

*** Sometimes we see a doublet in the BioAnalyzer image of the final gel purified library. We take the height of the first peak to represent the library insert size when determining molarity. These doublets are not visible on gels, the libraries sequence fine and show inserts surrounding the first peak size.*