

Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: LI1 Mouse

RNA ID: Limb 15-1

Library ID: LID46985

Protocol ID:

Cold Spring Harbor Laboratory

Genome Center

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LAB MEMBERS

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Mouse Tissue Total RNA isolation

1. Remove the tissue sample from storage. Determine the amount of tissue.
2. Place it directly into 3ml QIAzol Lysis Reagent in a 15ml Falcon tube for disruption and homogenization.
3. Homogenize immediately using the TissueRuptor.
4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
5. Separate the homogenization to 3 1.5ml eppendorf tubes (1ml/tube). Add 200µl chloroform to the tube containing the homogenate and cap it securely. Shake the tube vigorously for 15 s.
6. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.
7. Centrifuge for 15 min at 12,000 x g at 4°C.
8. Transfer the upper aqueous phase to a new collection tube. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.
9. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube. Close the lid gently and centrifuge at 8000 x g (10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.*
10. Repeat step 9 using the remainder of the sample. Discard the flow-through.*
11. Add 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the column. Discard the flow-through.
12. Pipet 500 µl Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the column. Discard the flow-through.
13. Add another 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at 8000 x g (10,000 rpm) to dry the RNeasy Mini spin column membrane.
14. Place the RNeasy Mini spin column into a new 2 ml collection tube, and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.
15. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube. Pipet 50 µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently, wait for 1min and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.
16. Repeat step 15 with a second volume of 50 µl RNase-free water. Elute into the same collection tube.
17. Proceed to ethanol precipitation.

Ethanol Precipitation

1. Add 2.5 volumes of 100% ethanol and 1/10 volumes of NaOAc PH 5.5 (Ambion Cat. # AM9740) to the eluted RNA.
2. Freeze in -80°C for at least 30 min.
3. Centrifuge for 35 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 1 mL 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 3-5 min making sure that the pellet is dry.

8. Resuspend the pellet with RNase-free water.

Separating the large RNA Procedure

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 the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. Up to 700 μ L can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 μ L, apply the mixture in successive applications to the same filter. Centrifuge for 30 seconds to pass the mixture through the filter. Centrifuge at RCF 5,000 x g (typically 5,000 rpm). Collect the filtrate. If the RNA mixture is >700 μ 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 (This is for small RNA.).
5. Apply 700 μ L miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~1 min at RCF 5,000 x g, or use vacuum to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.
6. Apply 500 μ L Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.
7. Repeat with a second 500 μ L aliquot of Wash Solution 2/3. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min at RCF 10,000 x g to remove residual fluid from the filter.
8. Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 80 μ L of 95°C Elution Solution, and close the cap. Incubate at room temperature for ~2 min. Spin for 1 min at RCF 10,000 x g to recover the RNA. Repeat step 8 with a second aliquot of preheated Elution Solution.
9. Transfer the RNA solution to a new RNase free 1.5ml tube. Follow by Ethanol Precipitation.

Ethanol Precipitation

1. Add 2.5 volumes of 100% ethanol and 1/10 volumes of NaOAc PH 5.5 (Ambion Cat. # AM9740) to the eluted RNA.
2. Freeze in -80°C for at least 30 min.
3. Centrifuge for 35 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 1 mL 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 3-5 min making sure that the pellet is dry.

8. Resuspend the pellet with RNase-free water.

DNase Digest (same for Small and Large RNA) Clean up

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.

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 supernatant is 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. Bring volume up to 250uL and repeat the entire Oligotex procedure again with the remaining RNA.

12. EtOH precipitate.

RIBOMINUS TREATMENT (Ribominus Kit Invitrogen Cat. # A10837):

Hybridization Step

Instructions are provided below to perform hybridization for 1–10 ug of your total RNA sample with the RiboMinus™ Eukaryote Probe. To process >10 ug total RNA sample, divide your sample into two samples, each containing <10 ug total RNA.

1. Set a water bath or heat block to 70–75°C.

2. To a sterile, RNase-free 1.5 mL microcentrifuge tube, add the following:

Total RNA (1–10 ug): <10 uL

RiboMinus™ Probe (15 pmol/L): 10 uL

Hybridization Buffer: 100 uL

3. Incubate the tube at 70–75°C for 5 minutes to denature RNA.

4. Allow the sample to cool to 37°C slowly over a period of 30 minutes by placing the tube in a 37°C water bath (a heat block works as well). To promote sequence-specific hybridization, it is important to allow slow cooling. **Do not** cool samples quickly by placing tubes in cold water.

5. While the sample is cooling down, proceed to **Preparing Beads**.

*An earlier version of this protocol says to use RNA in less than 20ul, add 10ul of probe and 300ul hybridization buffer, this larger volume means you need to precipitate the ribominused RNA in a 2ml tube at the end. Either way works. It doesn't change anything else expect the supernatant volumes and the precipitation tube size.

Preparing Beads

1. Resuspend RiboMinus™ Magnetic Beads in its bottle by thorough vortexing.

2. Pipet 750 uL of the bead suspension into a sterile, RNase-free, 1.5 mL microcentrifuge tube.

3. Place the tube with the bead suspension on a magnetic separator for 1 minute. The beads settle to the tube side that faces the magnet. Gently aspirate and discard the supernatant.

4. Add 750 uL sterile, DEPC Water to the beads and resuspend beads by pipetting

5. Place tube on a magnetic separator for 1 minute. Aspirate and discard the supernatant.

6. Repeat Steps 4–5 once.
7. Resuspend beads in 750 μ L Hybridization Buffer and transfer 250 μ L beads to a new tube and maintain the tube at 37°C for use at a later step.
8. Place the tube with 500 μ L beads on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
9. Resuspend beads in 200 μ L Hybridization Buffer and keep the beads at 37°C until use.

Removing rRNA

1. After the incubation at 37°C for 30 minutes of the hybridized sample (above), briefly centrifuge the tube to collect the sample to the bottom of the tube.
2. Transfer the sample (~120 μ L- this will be ~330ul with the older protocol) to the prepared RiboMinus™ Magnetic beads from Step 9 (**Preparing Beads**, above). Mix well by pipetting up and down
3. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
4. Place the tube on a magnetic separator for 1 minute to pellet the rRNA-probe complex. **Do not discard the supernatant. The supernatant contains RiboMinus™ RNA.**
5. Place the tube with 250 μ L beads from Step 7 (**Preparing Beads**, above) on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
6. To this tube of beads, add ~320 μ L (~500ul with older protocol) supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or low speed vortexing.
7. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
8. Place the tube on a magnetic separator for 1 minute to pellet the rRNA-probe complex. **Do not discard the supernatant as the supernatant contains RiboMinus™ RNA.**
9. Transfer the supernatant (~ 320 μ L- ~500ul with older protocol) containing **RiboMinus™ RNA** to a small filter column and spin at max speed for 2 minutes to remove any remaining magnetic particles.
10. Transfer flow through (ribominus RNA) to a new tube (1.5ml for small volume, 2ml for large volume)
11. EtOH precipitate with glycoblue

SPIKE-INS: NIST Spike-Ins beta set: Pool 14. 2 ng added to 100 ng of RNA. Use “corrected fasta” to map against.

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, pool 14 NIST beta) in 4.75ul or less of RNase free H2O 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 H2O 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 0.1M 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 H2O)
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)
0.5ul 0.1 M MgCl₂ (Ambion Cat. # AM9530G)
1ul DTT (Invitrogen Cat. # sold with SS III)
2ul dUTPs (Roche dUTP Cat. # 13796926 dNTPs Cat. # 11969064001)
0.5ul 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 H₂O

75ul final reaction volume

2 hours 16 degrees, 4 degrees hold in PCR machine
Bringing volume to 100ul with H₂O, 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 H₂O
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 H2O), 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 H2O (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 (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 H2O (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 2 x 25ul

Measure library with Nanodrop (1ul) –very inaccurate.

Run High sensitivity DNA chip (1ul) (Agilent Cat. # 5067-4626)

Dilute to 10nM (do not have to use whole library)

Prior to cluster generation we add PhiX at 1%.

