Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Cell Line] H1 derived Neurons Biorep #2
RNA ID: 096WC
Sample ID: SID30046
Library ID: LID55973
Protocol ID:

Cold Spring Harbor Laboratory
Genome Center
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LAB MEMBERS

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CELL CULTURE: Cells are grown according to the ENCODE growth protocol and standards. Each bioreplicate grown and isolated independently.

RNA ISOLATION:

Kits: RNeasy mini kit (QIAGEN cat#:74106)
miRNeasy Mini kit (QIAGEN cat#:217004)
mirVana miRNA Isolation Kit (Cat #: AM1560)

Total RNA Purification

1. Regular harvest and count cells and centrifuge for 10 min at 1900 rpm 4°C.
2. Completely aspirate supernatant.
3. Resuspend all pellets in 10-30 mL of cold PBS (Thermo Scientific Cat. # SH30264.02) by pipetting up and down.
4. Centrifuge for 5 min at 2000 rpm 4°C.
5. Carefully aspirate the supernatant.
6. Add Buffer RLN to lyse plasma membrane. For the pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Carefully resuspend cells in the appropriate volume of cold (4C) Buffer RLN. Incubate on ice for 5 min.

Number of cells	RLN (4C) (ml)
$5X10^6$-$5X10^7$	0.5
$5X10^7$-$1X10^8$	1.0

7. Centrifuge lysate at 4C for 10 min at 3200rpm. Transfer supernatant to an RNase-free centrifuge tube (cytosol part), and keep the pellet (nuclear pellet).
8. Add 1ml (if the sample is in epprndorf tube) RLN to resuspend nuclear pellet. Centrifuge at 4C for 5 min at 3200rpm.
9. Discard the supernatant. Add 700ul QIAzol to the pellet. Homogenize the sample using a syringe and 18 guage needle. Pass sample through the needle until it becomes smooth and there are no thick globs.
Note: If number of cells is more than $1x10^7$ then transfer the homogenate to a 15ml Falcon tube and add more QIAzol.
10. Place the tube(s) containing the homogenate on the benchtop at room temperature (15-25°C) for 5 min.
11. Add 20% volumes of chloroform (Sigma Cat. # C2432-25ML) to the homogenate and cap it securely. Shake the tube vigorously for 15 s.
12. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 minutes.
13. Centrifuge the homogenate for 15 min at 12,000 x g at 4°C.
14. Transfer the upper, colorless, aqueous phase containing the RNA to a new collection tube.
15. Add 1.5 volume of 100% ethanol and mix thoroughly by vortexing or invert the tube several times. Do not centrifuge.
14. Pipet 700 µL of the sample including any precipitate that may have formed into an RNeasy Mini spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Repeat this step until the whole sample has been pipetted into the spin column and discard the flow-through each time.
15. Pipet 700 µL Buffer RWT into the RNeasy Mini spin column from step 15. Close the lid gently and centrifuge for 30 at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow through.
16. 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) to wash the spin column membrane. Discard the flow through.
17. Add another 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) to wash the spin column membrane. Discard the flow through and the collection tube.
18. Place the RNeasy Mini spin column in a new 2 mL collection tube. Open the lid and centrifuge at full speed for 1 min.
19. 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 one minute and then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the total RNA (containing large RNA).
20. If the expected RNA yield is >30 µg, repeat step 19 with an additional volume of 50 µL RNase-free water. Elute into the same collection tube.
21. 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 total RNA Procedure

1. Preheat Elution Solution to 95°C for use in eluting the RNA from the filter at the end of the procedure. If the 100% ethanol you plan to use for this procedure is stored cold, warm it to room temperature before starting the Final RNA Isolation.
2. Add 1.25 volume of 100% ethanol to the aqueous phase recovered from the organic extraction. Mix thoroughly by inverting the tube several times.
3. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pass the sample through a Filter Cartridge, and collect the filtrate. Up to 700 µL can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.
4. Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10,000 x g (typically 10,000 rpm). Spinning harder than this may damage the filters.
5. Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge from above and centrifuge for ~5–10 sec or use a vacuum to pull 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 µL 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 µL aliquot of Wash Solution 2/3.
8. 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 to remove residual fluid from the filter.
9. Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL of pre-heated (95°C) nuclease-free water to the center of the filter, and close the cap. Spin for ~20–30 sec at maximum speed to recover the RNA.
10. Transfer the RNA solution to a new RNase free ependof tube. Follow by the Separating large RNA procedure.

Separating 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. Keep the flow-through for the small RNA Isolation.
4. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pass the sample through a Filter Cartridge, and collect the filtrate. Up to 700 µL can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.
5. Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10,000 x g (typically 10,000 rpm). Spinning harder than this may damage the filters.
6. Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~1 min at RCF 5,000 x g. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.
7. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step. Repeat with a second 500 µL aliquot of Wash Solution 2/3.

8. 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.
9. Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL 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.
10. Repeat steps 9 with a second aliquot of preheated Elution Solution.
11. Transfer 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 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)

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	8 µL	4 µL
20 U/µL RNasin/anti-RNase	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 waterbath for 30 min.
3. Proceed to RNA Cleanup, which is different for Small and Large RNA.

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.

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 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 5 min making sure that the pellet is dry.
8. Resuspend the pellet with RNase-free water.

Protocol courtesy of Philippe Batut

Degradation of 5'-monophosphate RNAs (Terminator digest)

Start from 5µg total RNA (in ≤10µl H_2O), DNAseI-treated.

Note: I've successfully gone down to 2-3µg input per sample when pooling several libraries after the RT step.

The quality of the RNA should be checked at that point by running a Bioanalyzer RNA Nano chip – RNA integrity is absolutely crucial to generate high-quality libraries.

- Denature RNA 5 min @ 65ºC. Immediately put on ice for 2 min.

    Note: Proper denaturation is important for degradation efficiency (5' structures can protect transcripts from digestion).

- Prepare mix (20µl total):

RNA sample	10µl (5µg)
TEX buffer A	2µl
TEX (1U/µl)	3µl
H_2O	5µl

- 90min @ 30ºC

- RNACleanXP cleanup, roughly as per manufacturer's protocol. Briefly:

    - Add 30µl RNACleanXP & mix thoroughly by vortexing or pipetting

- Precipitate 5 min @ room temperature
- Place on magnet for 3 min & carefully remove supernatant
- Wash twice with 100µl Ethanol 70%
- Air-dry 2 min (but without completely drying out the beads)
- Elute with 9µl H_2O, resuspend beads well by pipetting, incubate 3 min @ RT
- Place on magnet 3-5 min (untils beads are well separated) & recover 8 µl of supernatant.

Reverse-transcription

Index Sequence pool:

Neuron 096WC: index1ATGCGT-index2ATAAGC-index3ATCTCC-index4TAACTC-index5ATAGAG

- Prepare mix:

TEX-treated RNA	8 µl
CAGEscan_RT_15N oligo (400µM)	1µl
TSCAGE_6N oligo (4mM)	1µl

- 10 min @ 65ºC.
- Immediately place on ice-water mix (or ice-cold metal block) for 2 min.
- Add RT reaction mix (30µl):

Invitrogen 1st strand buffer	7.5µl
dNTPs mix	1.9µl
Sorbitol/Trehalose mix	7.5µl
DTT (100mM)	1.9µl
Betaine (5M)	5.6µl
SuperScript III RT (200U/µl)	4µl

- Incubate in thermal cycler with the following program:

4ºC	10''

22ºC	1 min
42ºC	30 min
75ºC	15 min
4ºC	Hold

- RNACleanXP cleanup: Add 65µl RNACleanXP, wash 2x µl 70% EtOH, elute with 42µl H2O, recover 40µl.

(!) Samples can be frozen at -20ºC at this point. Careful about degradation, though: RNA integrity still matters at this point.

5' Cap oxidization

Periodate oxidization of the 2' and 3' hydroxyl groups of the ribose of the 5' cap.

(!) The terminal 3' nucleotide of the RNA will also be oxidized and biotinylated.

- Prepare 250mM NaIO4 solution: dissolve 26.7mg $NaIO_4$ in 500µl H_2O.

    (!) Always prepare fresh.

    (!) Light-sensitive: keep covered in aluminum foil, on ice.

- Add 2 µl of 1M NaOAc, pH 4.5 to RNA/cDNA solution.

    (!) The pH of this solution is critical.

- Add 2 µl of 250mM $NaIO_4$ and mix well.

- Incubate on ice in the dark (or in foil) for 45 min.

- Stop the reaction by adding 2µl of 40% glycerol. Mix well by pipetting.

- Add 14µl of 1M Tris-HCl, pH 8.5. Mix well by pipetting.

- RNACleanXP cleanup: Add 105µl beads suspension, wash 2x 200µl EtOH 70%, elute with 40µl H_2O.

5' Cap biotinylation

- Prepare 15mM Biotin solution: dissolve 4.2mg biotin hydrazide in 750µl H2O.

Biotin does not dissolve well in water: vortex 20-30 min @ RT

(!) Always prepare fresh. Solution should be kept on ice, covered in foil

- Add 4µl of 1M NaCitrate, pH 6.0

- Add 13.5µl of 15mM biotin solution and mix well by pipetting

- Incubate at room temperature for 14-15 hours in the dark.

(!) No cleanup after this step: directly proceed to RNAseI digest.

RNAseI digest

- Prepare RNaseI mix. Per reaction:

1M Tris-HCl, pH 8.5	6 µl
0.5M EDTA, pH 8.0	1 µl
RNaseI (10U/µl)	5 µl

- Add RNaseI mix (12µl) and mix well by pipetting

- Incubate 30 min @ 37°C

(!) When pooling many libraries I extend this to 60 min, out of slight paranoia.

- Incubate 5 min @ 65°C and immediately place on ice for 2 minutes

- RNACleanXP cleanup: Add 125µl beads suspension, wash 2x 200µl EtOH 70%, elute with 40µl H_2O.

Streptavidin pulldown ("Cap-trapping")

- During RNaseI digest and cleanup, prepare magnetic streptavidin beads as follows:
    - Resuspend beads suspension by vortexing vigorously
    - Transfer 100µl of suspension to a new 1.7µl tube
    - Add 1.5µl of 20 µg/µl E. coli tRNA and mix well
    - Incubate 30 min @ RT (vortex every 3 min)

- Prepare wash buffers:

(!) Buffers can be stored @ RT for several months.

Wash Buffer 1

Reagents	Volume	Final concentration
5 M NaCl	45 ml	4.5 M
0.5 M EDTA, pH 8.0	5 ml	50 mM
Total	50 ml	

Wash Buffer 2

Reagents	Volume	Final concentration
5 M NaCl	3 ml	0.3 M
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
H_2O	46.9 ml	
Total	50 ml	

Wash Buffer 3

Reagents	Volume	Final concentration
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
10% SDS	2 ml	0.4%

1 M NaOAc, pH6.1	25 ml	0.5 M
1 M Tris-HCl, pH 8.5	1 ml	20 mM
H_2O	21.9 ml	
Total	50 ml	

Wash Buffer 4

Reagents	Volume	Final concentration
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
1 M NaOAc, pH6.1	25 ml	0.5 M
1 M Tris-HCl, pH 8.5	0.5 ml	10 mM
H_2O	24.4 ml	
Total	50 ml	

- Finish preparing beads:

  - Place streptavidin beads on magnetic stand for 3 min and remove supernatant

  - Add 50µl Buffer 1, resuspend beads well by pipetting, separate on magnetic stand, remove supernatant. Repeat a 2nd time.

  - Resuspend beads in 80µl Buffer 1

- Add 80µl beads suspension to RNAseI-treated sample

- Incubate 30 min @ RT (mix by gentle vortexing every 3 min)

- Place on magnetic stand for 5 min and discard supernatant

- Wash with buffer 1: Add 150µl buffer 1, resuspend beads well by pipetting, place on magnetic stand for 3 min, discard supernatant.

- Wash with buffer 2 (same procedure)

- Wash twice with buffer 3

- Wash twice with buffer 4

- Make sure supernatant is completely removed

- Elution from beads:

    - Dispense 12µl of 1M Tris-HCl, pH 7.0 to a new tube and keep on ice

    - Add 65µl of 50mM NaOH to the RNA/cDNA-bound beads. Mix well by pipetting

    - Incubate 10 min @ RT. Vortex gently every 2-3 min.

    - Place on magnetic stand for 3 min

    - Transfer supernatant to tube containing Tris-HCl pH 7.0

- AMPureXP cleanup: Add 130µl beads suspension, precipitate 5 min, wash 2x 200ml EtOH 70%, air-dry 2 min, elute with 73µl H_2O.

PCR amplification

Template	73µl
Ex Taq buffer	10µl
dNTPs (2.5mM)	8µl
CAGEscan_erF (10µM)	4µl
CAGEscan_erR (10µM)	4µl
Ex Taq (5U/µl)	1µl

PCR program:

95ºC	1 min 15''
55ºC	10''

68ºC 2 min

95ºC 15"

65ºC 10"

68ºC 2 min

Repeat the *last 3 steps* another 15 times (176 cycles total)

68ºC 5 min

Size selection by differential precipitation

(!) Make sure you actually recover exactly 100µl from the PCR: the exact volumes and ratios determine the size selection range and the efficiency of recovery.

- First precipitation: Precipitate (and remove) large inserts

  AMPureXP-to-sample ratio: 0.52 : 1

  - Add 52µl AMPureXP to 100µl of PCR reaction product
  - Precipitate 5 min, place on magnet 3 min
  - Remove and save the supernatant (needed in the next step)

- Second precipitation: Precipitate & recover medium inserts, discard short ones

  AMPureXP-to-sample ratio: 0.70 : 1

  - Prepare "beads-enriched" AMPure suspension:
    . Transfer 80µl AMPure suspension to new tube
    . Place on magnet for 3 min & discard 60µl of supernatant
    . Resuspend beads well in the remaining 20µl

- Add 18µl of this enriched suspension to the supernatant from the first round of precipitation & mix well

- Precipitate 5 min, separate on magnet, discard supernatant

- Wash 3x 300µl EtOH & air-dry until no ethanol remains (but do not overdry the beads)

- Elute with 20µl H2O, incubate 5 min @ RT, recover supernatant.

Quality control & Quantification:

Run the final library on a Bioanalyzer High Sensitivity DNA chip for quality control and preliminary quantification.

Expected size range: ~ 300-1000bp

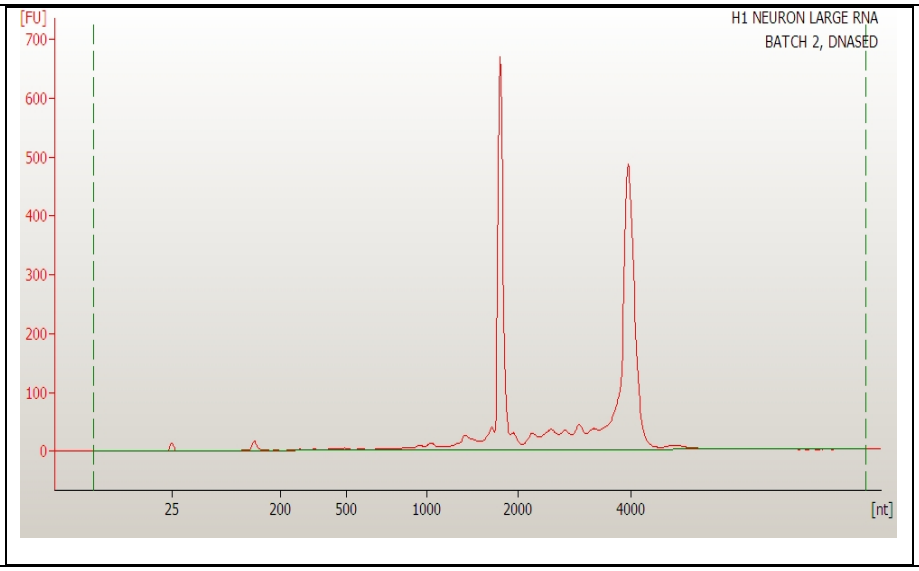
The library should be quantified by qPCR before sequencing.

Sequencing primers: Read 1: CAGEscan_r1Seq

Read 2: SBS8

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

Prior to cluster generation we add PhiX at 1%.

BioAnalyzer: Total RNA	
	

	BioAnalyzer: Final Library*