

Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Tissue] Whole Cell Liver Tissue Biorep #2

RNA ID: 099WC

Sample ID: SID30048

Library ID: LID55974

Protocol ID:

Cold Spring Harbor Laboratory

Genome Center

500 Sunnyside Blvd

LAB MEMBERS

Wet lab: Meagan Fastuca, Carrie A. Davis, Jorg Drenkow, Lei Hoon See, Huaien Wang, Kim Persaud.

Computational Lab: Alex Dobin, Sonali Jha, Wei Lin, Felix Schlesinger, Chris Zaleski.

PI: Tom Gingeras

Tissue Stabilization: Tissues were received frozen at -80C.
Precool RNAlater ICE at -80 C, add 10 vol -80C RNAlater ICE to tissue sample.
Mix and immediately transfer to -20C and store for at least 16 hours.

RNA ISOLATION:

Human Tissue Total RNA isolation

1. Remove the tissue sample from storage. Determine the amount of tissue.
2. Place it directly into predetermined amount of QIAzol Lysis Reagent in a 15ml Falcon tube for disruption and homogenization. (700 μ l QIAzol per 25 mg tissue)
3. Homogenize immediately using the TissueRuptor for 75 sec.
4. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
5. Separate the homogenization to 1.5ml eppendorf tubes (1ml/tube). Add 200 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l RNasefree 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 μ l 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 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. 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 µL, 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 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, 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 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. 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 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. 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)

<i>Reagents</i>	<i>100 µL Sample (100 µg RNA max)</i>	<i>50 µL Sample (50 µg RNA max)</i>
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
<i>Total Volume</i>	<i>100 µL</i>	<i>50 µL</i>

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 ($\geq 10,000$ 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 ($\geq 10,000$ 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 ($\geq 10,000$ 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 H₂O), 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 @ 65°C. 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 ₂ O	5µl

- 90min @ 30°C

- 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 H₂O, 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:

human liver 099WC: index1GGATAC-index2TTAACG-index3GAGTGC-index4AAGGAC-index5CGCGTT

- Prepare mix:

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

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

Invitrogen 1 st 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:

4°C	10"
22°C	1 min
42°C	30 min
75°C	15 min
4°C	Hold

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

(!) Samples can be frozen at -20°C 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 NaIO₄ in 500µl H₂O.

(!) 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 NaIO₄ 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 H₂O.

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 H₂O.

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 ₂ O	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 ₂ O	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 ₂ O	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 200µl EtOH 70%, air-dry 2 min, elute with 73µl H₂O.

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:

95°C 1 min 15"

55°C 10"

68°C 2 min

95°C 15"

65°C 10"

68°C 2 min

Repeat the *last 3 steps* another 15 times (17 cycles total)

68°C 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	
-------------------------------	--

