Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Tissue] SM-9IMHG rep 1, Artery – Aorta total RNA
RNA ID: 340WC
Library ID: 295918
Composite Library ID: 295920
Protocol ID: Long RNA T-U

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RNA extraction from Human Tissue

Reagents : RNeasy Fibrous Tissue Mini Kit (Qiagen)

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNasefree water provided in the RNase-Free DNase Set box. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. **Do not vortex.**
For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

1. Heat a water bath or heating block to 55°C for proteinase K digestion.
2 On dry ice, excise a piece of tissue < 30mg, keeping the tissue frozen at all times. Weigh the tissue.
3. Add 300µl Buffer RLT with β-ME to the tissue
4. Then add 1.5ul reagent DX (Qiagen) to reduce foaming of sample.
5. Add 1/3 volume of Zirconia beads (1mm diameter, Biospec Products) to the tube and close the tube tightly. Use parafilm to wrap around the lid of the tube to prevent leakage.
6. Position the tubes in the outermost rack positions on the Tissue Lyser. Set the Tissue Lyser to 30Hz and beat for 5 min.
TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.
7. Carefully pipet the lysates into new microcentrifuge tubes (not supplied).
Do not reuse the Zirconia beads.
8. Add 590 µl RNase-free water to the lysate. Then add 10 µl proteinase K solution, and mix thoroughly by pipetting.
9. Incubate at 55°C for 10 min.
10. Centrifuge at 20–25°C for 3 min at 10,000 x g.
A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.

11. Pipet the supernatant into a new 1.5 ml or 2 ml microcentrifuge tube (not supplied). Avoid transferring any of the pellet. If this is unavoidable, a small amount of pelleted debris may be carried over without affecting the RNeasy procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.
12. Add 0.5 volumes of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge. Precipitates may be visible after addition of ethanol. This does not affect the procedure.
13. Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge at 20–25°C for 15 s at _8000 x g (_10,000 rpm). Discard the flowthrough.* Reuse the collection tube in step 14.
14. Repeat step 13 using the remainder of the sample. Discard the flow-through.*
15. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at _8000 x g (_10,000 rpm) to wash the membrane. Discard the flow-through.*
Reuse the collection tube in step 15.
Optional: If on-column DNase digestion is not desired, add 700 µl Buffer RW1 instead, centrifuge for 15 s at _8000 x g, and discard the flow-through* (but not the collection tube). Proceed to step 16.
* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.
16. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.
17. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.
Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.
18. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) at 20–25°C. Discard the flowthrough.* Reuse the collection tube in step 18.
19. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 15 s at _8000 x g (_10,000 rpm) to wash the membrane. Discard the flow-through.
Reuse the collection tube in step 19.
Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).
20. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge at 20–25°C for 2 min at _8000 x g (_10,000 rpm) to wash the membrane.

The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
21. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 20.
* Flow-through contains Buffer RLT and is therefore not compatible with bleach.
22. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the RNeasy spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at _8000 x g (_10,000 rpm) at 20–25°C.
23. Repeat step 22 using another 30–50 µl RNase-free water, or using the eluate from step 19 (if high RNA concentration is required). Reuse the collection tube from step 22. If the expected RNA yield is >30 µg, there is no need to repeat step 22.
If using the eluate from step 22, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.
23. Proceed with Ethanol precipitation.

Ethanol Precipitation (same for Total, Small and Large RNA)

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 2hrs.
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 µL 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.

Long and Small RNA Separation

MirVana kit: Ambion MirVana miRNA Isolation Kit Cat # AM1561

1. Add 5 volumes of Lysis/Binding solution to total RNA.
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 1/3 volume of 100% ethanol to the RNA mixture from before.
6. Mix thoroughly by vortexing or inverting tube several times.
7. For each sample, place a Filter Cartridge into one of the collection tubes supplied.
8. Pipet up to 700 uL of the lysate/ethanol mixture (from the previous step) onto the filter cartridge.
9. Centrifuge for approximately 15 seconds at 5,000 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 the Long RNA using RNA cleanup section.)
10. Collect the filtrate (pool successive passes through the filter in a new tube) and record the volume.
11. Add 2/3 volume room temperature 100% ethanol to filtrate (flow-through) and mix thoroughly.
12. Pass 700 uL of each sample through a second filter cartridge and centrifuge for 15 seconds at 5,000 x g.
13. Discard the flow through.
14. Continue passing sample until the entire sample is through the filter.
15. 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 (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 (can also use vacuum).
4. Discard the flow through.
5. Repeat steps 3 and 4.
6. Spin the filter cartridge for 1 minute at 10,000g 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. Proceed to Ethanol Precipitation.

Ethanol Precipitation

See above

DNase I treatment (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 DNase 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 Long RNA. For long RNA, follow the Long RNA Purification procedure.

Ethanol Precipitation

See above.

Resuspend the pellet with 50 ul RNase-free water.

Determine RNA concentration with Nano Drop.

Ribo Zero Magnetic Separation Protocol (r-RNA Removal)

(Epicentre Ribo-Zero rRNA Removal Kit- Human/Mouse/Rat- Cat. No. MRZH11124)

A. Individual Washing Procedure Note:

1. Mix the Magnetic Beads well by pipetting or gentle vortexing.

2. For each reaction, pipet 225 µl of Magnetic Beads into a 1.5-ml RNase-free microcentrifuge tube. Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.

3. Place each 1.5-ml microcentrifuge tube on the magnetic stand for at least 1 minute (until the solution appears clear).

4. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant. ***Caution:*** *The supernatant contains 0.1% sodium azide.*

5. Remove the 1.5-ml microcentrifuge tube from the magnetic stand and add 225 µl of RNase-Free Water to each tube. Mix well by repeated pipetting or vortexing at medium speed.

6. Repeat steps 3, 4 and 5 (i.e. wash the beads a total of 2 times with RNase-Free water).

7. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add 65 µl of Magnetic Bead Resuspension Solution to each tube. Mix well by repeated pipetting or vortexing at medium speed.

8. Optional: Add 1 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.

9. Store the microcentrifuge tubes at room temperature until required 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:

RNase-Free Water	x µl
Ribo-Zero Reaction Buffer	4 µl
Total RNA sample	5 µg
Ribo-Zero rRNA Removal Solution	10 µl
Total volume	40 µl

2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. Store the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer at –70°C to –80°C.

3. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

C. Magnetic Bead Reaction and rRNA Removal

Required in Part C: 50°C water bath or heating block for 2.0-ml tubes.

1. Using a pipette, add the treated RNA from Part B to the 1.5-ml microcentrifuge tube containing the washed Magnetic Beads and, without changing the pipet tip, ***immediately and thoroughly mix the contents of the tube by pipetting at least 10 times. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.*** Repeat this process for each sample.***Important!*** *Always add the treated RNA sample to the washed Magnetic Beads and immediately mix by pipetting. Never add the Magnetic Beads to the treated RNA sample. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact the efficiency of the rRNA removal.*

2. Incubate the 1.5 ml microcentrifuge tube at room temperature for 5 minutes.

3. Following incubation, mix the reactions by vortexing at medium speed for 10 seconds and then place at 50°C for 5 minutes in an appropriate water bath or heating block. Avoid any significant condensation during this incubation step.

4. After the 5-minute incubation at 50°C, remove the microcentrifuge tubes and immediately place them on a magnetic stand for at least 1 minute (until the solution appears clear).

5. Carefully remove each supernatant (85-90 µl) containing the RNA and transfer to a labeled 1.5-ml RNase-free microcentrifuge tube. ***Important!*** *The supernatant contains rRNA-depleted RNA*

.***Optional:*** *If a small amount of Magnetic Beads are still visible in the supernatant, place the collected supernatant onto the magnetic stand for 1 minute. Remove the supernatant containing the rRNA-depleted RNA and transfer to a new 1.5-ml RNase-free microcentrifuge tube.*

6. Ethanol precipitate and resuspend in 10 µl for library construction.
7. Use 1.5 µl for running on a Pico-chip on the Bioanalyzer.

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.

Primer and adapter sequences:

index 2 :ACGTAA

Multiplexing Adapters1
5' P-GATCGGAAGAGCACACGTCT
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Multiplexing PCR Primer 1.01
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Multiplexing PCR Primer 2.01
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Use entire RNA sample from ribo zero procedure.

cDNA- 1st strand: Mix

5.5 ul sample r- RNA
2ul 50ng/ul random primers
2.5 50uM oligo-DT primer
Up to 12.5ul with RNAse free H20 if needed

98° 2 min
70° 5 min
0.1°/s ramp to 15°
15° 30 min
0.1°/s ramp to 25°
25° 10 min
0.1°/s ramp to 42°
42° 45 min
0.1°/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
1.25ul .1M MgCl2
1.25ul 10mM dNTPs
2.5ul .1M DTT
1.25ul RNAse inhibitor

_____
22.5ul (total at this point)

After 30 minutes at 15 degrees, pause program and add (**while still in 15° hold !**):

1.25ul Actinomycin-D (we have a 1mg/ml stock, dilute to 120ng/ul in 10mM Tris-Cp pH 7.6 before use)
1.25ul Superscript III

_____
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
Elute 2 x 15ul EB

2nd Strand Synthesis

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

21.5 ul	RNase free H20
2 ul	5X 1st Strand Buffer
15 ul	5X 2nd Strand Buffer
0.5 ul	MgCl2
1 ul	0 1 M DTT
2 ul	10 mM ea dUNTPs (dU, dC, dG, dA)
0.5 ul	E. coli DNA ligase
2 ul	E. coli DNA polymerase I
0.5 ul	RNAse H

_____
45 ul

Mix:
30ul first strand reaction
45ul second strand mix

_____
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 15ul
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)

Add 20 ul EB buffer (fragmentation takes place in 50ul).

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 (using pipette)
Place on machine (snaps in) and run program degas45snapcap100ul (45s sonication)
Run Bioanalyzer- high sensitivity DNA chip to check fragment size (peak should be 200-300)

End-Repair cDNA

48ul sample
27ul H20
10ul T4 DNA ligase buffer with 10mM ATP ("10X ER")
4ul dNTP mix 10mM
5ul T4 DNA polymerase 3U/ul (NEB)
1ul Klenow DNA polymerase 5U/ul (NEB)
5ul T4 PNK 10U/ul (NEB)

_____
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
10ul dATP (1mM)
3ul Klenow fragment 3' to 5' exo- 5U/ul
_____
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
1ul index adapter oligo mix
5ul DNA ligase 1U/ul (Enzymatics)
_____
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 ul 500 mM KCl
1 ul UNG

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

Add 10ul loading buffer

Run on 2% Ultra-pure agarose gel for 2 hours, 70V (use 100bp ladder)
Cut out 250bp band, and another band just slightly larger (freeze larger slice, -20)

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

Use Qiaquick gel extraction kit, elute 2 x 15ul

PCR Amplification:

15ul eluted cDNA from gel-extraction (freeze remaining cDNA)- If you suspect you need more or less for good amplification, use more or less
2ul PE InPE primer 1 25 uM
2ul PE InPE primer 2.0 05 uM
2ul index primer mix , 25 uM
50ul HF Phusion Mix
29ul H20 (adjust this volume according to how much cDNA was used)

_____
100ul final PCR volume

98° 1 min

19 cycles of:
98° 10s
60° 30s
72° 30s

72° 5 min
4° hold

Purification using AMPure XP beads

Perform the following steps, at room temperature, to concentrate your DNA sample.
1. Add 0.75X volume of AMPureXP® magnetic beads to PCR reaction. (80 ul per 100 ul PCR reaction)
Refer to the provider's instructions regarding proper use and storage of AMPureXP magnetic beads. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly (since the bead mixture is viscous and precise volumes are critical to the purification process).
2. Mix the bead/DNA solution thoroughly. Mix the beads with the DNA by pipetting up and down or inverting the tube until the solution is homogenous.
3. Quickly spin down the tube (1 second) to collect the beads. Do not pellet beads.

4. Allow the DNA to bind to beads for 5 min.
5. Spin down the tube (1 second) to collect beads. Do not pellet beads.
6. Place the tube in a magnetic bead rack for approximately 3 minutes to collect the beads to the side of the tube. The bead pellet is adequately formed when the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added. Do not remove the tube from the magnetic rack.
7. Slowly pipette off cleared supernatant and discard. Avoid disturbing the bead pellet. Since the AMPureXP buffer is viscous, some beads may slide down the side of the tube during aspiration of this buffer. If this occurs, it is preferable to leave a small volume of buffer behind to avoid aspirating beads; this residual buffer will be adequately removed during subsequent 70% ethanol washes.
8. Wash beads with 500 ul freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
• Do not remove the tube from the magnetic rack. Slowly dispense the 70% ethanol against the side of the tube opposite the beads.
• Do not disturb the bead pellet.
• After one minute, pipette and discard the 70% ethanol.
9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
• Remove tube from magnetic rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
• Place the tube back on magnetic rack.
• After 30 seconds, slowly pipette off any remaining 70% ethanol.
• Remove the tube from magnetic rack and allow beads to airdry (tube caps open) for up to 5 minutes. Beads can also be dried at 37C for about 4 min.
11. Elute the DNA off the beads.
• Thoroughly resuspend beads in 25 – 40 ul EB buffer (Qiagen) by pipetting up and down at least 20 times and/or vortexing. If beads appear over-dried or cracked, pipette vigorously to resuspend beads).
• Incubate the Elution Buffer with the beads for at least 2 minutes.
• Spin the tube down to pellet beads.
• Place the tube back on the magnetic tube rack and allow beads to magnetize to the side of the tube.
• After 30 seconds, pipette the eluted DNA into a Qiagen spin column and spin at 12000 rpm For one minute.

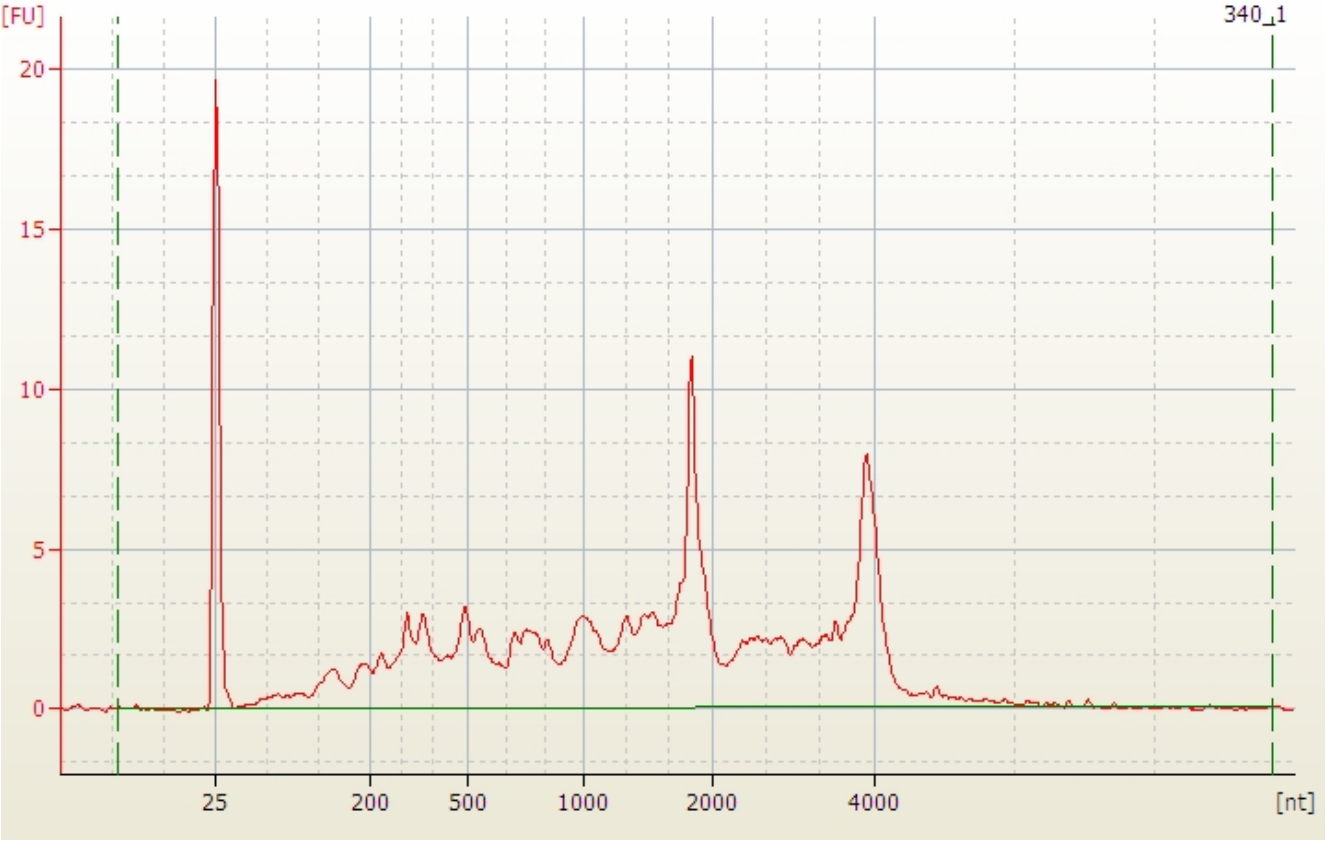
Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul)

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

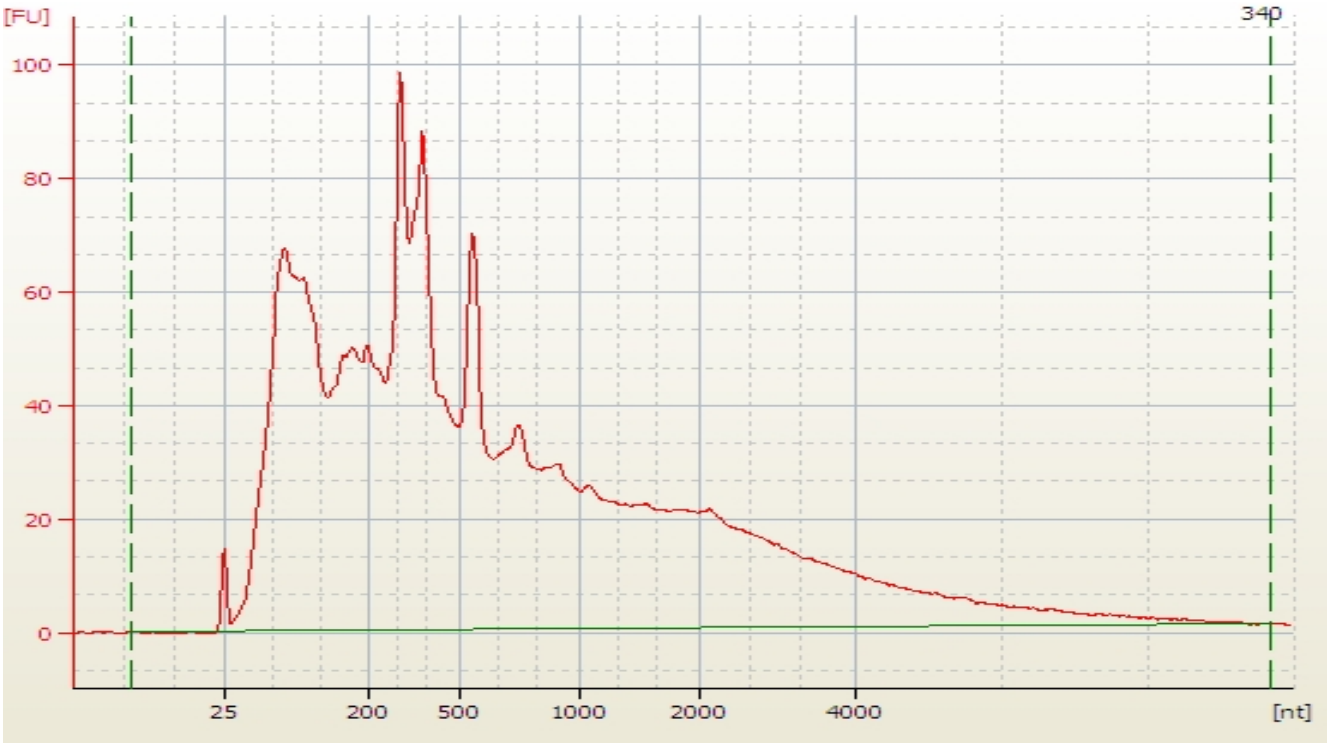
Mix both libraries 1:1 before submitting them for sequencing

Prior to cluster generation we add PhiX at 1

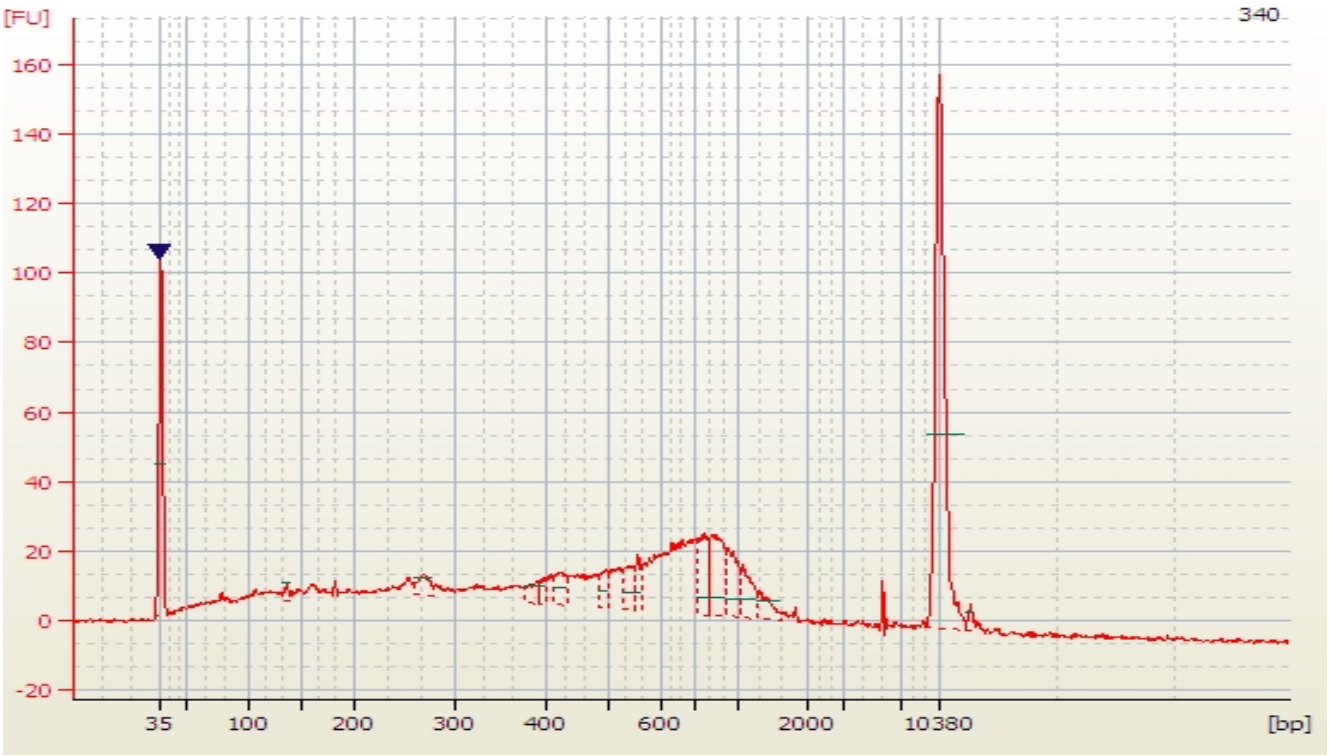
BioAnalyzer: Total RNA



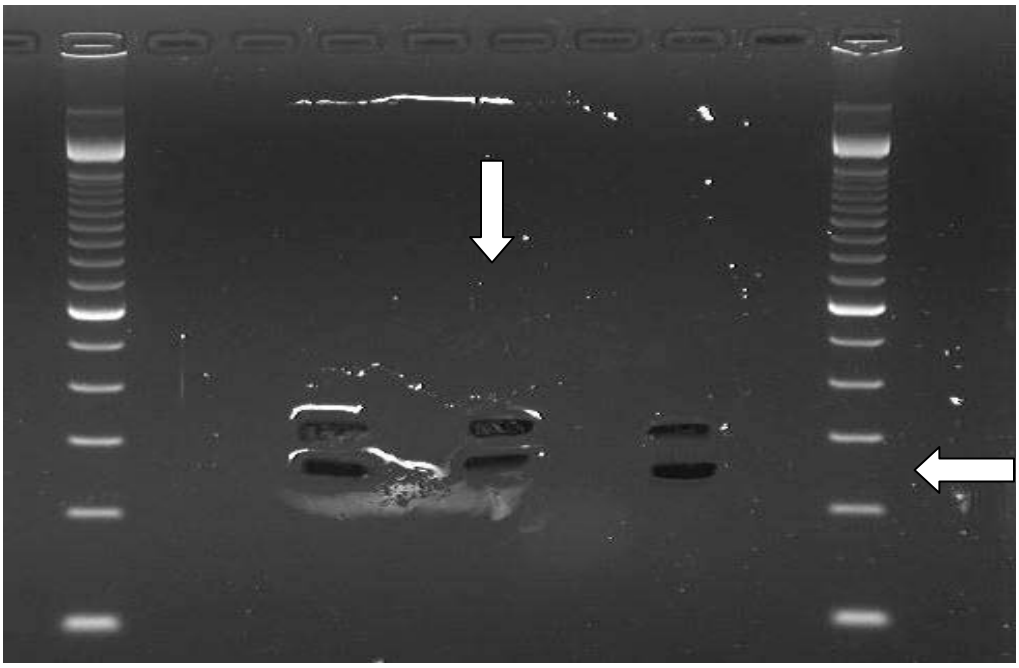
BioAnalyzer: ribo Zero RNA:



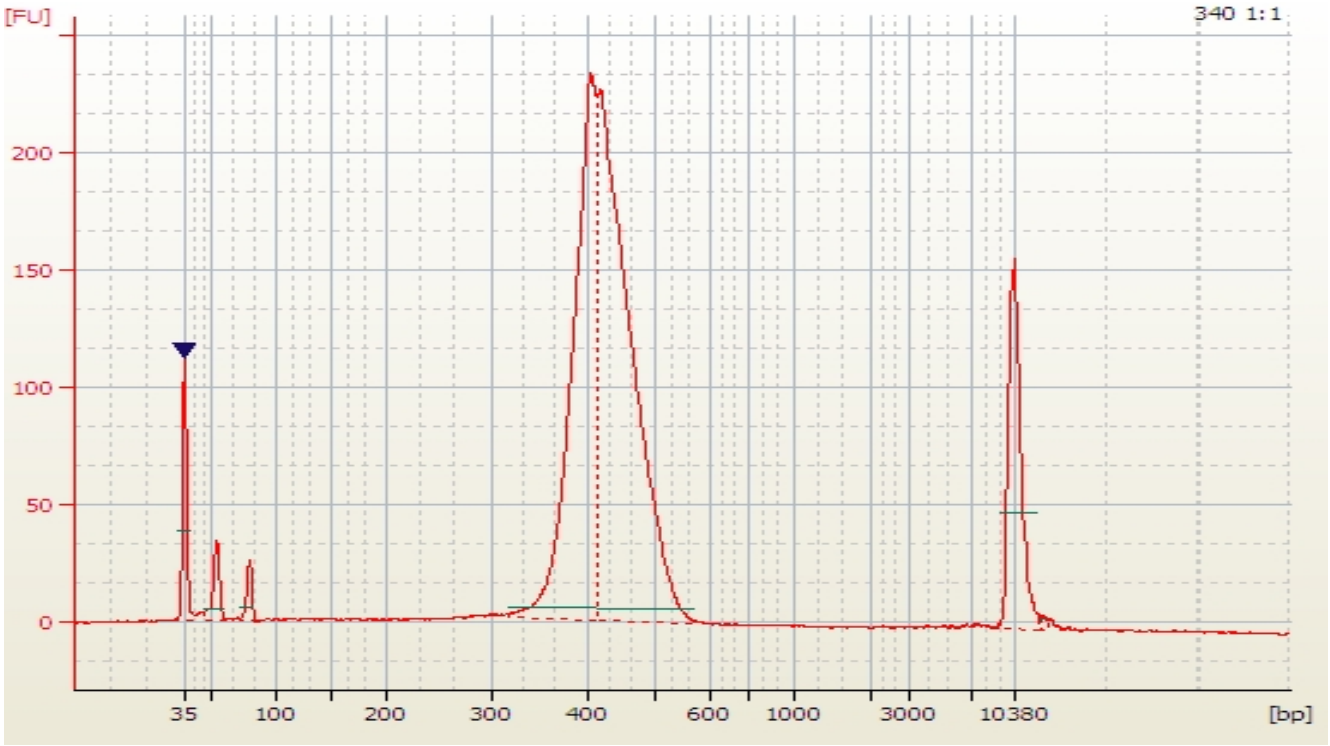
BioAnalyzer: cDNA:



Gel size selection:



BioAnalyzer: Final



* *Sometimes we see a doublet in the BioAnalyzer image of the final 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.*