Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: ENCBS328AAA
Cell Line: HSMM (Skeletal Muscle Myoblast)
RNA ID: 236WC
Library ID: 291757
Composite Library ID: 291761
Protocol ID: Long RNA T-U

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LAB MEMBERS

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RNA was sent to us from University of Washington.

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 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 Long RNA. For long RNA, follow the Long RNA Purification procedure.

Long RNA Purification

L1. Pipet 700µl Buffer RWT into the RNeasy Mini spin column from step 10. Close

the lid gently and centrifuge for 15 s at ≥10,000 rpm to wash the spin column membrane. Discard the flow-through.

L2. Add 500µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and

centrifuge for 15 s at ≥10,000 rpm to wash the spin column membrane. Discard the flow-through.

L3. Pipet another 500µl Buffer RPE into the RNeasy Mini spin column. Close the lid

gently and centrifuge for 15 s at ≥10,000 rpm to wash the spin column membrane. Discard the flow-through and the collection tube.

L4. Place the RNeasy Mini spin column in a new 2 ml collection tube. Open the lid

and centrifuge at full speed for 1 min.

L5. Place the RNeasy Mini spin column into a new 1.5 ml collection tube. Pipet 30–50µl RNase-free water directly onto the spin column membrane. Close the

lid gently and centrifuge for 1 min at ≥10,000 rpm to elute the total RNA.

L6. If the expected RNA yield is >30 µg, repeat step L5 with a second volume of

30–50µl RNase-free water. Elute into the same collection tube.

L7. 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 500 uL 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.

Ribo Zero Magnetic Protocol (r-RNA Removal)

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

Washing Beads Procedure

1. For each Ribo-Zero reaction, 225 µl of the Magnetic Beads is required.
Note: Mix the Magnetic Beads well by pipetting or gentle vortexing.
2. Pipet 225 ul Magnetic Bead suspension slowly into a 1.5-ml microcentrifuge tube to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.
3. Place the 1.5-ml microcentrifuge tube containing the Magnetic Beads 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 stand and add 225 ul of RNase- Free Water. Mix well by repeated pipetting or by 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 ul of Magnetic Bead Resuspension Solution. Mix well by repeated pipetting or by vortexing at medium speed.
Note: The volumes of the beads and Resuspension Solution are additive. Although the washed beads are resuspended in 60 µl per reaction, each reaction uses 65 µl of resuspended beads.

8. 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.

Treatment of RNA with Removal Solution

In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine:

4 ul	Ribo Zero Reaction Buffer
26 ul	5 µg Total RNA sample
10 ul	Ribo-Zero rRNA Removal Solution
40 ul	Total volume.

1.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.
2. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

Magnetic Bead Reaction and rRNA Removal

Using a pipette, add the treated RNA 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. Place the supernatant (RNA solution) on ice and immediately proceed to ethanol precipitation using same procedure as previously described.
7. Resuspend in 10 uL and remove 1.5 uL to run on a the Agilent Bioanalyzer on an RNA Pico chip.
Adjust the volume to 6 ul.

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:

Barcode1= ACACAC
Barcode2= CACACA

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)

2ul 5X 1st Strand Buffer
15ul 5X 2nd Strand Buffer
.5ul MgCl2
1ul DTT
2ul dUNTPs
.5ul E. coli DNA ligase
2ul E. coli DNA polymerase I
.5ul RNAse H

_____
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)

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
1.7ul 500 mM KCl
1ul 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)

If you do 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 AC, 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 0.8X 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 by shaking in a VWR® vortex mixer at 2000 rpm (room temperature) for 10 minutes. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

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 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.

• Use a sufficient volume of 70% ethanol to completely cover the bead pellet (500 ul). 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.

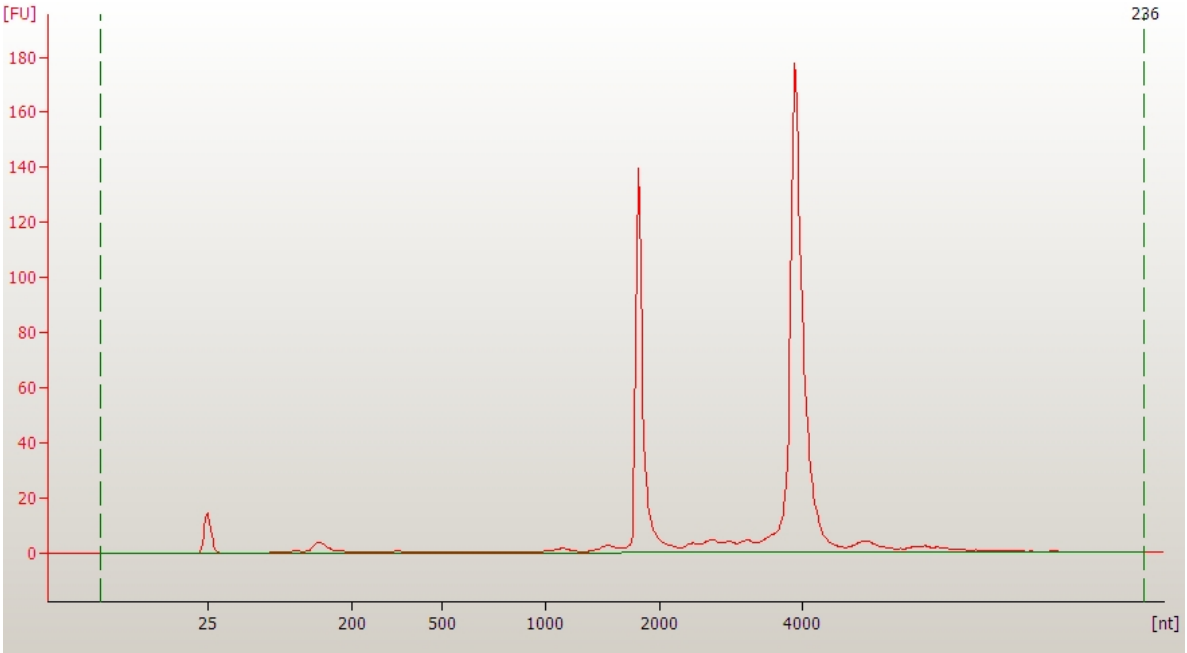
Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul)

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

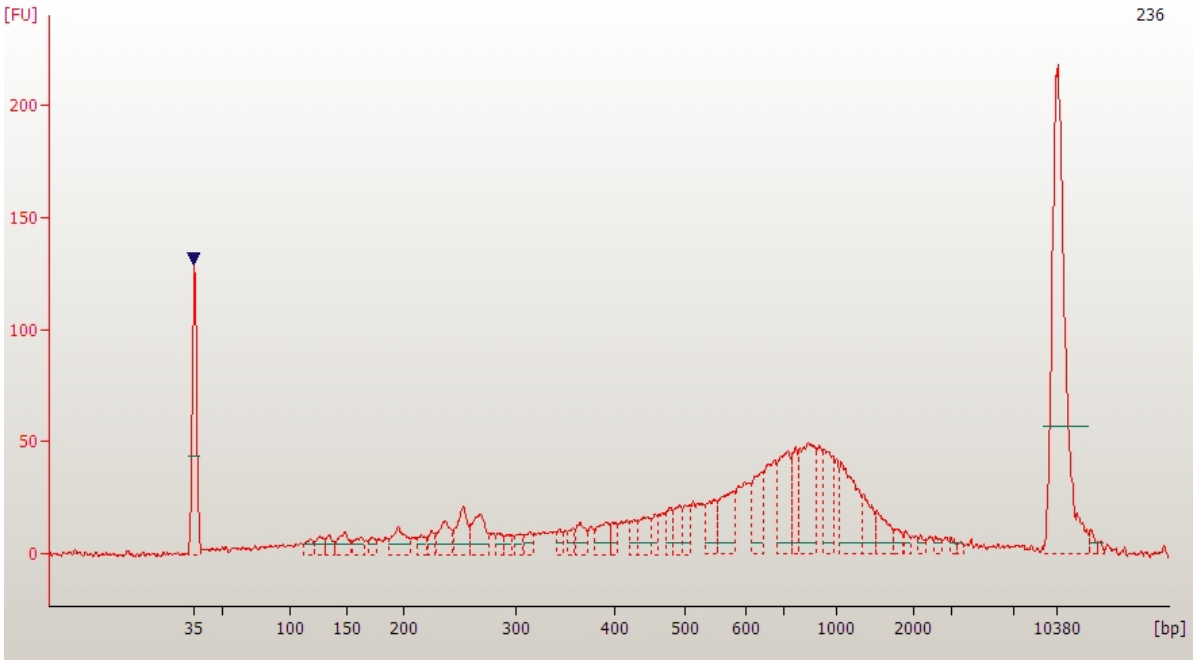
Mix both libraries 1:1 before submitting them for sequencing

Prior to cluster generation we add PhiX at 1

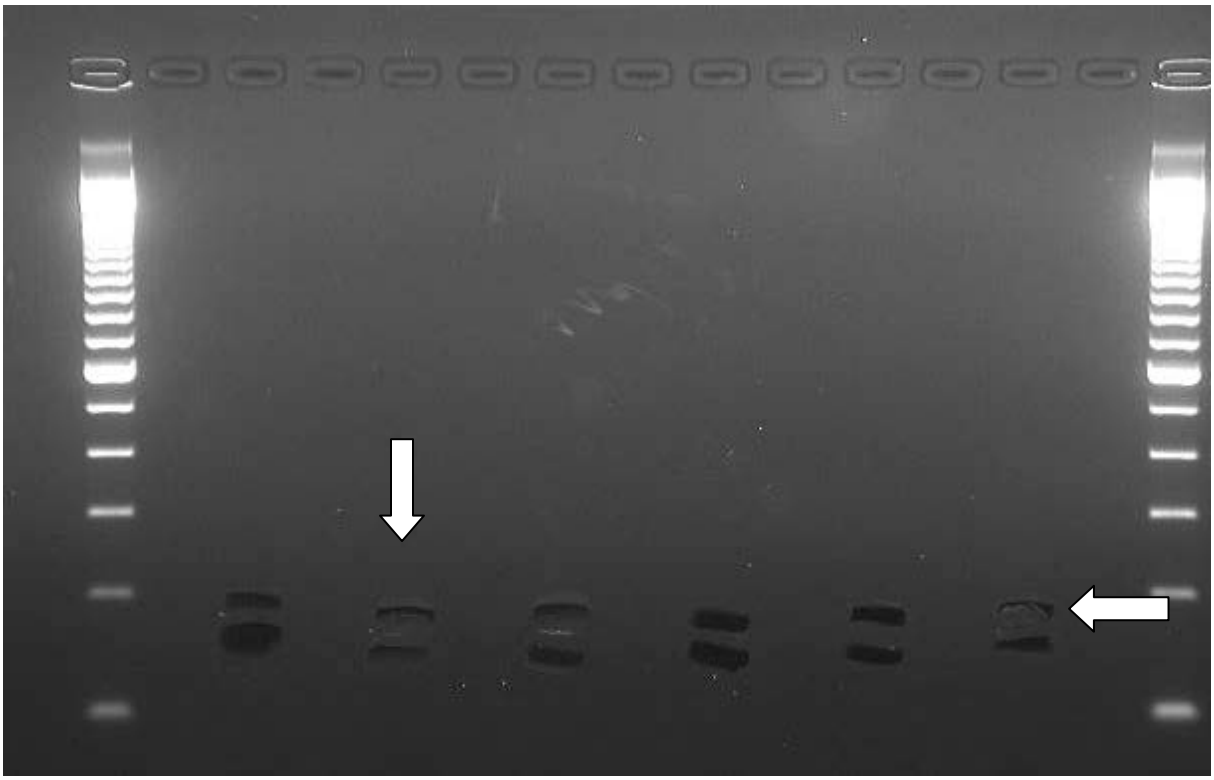
BioAnalyzer: Total RNA



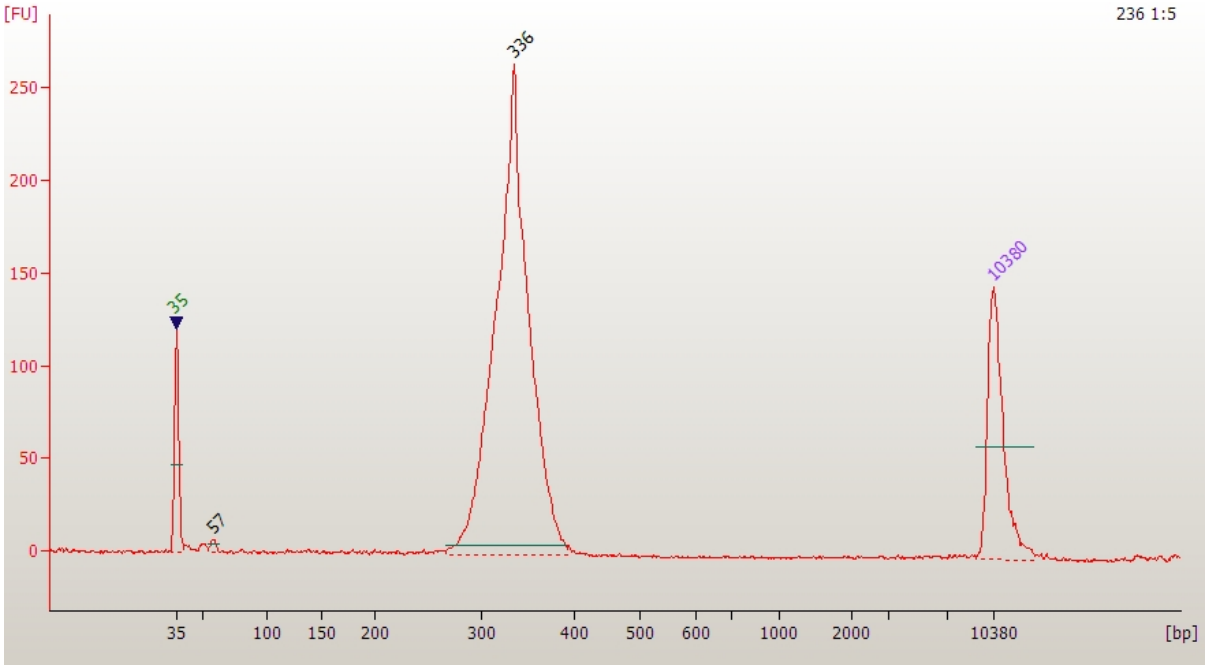
BioAnalyzer: cDNA



Gel Size Selection:



BioAnalyzer: Final Library:



* *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.*