Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Cell Line] Whole Cell IMR90 RNA Biorep #2
RNA ID: 0046WC
Library ID: LID45239
Protocol ID:

Cold Spring Harbor Laboratory
Genome Center
500 Sunnyside Blvd
Woodbury, NY 11797

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

CELL CULTURE: Cells received from John Stam.

RNA ISOLATION:

Kits: mirVana miRNA Isolation Kit (Cat #: AM1560)

1. Thaw the cells which are in the RNAlater Buffer. Transfer the cells and RNAlater buffer to a new 15ml Falcon RNase free tube. Add 6 times volume Lysis/Binding Solution to the tube, mix them well by votex.
2. Homogenize the sample using a syringe and 18 guage needle. Pass sample through the needle about twenty times.
3. Add 1/10 volume of miRNA Homogenate Additive to the homogenate, and mix well by vortexing or inverting the tube several times.
4. Leave the mixture on ice for 10 min.
5. Add a volume of Acid-Phenol:Chloroform that is equal to the lysate volume before addition of the miRNA Homogenate Additive. For example, if the original lysate volume was 300 µL, add 300 µL Acid-Phenol:Chloroform. (Be sure to withdraw from the bottom phase in the bottle of Acid-Phenol:Chloroform, because the upper phase consists of an aqueous buffer.)
6. Vortex for 30–60 sec to mix. Leave at room temperature for 2 minutes.
7. Centrifuge for 5 min at maximum speed (10,000 x g) at room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation.
8. Carefully remove the aqueous (upper) phase without disturbing the lower phase, and transfer it to a fresh tube (DO NOT DISCARD). Note the volume removed.

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℃ 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℃ for at least 30 min.
3. Centrifuge for 35 min at max speed at 4℃.
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℃ 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℃ for at least 30 min.
3. Centrifuge for 30 min at max speed at 4℃.
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.

RIBO-Zero TREATMENT:

1 Remove the Ribo-Zero Core Kit from 4℃ storage and allow the tubes to warm to room Temperature. These components must be at room temperature for use in Step A3
Important! Allow the components of the Ribo-Zero Core Kit to equilibrate to room temperature for use in Step A3.
2 Remove the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) from –70℃ to –80℃ storage, thaw the tubes, and place them on ice.
3 Vigorously mix the room-temperature Microspheres for 20 seconds by vortexing to produce a homogeneous suspension. The Microspheres are capable of withstanding vigorous vortex mixing and remain as a homogeneous suspension for several minutes.
4 For each reaction, pipette **130 µl of Microspheres** into a separate 2-ml Microsphere Wash Tube. Aspirate the Microspheres suspension SLOWLY to avoid air bubbles and to ensure pipetting the full required volume Return the unused Microspheres to 4℃
Important! Prepare the Microspheres for each RNA sample separately DO NOT batch-wash the Microspheres for multiple samples
5 Centrifuge the dispensed Microspheres at 12,000 x g in a bench-top microcentrifuge for 3 minutes. Remove each tube from the microcentrifuge, keeping it in the same orientation as was in the microcentrifuge, and carefully pipette off and discard the supernatant, without disturbing the Microsphere pellet
Caution: The supernatant contains 0 1% sodium azide. Discard the supernatant according to local ordinances
6 Wash the Microspheres by adding **260 µl of Microsphere Wash Solution** to each tube
Vigorously vortex (at maximum speed) the tube(s) to resuspend the Microspheres
Centrifuge the tube(s) at 12,000 x g for 3 minutes in a bench top microcentrifuge
Remove each tube from the microcentrifuge, keeping it in the same orientation as in the microcentrifuge, and carefully pipette off and discard all of the supernatant without disturbing the Microsphere pellet
7. Add **130 µl** of **Microsphere Resuspension Solution** to each tube and resuspend the Microspheres by vigorous vortex mixing at maximum speed until a homogeneous

suspension is produced.
8 Add **2 µl** of **RiboGuard RNase Inhibitor** to each tube of resuspended Microspheres Vortex briefly (10 seconds) and store the tubes at room temperature for use in Step C (page 8)

B. Treat 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:

x µl RNase-Free Water
8 µl Ribo-Zero Reaction Buffer
10 µg Total RNA Sample
20 µl Ribo-Zero rRNA Removal Solution
1 ul NIST spikes
40 µl Total Volume

2 Gently mix the reaction(s) and incubate at **68°C for 10 minutes**. During the incubation return the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer to storage at –70°C to –80°C. Hint: During the incubation, familiarize yourself with Step C1

3 Remove the reaction tube(s) and incubate each at **room temperature for 15 minutes**

C Microsphere Reaction and rRNA Removal

Required in Step C: 50°C water bath or heating block for 2-ml tubes
1 Briefly mix by vortexing (at medium speed for about 20 seconds) the washed, room-temperature Microspheres in the 2-ml Wash Tube from Step A8 If necessary, pulse-centrifuge (5 seconds) to collect the Microsphere suspension in the bottom of the tube, then resuspend by pipetting the slurry several times It is important to have a homogeneous slurry before adding the hybridized RNA from Step B3
2 Using a pipet, add the hybridized RNA sample from Step B3 to the resuspended Microspheres in the 2-ml Wash Tube and, without changing the pipet tip, immediately mix the contents of the tube by rapidly pipetting 10-15 times Then, immediately mix by vortexing (at medium speed) the contents of the tube for 5 seconds and place at room temperature before proceeding to the next sample
Important! ALWAYS add the RNA sample to the resuspended Microspheres in the 2-ml Wash Tube and immediately and rapidly mix by pipetting the contents of the tube NEVER add the Microspheres to the RNA sample
3 Incubate the tubes at **room temperature for 10 minutes** with vortex mixing (at medium speed) for 5 seconds every 3 to 4 minutes
Important! DO NOT use a shaker platform as this does not provide sufficient mixing
4 At the end of the 10-minute incubation at room temperature, mix by vortexing (at medium speed) the sample for 5 seconds and then place at **50°C for 10 minutes** in a water bath, heating block, or other temperature-controlled device
5 After 10 minutes at 50°C, immediately transfer the RNA-microspheres suspension to a Microsphere Removal Unit (filtration unit; provided in the Ribo-Zero Core Kit box) and centrifuge at 12,000 x g for one minute at room temperature Save the eluate that is in the collection tube and discard the filter unit with the microspheres
Important! The eluate contains the rRNA-depleted sample!

SPIKE-INS: NIST Spike-Ins beta set: Pool 14. 1 ng added to RNA sample. 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 A+ r- RNA purified from total RNA.

cDNA- 1st strand: Mix

4.75 ul sample r- RNA
2ul 50ng/ul random primers
2.5 50uM oligo-DT primer
Up to 11.25ul with RNAse free H20 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
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 (**before temp. ramp!**):

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)

1ul 5X 1st Strand Buffer
15ul 5X 2nd Strand Buffer
.5ul MgCl2
1ul DTT
2ul dU/dNTPs
.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 degas60snapcap100ul (60s 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 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 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, 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
1ul PE primer 2
50ul HF Phusion Mix
33ul H20 (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 1 x 15ul

Add 10ul loading dye, run on 2% gel at 70V for 2 hours

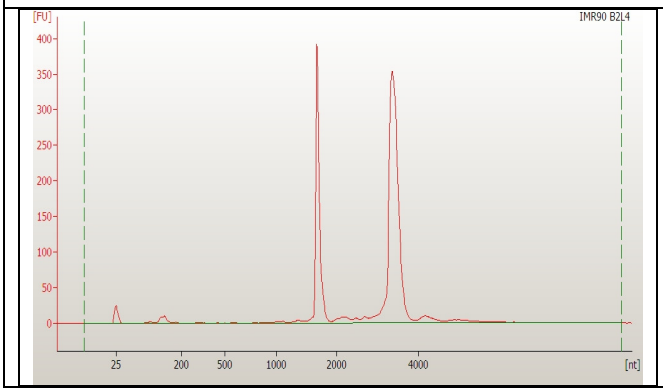
Cut distinct band (should be ~100bp larger than cDNA band)
You may have more than 1 band at this point, cut whichever band is ~100bp larger than your cDNA cut was

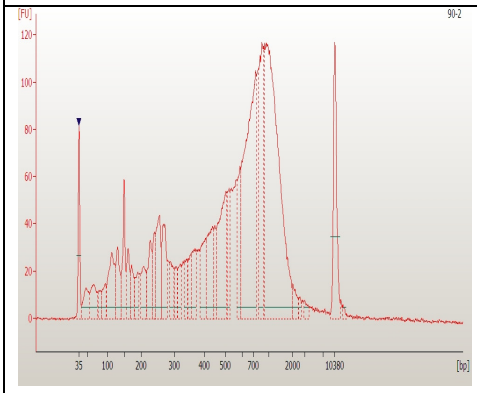
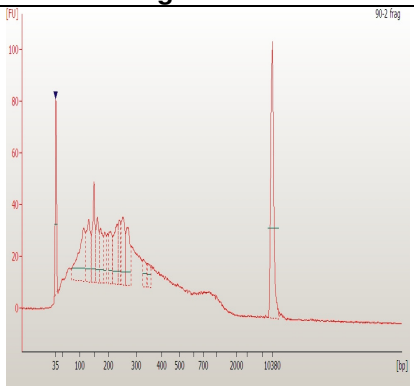
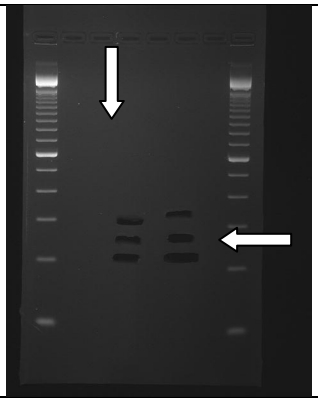
Gel purify as before
Elute 2 x 15ul
Precipitate
Resuspend 25ul H20

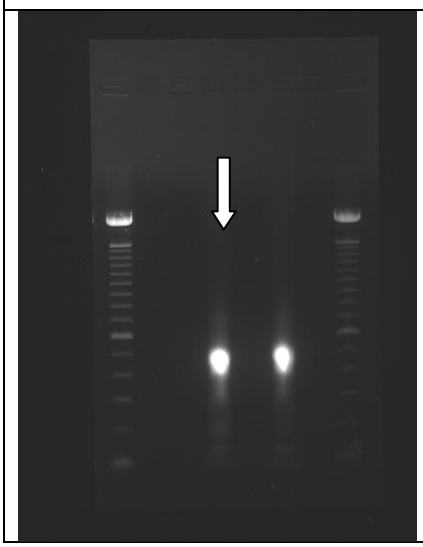
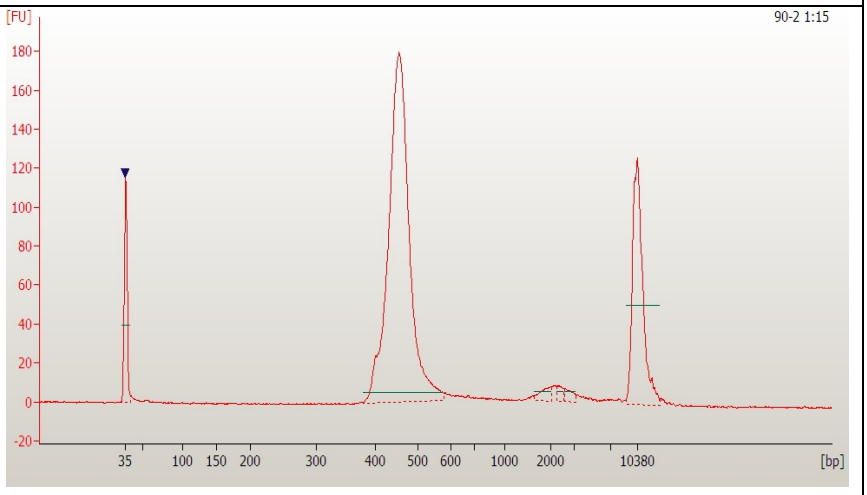
Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul)
Measure concentration also with Tecan (pico green) (1ul per dilution)
KAPA Biosystems qPCR (1ul) per dilution

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

Prior to cluster generation we add PhiX at 1%.

BioAnalyzer: Total RNA	BioAnalyzer: After oligo-dT	BioAnalyzer: After Ribominus
	N/A	N/A

BioAnalyzer: cDNA	BioAnalyzer: Post-Fragmentation	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.*