Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Cell Line] Whole Cell HUAEC_8061702.1P
RNA ID: 182WC
SID: SID38212
Library ID: LID56944
Protocol ID:

Cold Spring Harbor Laboratory
Genome Center
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LAB MEMBERS

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CELL CULTURE: Cells received from Promocell in RNAlater.

MirVana RNA Isolation- Long and Small RNA Separation

1. Add 1.2 mL Lysis/Binding solution to 100-10^7 cells containing around 200 uL of RNA later (add more lysis/binding solution for greater volumes of RNA later).

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 an equal volume of Acid-Phenol:Chloroform to the volume of the lysate before the addition of miRNA Homogenate Additive.

6. Vortex for 30-60 seconds to mix.

7. Centrifuge for 5 minutes 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 not repeat centrifugation.

8. Carefully remove the aqueous (upper) phase without disturbing the lower phase and transfer it to a fresh tube. Note the volume of the aqueous phase removed.

Long and Small RNA Separation

*Preheat nuclease free water to 95°C for elution steps.

1. Add 1/3 volume of 100% ethanol to the aqueous phase recovered previously from step 8.

2. Mix thoroughly by vortexing or inverting tube several times.

3. For each sample, place a Filter Cartridge into one of the collection tubes supplied.

4. Pipet up to 700 uL of the lysate/ethanol mixture (from the previous step) onto the filter cartridge.

5. Centrifuge for approximately 15 seconds at 10,000 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 Long RNA using RNA cleanup section.)**

6. Collect the filtrate (pool successive passes through the filter in a new tube) and record the volume.

7. Add 2/3 volume room temperature 100% ethanol to filtrate (flow-through) and mix thoroughly.

8. Pass 700 uL of each sample through a second filter cartridge and centrifuge for 15 seconds at 10,000 x g.

9. Discard the flow through.

10. Continue passing sample until the entire sample is through the filter.

11. 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 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. Store eluate with RNA at -20°C or colder.

SPIKE-INS: 1ul of a 1/10 dil of ERCC spike-ins (Ambion) added to 5 ug of total RNA. Use "corrected fasta" to map against.
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Ribo Zero procedure:

Input Total RNA: 5 ug

A kit reaction will remove the 28S, 18S, 5.8S, and 5S rRNA from to 1-5 µg of input human, mouse, or rat total RNA. The total RNA preparation should be free of salts (e.g., Mg2+ or guanidinium salts), and organics such as phenol and ethanol. We

recommend that the sample be dissolved in RNase-Free Water or TE Buffer. Use Table 1 to determine the maximum volume in which the total RNA sample can be dissolved.
Note: This table is replicated in the reaction protocol on page 7.
Table 1. Volumes of Ribo-Zero™ Ribosomal RNA Removal Solution.

Amount of Input Total RNA	Maximum Volume of Total RNA That Can Be Added to Each Reaction	Volume of Ribo-Zero rRNA Removal Solution Used per Reaction
1-2.5 µg	28 µl	8 µl
>2.5-5 µg	26 µl	10 µl

Ribo-Zero rRNA Removal Solution (Human/Mouse/Rat):
The volume of Ribo-Zero rRNA Removal Solution used in a reaction is dependent on the amount of input total RNA (Table 1).
Note: It is important to quantify the amount of total RNA in the sample as accurately as possible in order to use the appropriate amount of Ribo-Zero rRNA Removal Solution in Part B.

Ribo-Zero Microspheres:
The Ribo-Zero Microspheres must be washed prior to use. It is critical to resuspend the Ribo-Zero Microspheres into a homogeneous slurry before dispensing them into the 2-ml Wash Tube(s) (provided in the kit). The best way to resuspend the Microspheres is by vigorous vortex mixing. The Microspheres are capable of withstanding vigorous vortex mixing and remain in homogeneous suspension for several minutes after mixing. When treating multiple total RNA samples, we strongly recommend that the Microspheres be prepared separately for each sample. Do not batch-wash the Microspheres for multiple samples.

A. Prepare the Ribo-Zero Microspheres
The Ribo-Zero Microspheres must be washed using the Ribo-Zero Microsphere Wash Solution and then resuspended in the Ribo-Zero Microsphere Resuspension Solution before use.

Required in Part A

Component Name	Tube Label	Tube Color
Ribo-Zero Microspheres	Microspheres	Colorless
Ribo-Zero Microsphere Wash Solution	Microsphere Wash Solution	Green
Ribo-Zero Microsphere Resuspension Solution	Resuspension Solution	Red
RiboGuard RNase Inhibitor (100 U/µl)	RiboGuard RNase Inhibitor	Blue
Microsphere Wash Tube (2 ml)	Microsphere Wash Tube	Colorless

1. Remove the Ribo-Zero Core Kit from 4°C storage and allow the tubes to warm to room temperature. These components must be at room temperature for use in Part A, Step 3.
Important! Allow the components of the Ribo-Zero Core Kit to equilibrate to room temperature for use in Part A, Step 3.
2. Remove the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) from –70°C to –80°C 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 65 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°C.

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 130 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 65 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 1 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 Part C.
B. Treat the Total RNA Sample with Ribo-Zero rRNA Removal Solution

Required in Part B
Component Name Tube Label Tube Color
Ribo-Zero Reaction Buffer Ribo-Zero Reaction Buffer Blue
Ribo-Zero rRNA Removal Solution rRNA Removal Solution (H/M/R) Blue
(Human/Mouse/Rat)
RNase-Free Water RNase-Free Water Colorless
Additionally required for each reaction (provided by user):
0.2-ml or 0.5-ml microcentrifuge tube (RNase-free)
Incubation temperatures performed in Part B: 68°C and room temperature.

1. The maximum volume of the RNA sample and the volume of the Ribo-Zero rRNA Removal Solution used per reaction is dependent on the amount of total RNA in the sample (see Table below).
Amount of Input Total RNA

(see table above)

Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat)

In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine in the order given:
x µl RNase-Free Water
0.5 ul NIST controls pool 13
4 µl Ribo-Zero Reaction Buffer
5 µg Total RNA Sample (see Table above)
10 µl Ribo-Zero rRNA Removal Solution (see Table above)

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.
Note: During the incubation, familiarize yourself with Part C, Step 1.
3. Remove the reaction tube(s) and incubate each at room temperature for 15 minutes.
C. Microsphere Reaction and rRNA Removal
Required in Part C: 50°C water bath or heating block for 2.0-ml tubes.
1. Briefly mix by vortexing (at medium speed for about 20 seconds) the washed, room-temperature Microspheres in the 2.0-ml Wash Tube from Part A, Step 8. 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 Part B, Step 3.
2. Using a pipet, add the hybridized RNA sample from Part B, Step 3 to the resuspended Microspheres in the 2.0-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 1 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!
techhelp@epicentre.com • (800) 284-8474 9
Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat)

D. Purification the rRNA-Depleted Sample
The rRNA-depleted sample can be purified by ethanol precipitation or by a column method. Part D.1 details the ethanol precipitation procedure and Part D.2 provides guidance for column purification using a RNA Clean & Concentrator-5 Column (Zymo Research; Cat. Nos. R1015, R1016).

Ethanol Precipitation
1. Adjust the volume of each sample to 180 µl using RNase-Free Water.
2. Add 18 µl of 3 M Sodium Acetate to each tube.
3. Add 2 µl of Glycogen (10 mg/ml) to each tube and mix by gentle vortexing.
4. Add three volumes (600 µl) of ice-cold 100% ethanol to each tube and mix thoroughly by gentle vortexing.
5. Place the tubes at –80°C for at least 1/2 hour.
6. Centrifuge the tubes at >12,000 x g in a microcentrifuge for 30 minutes. Carefully remove and discard the supernatant.
7. Wash the pellet with ice-cold 70% ethanol and centrifuge at >12,000 x g for
5 minutes. Carefully remove and discard the supernatant.
8. Repeat Step 7 (above) one more time.
9. Centrifuge briefly to collect any residual supernatant. Carefully remove and discard the supernatant and allow the pellet to air dry at room temperature for 5 minutes.
10. Dissolve the pellet in 5.5 Water. The rRNA-depleted RNA can be used immediately or stored at –70°C to –80°C.

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 all the RNA from the 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 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)

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

_____
23.5ul

Mix:
30ul first strand reaction
23.5ul second strand mix
21.5ul 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
2ul PE primer 1 25 uM
2ul PE primer 2 25 uM
50ul HF Phusion Mix
31ul H20 (adjust this volume according to how much cDNA was used)
_____
100ul final PCR volume

98° 1 min

16 cycles of:

98° 10s
60° 30s
72° 30s

72° 5 min
4° hold

Purification using AMPure XP beads

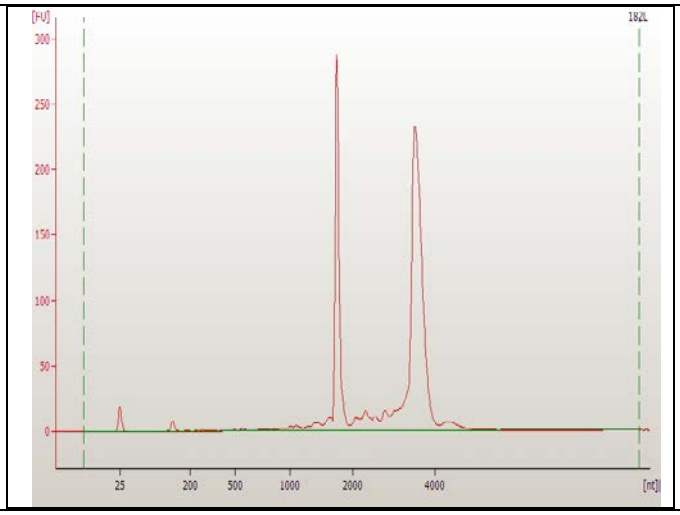
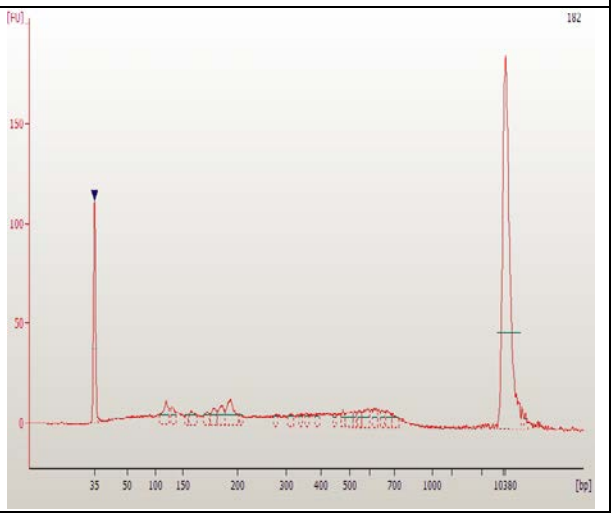
Perform the following steps, at room temperature, to concentrate your DNA sample.
1. Add 0.8X volume of pre-washed AMPureXP® magnetic beads to PCR reaction. (80 ul per 100 ul PCR reaction)
Refer to the provider's instructions regarding proper use and storage of AMPureXP magnetic beads. Before using, 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.

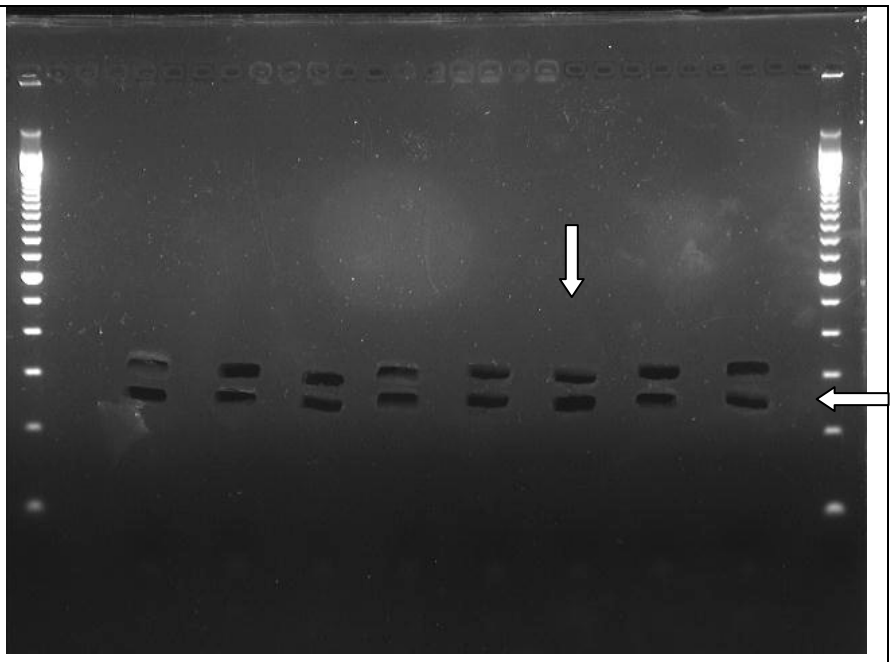
Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul)

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

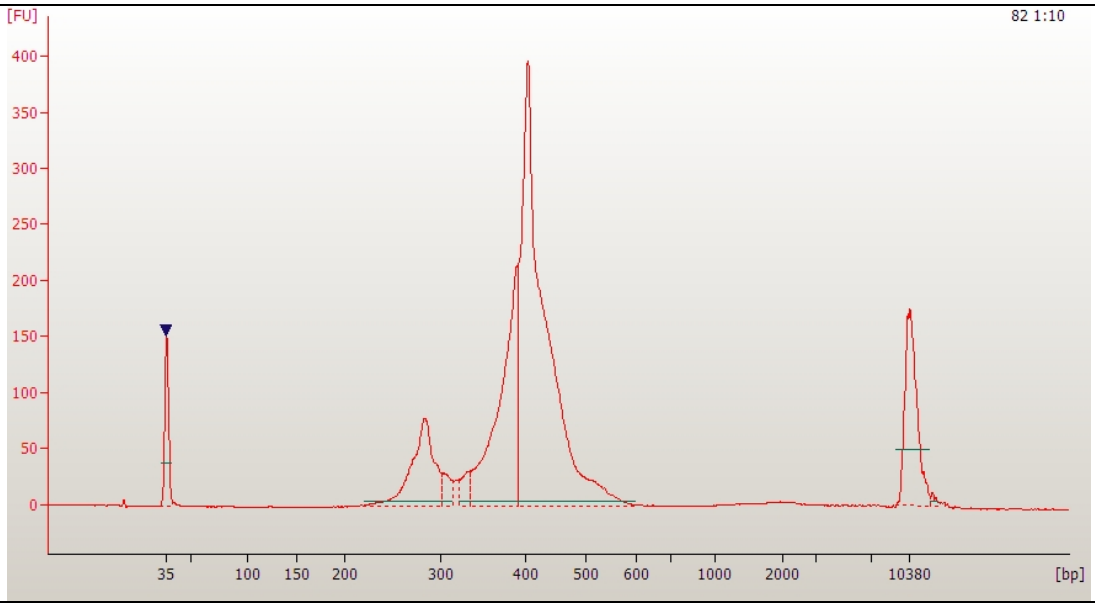
Prior to cluster generation we add PhiX at 1%.

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.*