Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: GM12878 A+ long RNA library

RNA ID: 191WC

Sample ID: SID38809

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Cell culture

Cells were prepared by UCHC and arrived as frozen cell pellets. Both long and small RNA were extracted from the cells using the Qiagen miRNeasy kit (Qiagen Cat # 217004).

RNA Isolation

Kits: miRNeasy Mini kit (QIAGEN cat#:217004)
RNeasy MinElute cleanup kit (QIAGEN cat#:74204)

1. Add QIAzol Lysis Reagent to the pellet and vortex to mix.
2. If number of cells is ≤ $3x10^{6,}$ vortex for 1 min to homogenize the cells.
If number of cells is >$3x10^{6,}$ homogenize by placing 700 µL of sample into a QIAshredder homogenizer and centrifuge for 2.5 min at maximum speed.
3. Place the tube(s) containing the homogenate on the benchtop at room temperature (15-25°C) for 5 min.
4. Add 20% volume of chloroform to the homogenate and cap it securely. Shake the tube vigorously for 15 s.
5. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 min.
6. Centrifuge the homogenate for 15 min at 12,000 x g at 4°C.
7. Transfer the upper, colorless, aqueous phase containing the RNA to a new collection tube.
8. Add an equal volume of 70% ethanol and mix thoroughly by vortexing. Do not centrifuge.
9. Pipet 700 µL of the sample including any precipitate that may have formed into an RNeasy Mini spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Repeat this step until the whole sample has been pipetted into the spin column and discard the flow-through each time.
10. Pipet the flow-through (which contains miRNA) into a 2 mL RNase free tube. The spin column contains the large RNA.

To purify small RNA fraction proceed to **Small RNA Purification.
To purify large RNA fraction proceed to Total RNA **(Containing Large RNA) Purification.

Small RNA Purification

S1. Add 450 µL of 100% ethanol (0.65 volumes) to the flow-through from step 15 and mix thoroughly by vortexing. Do not centrifuge.
S2. Pipet 700 µL of the sample into an RNeasy MinElute spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 30 s at room temperature. Discard the flow through. Repeat this step until the whole sample has been pipetted into the spin column and discard the flow-through each time.
S3. Add 700 µL Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow through.

S4. Pipet 500 µL Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
S5. Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the spin column membrane. Discard the flow-through and the collection tube.
S6. Place the RNeasy MinElute spin column into a new 2 mL collection tube, making sure that the column does not come in contact with the flow through. Open the lid and centrifuge for 5 min at ≥8000 x g (≥10,000 rpm).
S7. Place the RNeasy MinElute spin column into a 1.5 mL collection tube and pipet 20 µL RNase free water onto the spin column membrane. Close the lid gently and wait 1 min. Then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the small RNA fraction. Repeat with a second volume of 20 µL RNase free water.
S8. Proceed to ethanol precipitation.

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 (same for Small and Long 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 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 RNA pellet.
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 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.

Small RNA Cleanup

1. Make sure there is a max of 45 µg RNA in the 100 µL RNA sample.
2. Add 350 µL Buffer RLT to the 100 µL sample of RNA. Vortex to mix well.
3. Add 675 µL of 100% ethanol to the reaction and mix by inverting.
4. Transfer 700 µL of sample into an RNeasy MinElute column 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. Repeat this step with the remaining sample.
5. Add 700 µL Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow through.
6. Pipet 500 µL Buffer RPE into the RNeasy MinElute spin column. Close the lid gently and centrifuge for 30 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
7. Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the spin column membrane. Discard the flow-through and the collection tube.
8. Place the RNeasy MinElute spin column into a new 2 mL collection tube, making sure that the column does not come in contact with the flow through. Open the lid and centrifuge for 5 min at ≥8000 x g (≥10,000 rpm).
9. Place the RNeasy MinElute spin column into a 1.5 mL collection tube and pipet 20 µL RNase free water onto the spin column membrane. Close the lid gently and wait 1 min. Then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm).
10. Repeat step 9 with a second volume of 20 µL RNase free water.
11. Proceed to ethanol precipitation.

Poly-A+ mRNA Selection

Kit: Oligotex mRNA mini kit (Qiagen cat# 70022)

The batch protocol has been used for the recent library production, but from other experience the spin column protocol (listed in the handbook prior to the batch protocol) gives the same results (as far as bioanalyzer image goes). Do the poly-A+ selection twice.

Important notes before starting
• This protocol may be necessary if you are using impure total RNA or if you are unsure about the purity of your total RNA. Many isolation procedures do not remove contaminants such as protein that can clog Oligotex spin columns. Better results are generally obtained with purer starting material.

• Heat Oligotex Suspension to 37°C in a water bath or heating block. Mix by vortexing, and then place at room temperature.
• Heat a water bath or heating block to 70°C, and heat Buffer OEB.
• Review the introductory material on pages 12–19 before starting.
• If working with RNA for the first time, please read Appendix A (page 76).
• Determine the amount of total RNA in the RNA sample (see "Quantification of starting RNA", page 18).

• Buffer OBB may (and almost always does) form a precipitate upon storage. If necessary, redissolve by warming at 37°C for approximately 10 minutes, and then place at room temperature. You can wrap the OBB bottle in parafilm and carefully, partially, submerge it in the water bath, or aliquot the needed amount in 1.5ml tubes and use the heat block

• Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at 20 to 30°C (room temp).
• All centrifugation steps should be performed in a microcentrifuge at maximum speed (14,000–18,000 x g).

Procedure

1. Determine the amount of starting RNA. Do not use more than 3 mg total RNA. Pipet total RNA into an RNase-free 1.5 ml microcentrifuge tube, and adjust the volume with RNase-free water (if necessary) to the volume indicated in Table 5.

Note: The initial volume of the RNA solution is not important so long as the volume can be brought up to the indicated amount with RNase-free water. If starting with precipitated RNA, dissolve the RNA pellet in the appropriate amount of RNase-free water by heating the tube for 3 min at 60°C followed by vortexing for 5 s and sharply flicking the tube. Repeat at least twice.

2. Add the appropriate volume of Buffer OBB and Oligotex Suspension (see Table 5). Mix the contents thoroughly by pipetting or flicking the tube.

Table 5. Buffer amounts for Oligotex mRNA Batch Protocol

Total RNA	Add RNAse free water to:	Buffer OBB (ul)	Oligotex Suspension (ul)	Prep size
≤0.25 mg	250 ul	250	15	Mini
0.25–0.50 mg	500ul	500	30	Midi
0.50–0.75 mg	500ul	500	45	Midi
0.75–1.00 mg	500ul	500	55	Midi
1.0–1.5 mg	650ul	650	85	Maxi
1.5–2.0 mg	650ul	650	115	Maxi
2.0–2.5 mg	650ul	650	135	Maxi
2.5–3.0 mg	650ul	650	175	Maxi

*We generally use slightly more than the recommended amount of beads (~5ul)

3. Incubate the sample for 3 min at 70°C in a water bath or heating block.
This step disrupts secondary structure of the RNA.
4. Remove sample from the water bath/heating block, and place at 20 to 30°C for
12 min (manual says 10, we say 12).
This step allows hybridization between the oligo dT30 of the Oligotex particle and the poly-A tail of the mRNA.
5. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000 x g), and carefully remove the supernatant by pipetting.
Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure. Note: Save the supernatant until certain that satisfactory binding and elution of poly A+ mRNA has occurred.
6. Resuspend the Oligotex:mRNA pellet in 1 ml Buffer OW2 by vortexing or pipetting (pipetting works better, be sure to resuspend well) Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed, and carefully remove the supernatant by pipetting.
Loss of the Oligotex resin can be avoided if approximately 50 µl of the supernatantis left in the microcentrifuge tube. The remaining solution will not affect the procedure.
7. Repeat step 6 once.
8. Add 20–100 µl hot (70°C) Buffer OEB. Pipet up and down 10-15 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A+ mRNA, to a small spin column, close column and set aside.
*We always use 100ul, it gives better yields.
Note: The volume of Buffer OEB used depends on the expected or desired concentration of poly A+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. Remember that small volumes cool down quickly. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with Oligotex and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.

9. To ensure maximal yield, add another 20–100 µl hot (70°C) Buffer OEB to the Oligotex pellet. Pipet up and down 10-15 times to resuspend the resin, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant, containing the eluted poly A+ mRNA, into the spin filter with the previous 100ul of eluate.
10. Spin filter column for 2 min at 18000xg to remove any remaining Oligotex suspension from the A+ RNA.
11. EtOH precipitate.

Ribominus

Hybridization Step
Instructions are provided below to perform hybridization for 1–10 µg of your total RNA sample with the RiboMinus™ Eukaryote Probe. To process >10 µg total RNA sample, divide your sample into two samples, each containing <10 µg total RNA.

1. Set a water bath or heat block to 70–75°C.
2. To a sterile, RNase-free 1.5 mL microcentrifuge tube, add the following:
Total RNA (1–10 µg): <10 µL
RiboMinus™ Probe (15 pmol/L): 8 µL
NIST spike-in : 1 µL
Hybridization Buffer: 100 µL

3. Incubate the tube at 70–75°C for 5 minutes to denature RNA.
4. Allow the sample to cool to 37°C slowly over a period of 30 min by placing the tube in a 37°C water bath (a heat block works as well). To promote sequence-specific hybridization, it is important to allow slow cooling. **Do not** cool samples quickly by placing tubes in cold water.
5. While the sample is cooling down, proceed to **Preparing Beads**.

*****An earlier version of this protocol says to use RNA in less than 20 µL, add 10 µL of probe and 300 µL hybridization buffer, this larger volume means you need to precipitate the ribominused RNA in a 2 mL tube at the end. Either way works. It doesn't change anything else expect the supernatant volumes and the precipitation tube size.

Preparing Beads
1. Resuspend RiboMinus™ Magnetic Beads in its bottle by thorough vortexing.
2. Pipet 750 µL of the bead suspension into a sterile, RNase-free, 1.5 mL microcentrifuge tube.
3. Place the tube with the bead suspension on a magnetic separator for 1 min. The beads settle to the tube side that faces the magnet. Gently aspirate and discard the supernatant.
4. Add 750 µL sterile, DEPC Water to the beads and resuspend beads by pipetting
5. Place tube on a magnetic separator for 1 min. Aspirate and discard the supernatant.
6. Repeat Steps 4–5 once.
7. Resuspend beads in 750 µL Hybridization Buffer and transfer 250 µL beads to a new tube and maintain the tube at 37ºC for use at a later step.

8. Place the tube with 500 µL beads on a magnetic separator for 1 min. Aspirate and discard the supernatant.
9. Resuspend beads in 200 µL Hybridization Buffer and keep the beads at 37°C until use.

Removing rRNA
1. After the incubation at 37°C for 30 min of the hybridized sample (above), briefly centrifuge the tube to collect the sample to the bottom of the tube.
2. Transfer the sample (~120 µL - this will be ~330 µL with the older protocol) to the prepared RiboMinus™ Magnetic beads from Step 9 (**Preparing Beads**, above). Mix well by pipetting up and down
3. Incubate the tube at 37°C for 15 min. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
4. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. **Do not discard the supernatant. The supernatant contains RiboMinus™ RNA.**
5. Place the tube with 250 µL beads from Step 7 (**Preparing Beads**, above) on a magnetic separator for 1 min. Aspirate and discard the supernatant.
6. To this tube of beads, add ~320 µL (~500 µL with older protocol) supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or low speed vortexing.
7. Incubate the tube at 37°C for 15 min. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
8. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. **Do not discard the supernatant as the supernatant contains RiboMinus**™ RNA.
9. Transfer the supernatant (~ 320 µL - ~500 µL with older protocol) containing **RiboMinus™ RNA** to a small filter column and spin at max speed for 2 min to remove any remaining magnetic particles.
10. Transfer flow through (ribominus RNA) to a new tube (1.5 mL for small volume, 2 mL for large volume)
11. Ethanol precipitate as before but add 1 µL glycoblue to facilitate the precipitation.
12. After drying the pellet, resuspend in 6.75µL H_2O.

Multiplexing Long RNA Library Protocol: T-U

1st strand cDNA Synthesis
6.75 ul RNA sample
2ul 50ng/ul random primers (Invitrogen Cat. # 48190-011)
2.5 50uM oligo-DT primer (Invitrogen Cat. # 18418-020)
1.25ul RNAse inhibitor (Ambion Cat. # AM2692)

12.5 ul total

Use AD-1st program on cycler (98° for 2 min; 70° for 5 min; 0.1°/s ramp to 15°; 15° for 30 min ; 0.1°/s ramp to 25°; 25° for 10 min ; 0.1°/s ramp to 42°; 42° for 45 min; 0.1°/s ramp to 50°; 50° for 15 min ; 75° for 15 min; 4° forever)

As soon as 15 degrees is reached (after ~16 min), pause program and add:

5 ul 5X First Strand Buffer (Invitrogen Cat. # sold with SS III)
1.25 ul 0.1 M $MgCl_2$ (Ambion Cat. # AM9530G)
1.25 ul 10 mM dNTPs (Invitrogen Cat. # 18427-013)
2.5 ul 0.1M DTT (Invitrogen Cat. # sold with SS III)

Add 10 uL of mix to each sample
22.5 ul total reaction so far

Dilute 1 mg/ml stock of Actinomycin D to 120 ng/ul by mixing:
1.5 (or 3) ul 1 mg/ul AD + 11 (or 22) ul 10 mM Tris pH 7.6 (Sigma Cat. # T2444-1L)

After 30 minutes at 15 degrees, pause program and add:

1.25 ul Actinomycin-D (120 ng/ul) (Invitrogen Cat. # A7592)
1.25 ul Superscript III (Invitrogen Cat. # 18080-044)

Add 2.5 ul of mix to each sample

25 ul 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 volumes Buffer PB (500 ul), mix and apply to Minelute spin column
Follow Qiagen Minelute cleanup protocol (Qiagen Cat. # 28006)
Elute 2 x 15 ul EB

2nd Strand cDNA Synthesis
1 ul 5X 1st Strand Buffer (Invitrogen Cat. # sold with SS III)
15 ul 5X 2nd Strand Buffer (Invitrogen Cat. # 10812-014)
0.5 ul 0.1 M $MgCl_2$ (Ambion Cat. # AM9530G)
1 ul DTT (Invitrogen Cat. # sold with SS III)
2 ul dU/dNTPs (Roche dUTP Cat. # 13796926 dNTPs Cat. # 11969064001)
0.5 ul E. coli DNA ligase (Invitrogen Cat. # 18052-019)
2 ul E. coli DNA polymerase I (Invitrogen Cat. # 18010-025)
0.5 ul RNAse H (Invitrogen Cat. # 18021-071)

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

22.5 ul RNAse free water

75ul final reaction volume

Use program 2nd on thermocycler: 2 hours at 16°; 4° forever

Bring volume up to 100 ul by adding 25 ul water
Add 500 ul of Buffer PB
Minelute cleanup, as before
Elute 2 x 26 ul of EB

Run a high sensitivity DNA chip on the bioanalyzer to determine fragmentation time.
If peak is around 1000 bp then sonicate for 60 s, if it is smaller sonicate for less time.

Fragment cDNA
Using Covaris sonicator:
Fill appropriate chambers with autoclaved DI water
Degas sonicator for 30 minutes prior to use

Transfer cDNA sample to the sonicator tube (Covaris Cat. # 520045)
Place on machine and run program "degas100ulsnapcap60s" (60s sonication)

Run a high sensitivity DNA chip to check fragment size

End-Repair cDNA
50 ul sample
Add 25 ul H20 to each sample

10 ul T4 DNA ligase buffer with 10mM ATP (New England Biolabs Cat. # B0202S)
4 ul dNTP mix 10mM (Invitrogen Cat. # 18427-013)
5 ul T4 DNA polymerase 3U/ul (New England Biolabs Cat. # M0203L)
1 ul Klenow DNA polymerase 5U/ul (New England Biolabs Cat. # M0210S)
5 ul T4 PNK 10U/ul (New England Biolabs Cat. # M0201L)

Add 25 ul of mix to each sample
100ul final volume

Place at room temperature for 30 min.

Add 500 ul of Buffer PB and minelute cleanup, elute 2 x 16ul

Addition of single <A> Base
32 ul eluted cDNA
5 ul NEBuffer2 (New England Biolabs Cat. # B7002S)

10 ul dATP (1mM) (Roche 11934511001)
3 ul Klenow fragment 3' to 5' exo- 5U/ul (New England Biolabs Cat. # M0212S)

Add 18 ul of mix to each sample
50 ul final volume

37 degrees, 30 min. (heat block)

Add 50 ul water, 500 ul Buffer PB, Minelute cleanup, elute 1 x 19ul

Adapter Ligation
19 ul eluted cDNA
25 ul 2x Rapid Ligation Buffer (Enzymatics Cat. # B101L)
1 ul Index Paired End adapter oligo mix (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
5 ul T4 DNA ligase 1U/ul (Enzymatics Cat. # L603-HC-L)

Add 31 ul of mix to each sample
50 ul final volume

Room temp, 15 min.

Add 50 ul water, 500 ul Buffer PB, Minelute cleanup, elute 1 x 15ul

UNG Treatment
15 ul eluted cDNA
1.7 ul 0.5 M KCl (Ambion Cat. # AM9640G)
1 ul UNG (Uracil N-Glycosylase) (Roche Cat. # N808-0096)

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

Add 10ul loading buffer
Run out samples on a 2% agarose gel.
Cut out 200 bp band, and another band just slightly larger (freeze larger slice at -20).
Then use the Qiaquick gel extraction kit (Qiagen Cat. #28706) and elute 2 x 15ul.

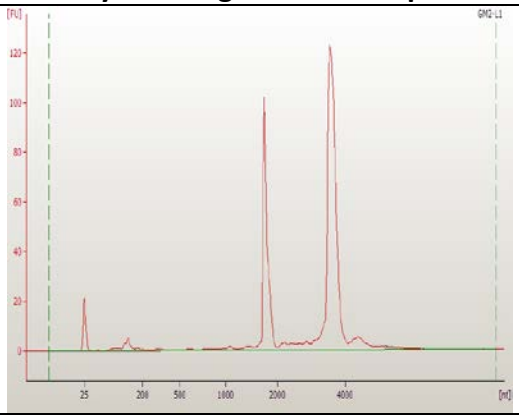
PCR Amplification
15 ul eluted cDNA from gel-extraction (can use more or less)
2 ul PCR Primer In PE 1.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
2 ul PCR Primer In PE 2.0 (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)

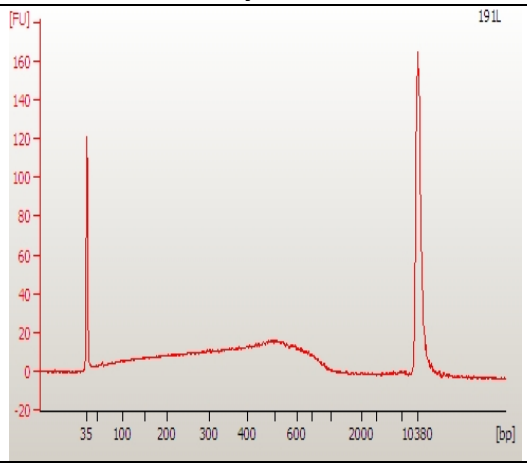
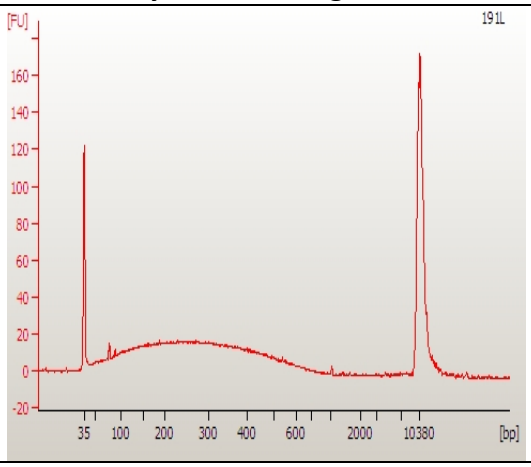
2 ul mix of 2 index primers *(PE 2 Index 1 and PE 2 Index 2 each diluted to 25uM)* (in Multiplexing Sample Preparation Oligonucleotide Kit Illumina Cat. # 1005709)
50 ul 2X HF Phusion Mix (New England Biolabs Cat. # M0531L)
29 ul water or up to 100 ul with water (depends on amount of DNA used)

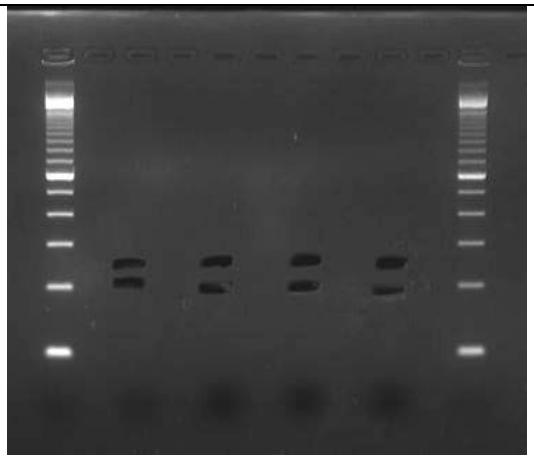
98° 1 min then 18 cycles (98° for 10s; 60° for 30s; 72° for 30s) then 72° for 5 min; 4° forever
Use AMPure XP Cleanup for PCR purification.

AMPure XP PCR Cleanup Protocol
(Agencourt AMPure XP beads Cat. # A63881)

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled. Add 80 uL Agencourt AMPure XP to sample.
2. Mix reagent and PCR reaction thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
3. Place the reaction tubes onto a magnetic stand for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
4. Aspirate the cleared solution and discard.
5. Dispense 200 µL of 70% ethanol in each reaction and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.
6. Let the beads air dry for 5 minutes off of the magnet.
7. Off of the magnet add 30 µL of elution buffer (Qiagen Elution Buffer was used) and pipette mix 10 times.
8. Place the tubes back on the magnet for 1 minute to separate beads from the solution.
9. Transfer the eluant to a new tube.
10. Measure sample concentration on ThermoScientific NanoDrop and run final libraries on Agilent Bioanalyzer to visualize final library.

Bioanalyzer: Long RNA after separation


Bioanalyzer: cDNA	Bioanalyzer: Post-Fragmentation
	

Gel Size Selection	Final Library
 Sample is in fourth well.