Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: [Cell Line] H1 derived Neurons polyA- Biorep #1,2
RNA ID: 0095WC, 0096WC
Library ID: LID48326
Protocol ID:

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CELL CULTURE: Cells are grown according to the ENCODE growth protocol and standards. Each bioreplicate grown and isolated independently.

RNA ISOLATION:

Kits: RNeasy mini kit (QIAGEN cat#:74106)
miRNeasy Mini kit (QIAGEN cat#:217004)
mirVana miRNA Isolation Kit (Cat #: AM1560)

Total RNA Purification

1. Regular harvest and count cells and centrifuge for 10 min at 1900 rpm 4℃.
2. Completely aspirate supernatant.
3. Resuspend all pellets in 10-30 mL of cold PBS (Thermo Scientific Cat. # SH30264.02) by pipetting up and down.
4. Centrifuge for 5 min at 2000 rpm 4℃.
5. Carefully aspirate the supernatant.
6. Add Buffer RLN to lyse plasma membrane. For the pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Carefully resuspend cells in the appropriate volume of cold (4C) Buffer RLN. Incubate on ice for 5 min.

Number of cells	RLN (4C) (ml)
$5X10^6$-$5X10^7$	0.5
$5X10^7$-$1X10^8$	1.0

7. Centrifuge lysate at 4C for 10 min at 3200rpm. Transfer supernatant to an RNase-free centrifuge tube (cytosol part), and keep the pellet (nuclear pellet).
8. Add 1ml (if the sample is in epprndorf tube) RLN to resuspend nuclear pellet. Centrifuge at 4C for 5 min at 3200rpm.
9. Discard the supernatant. Add 700ul QIAzol to the pellet. Homogenize the sample using a syringe and 18 guage needle. Pass sample through the needle until it becomes smooth and there are no thick globs.
Note: If number of cells is more than $1x10^7$ then transfer the homogenate to a 15ml Falcon tube and add more QIAzol.
10. Place the tube(s) containing the homogenate on the benchtop at room temperature (15-25℃) for 5 min.
11. Add 20% volumes of chloroform (Sigma Cat. # C2432-25ML) to the homogenate and cap it securely. Shake the tube vigorously for 15 s.
12. Place the tube containing the homogenate on the benchtop at room temperature for 2-3 minutes.
13. Centrifuge the homogenate for 15 min at 12,000 x g at 4℃.
14. Transfer the upper, colorless, aqueous phase containing the RNA to a new collection tube.
15. Add 1.5 volume of 100% ethanol and mix thoroughly by vortexing or invert the tube several times. Do not centrifuge.
14. 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.
15. Pipet 700 µL Buffer RWT into the RNeasy Mini spin column from step 15. Close the lid gently and centrifuge for 30 at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow through.
16. 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) to wash the spin column membrane. Discard the flow through.
17. Add another 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) to wash the spin column membrane. Discard the flow through and the collection tube.
18. Place the RNeasy Mini spin column in a new 2 mL collection tube. Open the lid and centrifuge at full speed for 1 min.
19. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube. Pipet 50 µL RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently. Wait one minute and then centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the total RNA (containing large RNA).
20. If the expected RNA yield is >30 µg, repeat step 19 with an additional volume of 50 µL RNase-free water. Elute into the same collection tube.
21. Proceed to ethanol precipitation.

Ethanol Precipitation

1. Add 2.5 volumes of 100% ethanol and 1/10 volumes of NaOAc PH 5.5 (Ambion Cat. # AM9740) 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.

Separating total RNA Procedure

1. Preheat Elution Solution to 95℃ 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℃) 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°C 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°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 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°C 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°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 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.

POLY-A+ SELECTION:

Poly A+ selection is done twice to insure purity.

Qiagen mRNA Isolation Protocol (using Oligotex mini kit)

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)

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. 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. **We save the supernatant always, as to save the A- fraction.**
Proceed to polyA- selection

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. Repeat procedure steps 1 to 9 once.
11. Spin filter column for 2 min at 18000xg to remove any remaining Oligotex suspension from the A+ RNA.
12. EtOH precipitate.

Poly A- selection:
Repeat above steps 2 – 5 two times to obtain pure polyA- fraction
At step 2 only add the appropriate amount of beads.
Precipitate the A- fraction, wash with 70 % EtOH, dry and reconstitute in water.

SPIKE-INS: Add 0.5 ul of NIST pool 13 controls to 5 ug RNA sample. Use “corrected fasta” to map against.

Ribo Zero procedure:

Input Total A- RNA: A total of 2 replicates have to be treated since 2 different sets of index primers are used in PCR rxn. Do not pool samples.

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 RNase-free microcentrifuge tube, combine in the order given:
x µl RNase-Free Water
0.5 ul NIST controls pool 13 (1 ng / ul)
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 of the A- r- RNA from the above step.
2 reactions have to be performed in parallel.

Primer and adapter sequences:

Bioreplicate 1
Sample A barcode1=ACACAC
Sample A barcode2=CACACA

Bioreplicate 2
Sample B barcode1=TGTGTG
Sample B barcode1=GTGTGT

Multiplexing Adapters1
5' P-GATCGGAAGAGCACACGTCT
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Multiplexing PCR Primer 1.01
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Multiplexing PCR Primer 2.01
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

cDNA- 1st strand: Mix

4.75 ul sample r- RNA
2ul 50ng/ul random primers
2.5 50uM oligo-DT primer
1.25ul RNAse inhibitor
Up to 12.5ul 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

_____
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 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 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 index adapter oligo mix
5ul DNA ligase 1U/ul (Enzymatics)

50ul final volume

Room temp, 30 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: Prepare 2 reactions (separate setup)

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 ea for 1st PCR and index primer mix TG, 25 uM ea for 2nd PCR
50ul HF Phusion Mix
29ul 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. **Purify both samples separately**
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,

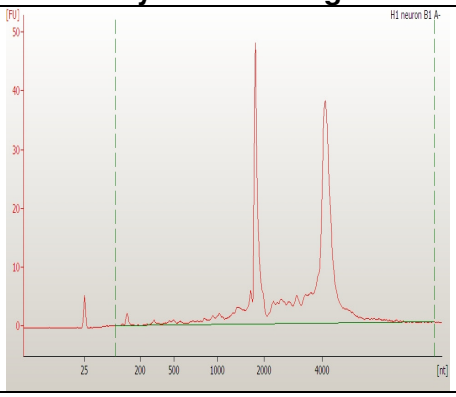
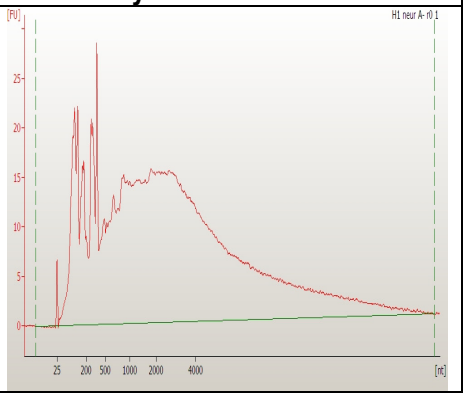
some beads may slide down the side of the tube during aspiration of this buffer. If this occurs, it is preferable to leave a small volume of buffer behind to avoid aspirating beads; this residual buffer will be adequately removed during subsequent 70% ethanol washes.
8. Wash beads with freshly prepared 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.

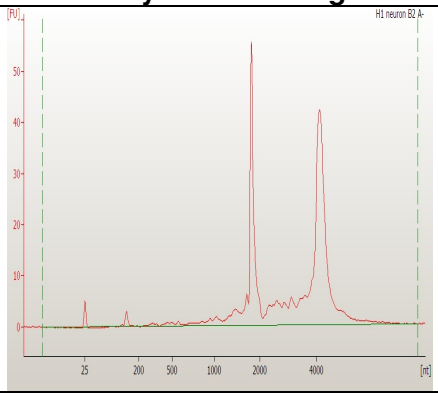
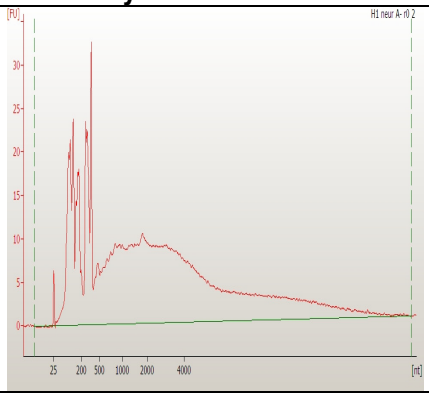
Measure library with Nanodrop (1ul) –very inaccurate.
Run High sensitivity DNA chip (1ul)

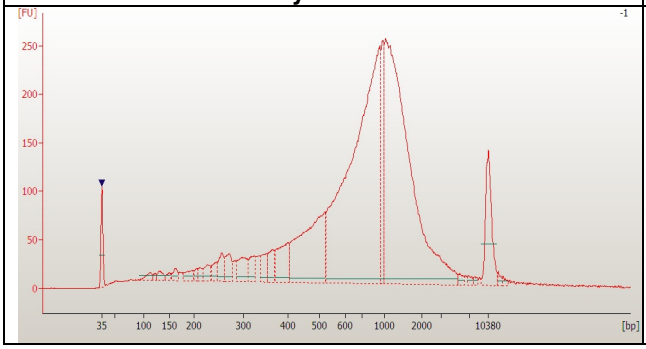
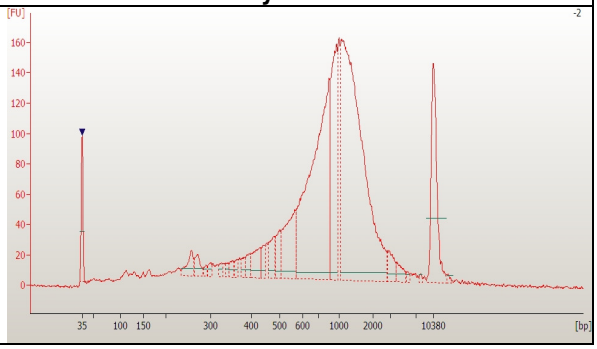
Dilute to 10nM (do not have to use whole library)

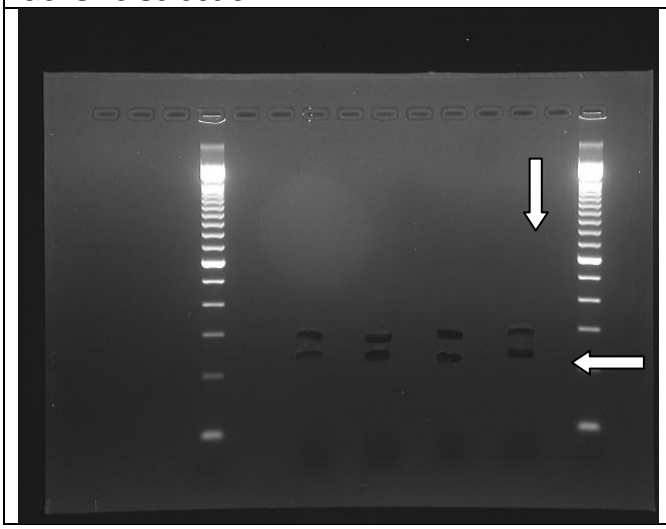
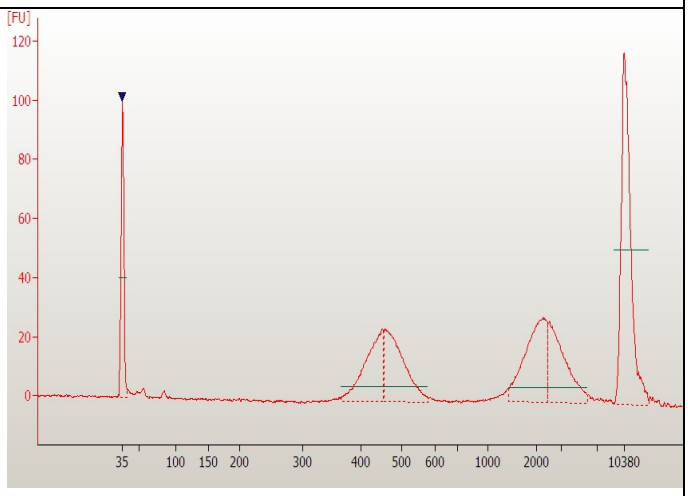
Mix both libraries 1:1 before submitting them for sequencing

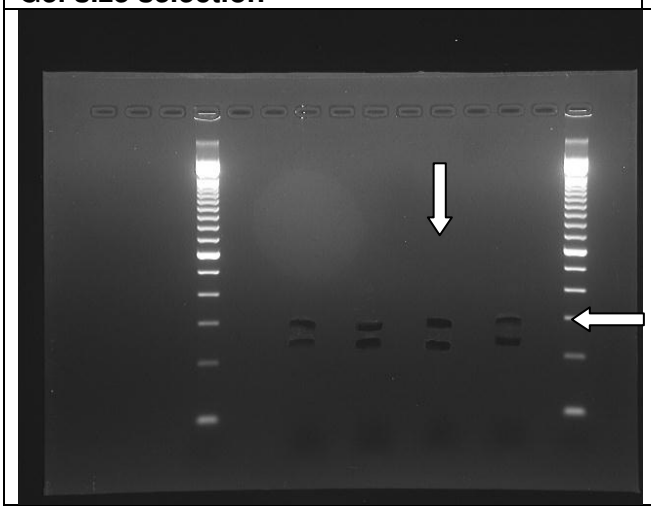
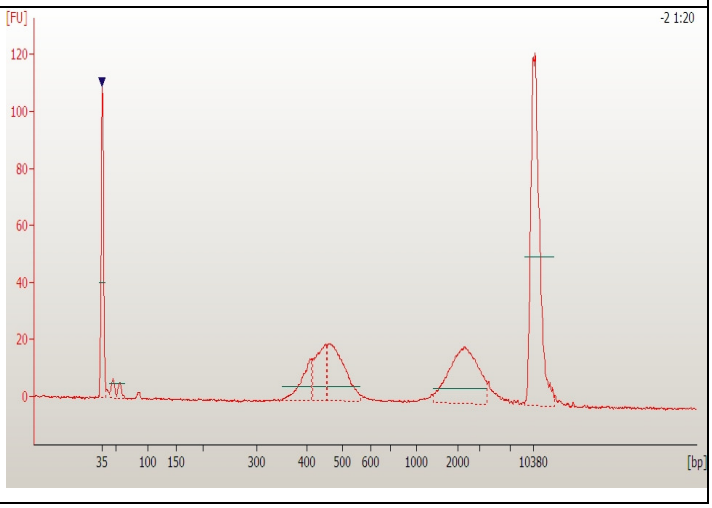
Prior to cluster generation we add PhiX at 1

BioAnalyzer: Total RNA 1	BioAnalyzer: After oligo-dT 1	BioAnalyzer: After Ribozero 1
		

BioAnalyzer: Total RNA 2	BioAnalyzer: After oligo-dT	BioAnalyzer: After Ribozero 2
		

BioAnalyzer: cDNA 1	BioAnalyzer: cDNA 2
	

Gel size selection	BioAnalyzer: Final Library* B1
	

Gel size selection	BioAnalyzer: Final Library* B2
	

* *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.*