Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description:
RNA ID: 013N
Library ID: LID44462
Protocol ID: 013Ns

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STRATEGY: This document contains information about small RNA libraries generated as part of the ENCODE Consortia. It describes the Small RNA Isolation and cloning methods used to generate stranded libraries that capture the 5' ends of RNAs <200 nucleotides in length. The libraries can then be sequenced on the Illumina platform. The 3' ends are A-tailed followed by ligating on a RNA linker to the 5' ends and RT-PCR.

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

Separate cytosol and nuclear from the whole cells

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 by pipetting up and down.
4. Centrifuge for 5 min at 2000 rpm 4℃.
5. Carefully aspirate the supernatant.

Number of Cells	RLN Buffer (4℃) (mL)
$5X10^6$- $5X10^7$	0.5
$5X10^7$- $1X10^8$	1.0
$5X10^7$- $2.5X10^8$	2.0
$2.5X10^6$- $5X10^8$	4.0

6. Add the appropriate amount of cold Buffer RLN to lyse plasma membrane. For the pelleted cells loosen them by pipetting up and down. Incubate on ice for 5 min.

7. Centrifugate lysate at 4℃ for 10 min at 3200 rpm. Transfer the supernatant (the cytosol) to an RNase free centrifuge tube and keep the nuclear pellet.

For the nuclear pellet, put 1ml RLN buffer and try to resuspend the pellet. Centrifuge at 4℃ for 5 min at 3200 rpm. Carefully discard the supernatant, add 800ul RLT (+BME 1:100, 1ml RLT buffer add 100ul BME). Homogenize the sample using a syringe and 18 guage needle. Pass the sample through the needle until it becomes smooth and there are no thick globs. It is important that the sample is homogenized completely. Freeze the lysate in -80℃.

RNA ISOLATION:

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

1. Thaw the frozen tubes from above. Transfer the solution to a new 15ml Falcon RNase free tube. Add 7 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℃ 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 the small 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.
4. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. 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. Centrifuge for 30 seconds to pass the mixture through the filter.

Centrifuge at RCF 5,000 x g (typically 5,000 rpm). Collect the filtrate. If the RNA mixture is >700 µL, transfer *the flow-through* to a fresh tube, and repeat until all of the RNA mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrateAfter 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.

5. Add 2/3 volume room temperature 100% ethanol to filtrate (i.e.flow-through).
6. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied. Pipet the filtrate/ethanol mixture (from the previous step) onto a second Filter Cartridge. 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. Centrifuge for ~1 min to pass the mixture through the filter. Centrifuge at RCF 5,000 x g (typically 5,000 rpm). Discard the flow-through, and repeat until all of the filtrate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.
7. 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, or use vacuum to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.
8. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.
9. Repeat with a second 500 µL aliquot of Wash Solution 2/3. 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.
10. Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 50 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. Repeat steps 9 with a second aliquot of preheated Elution Solution.
11. Transfer the RNA solution to a new RNase free 1.5ml tube. Follow by Ethanol Precipitation.

Ethanol Precipitation (same for Total, Small and Large 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 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 Total, 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 Total or Large RNA.

RNA Cleanup and Separation of Small RNA

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 and 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). Keep the flow-through which contains the small RNA. Then follow the small RNA protocol below.

Small RNA Separation

S1. Add another 450 µL of 100% ethanol to the flow-through and mix by inverting.
S2. Transfer 700 µL of the 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.
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). Repeat with a second volume of 20 µL RNase free water.
S8. Proceed to ethanol precipitation.

Ethanol Precipitation (same for Total, Small and Large 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℃ 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.

RIBOMINUS TREATMENT: In addition to the probes supplied with the Ribominus kit we also spike-in our own LNA probes against the 5S and 5.8S rRNA.

5S-LNAprobe-1 tt+Ccc+Agg+Cgg+Tct+Ccc+At
5S-LNAprobe-2 tc+Agg+Gtg+Gta+Tgg+Ccg+Tag
5.8S-LNAprobe-1 ct+Tca+Tcg+Acg+Cac+Gag+Cc
5.8S-LNAprobe-2 cg+Ctc+Aga+Cag+Gcg+Tagc

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℃.
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
Custom probes (5S, 5.8S 100uM each) : 1.5 µL
Hybridization Buffer: 100 µL
3. Incubate the tube at 70–75℃ 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 22.25 µL H_2O. Keep 1 µL for running a small RNA Bioanalyzer chip.

LIBRARY PROTOCOL:

The following primers and RNA Linker are needed to perform this procedure:

5'SBS3_Adapter (This is the RNA ligated onto the 5' end): "r" = ribose, RNA base
5'- rArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUNNNCG
A-Tail RT Primer (This is the primer used in the RT reaction):
5'-TCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTVN
PE 5' PCR (PCR Primer):
5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
PE 3' PCR (PCR Primer):

5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTC

Tobacco Acid Pyrophosphatase Reaction (TAP, Epicentre T19250)

1. Denature RNA at 85°C for 2 min or 65°C for 10 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
   a. RNA 21.25 µL
   b. 10X TAP reaction buffer 2.5 µL
   c. Anti-RNase (Ambion 20U/uL) 1 µL
   d. TAP (10 U/µL) 0.25 µL
3. Incubate at 37°C for 1hr.
4. Proceed to phenol chloroform extraction.

Phenol Chloroform Extraction

1. Add 1 volume phenol-chloroform 5:1 (pH 4.5) and vortex for 10 s.
2. Spin at 13000rpm 4°C for 20 min.
3. Transfer the upper phase to a new tube.
4. Add 1 volume chloroform and vortex for 10 s.
5. Spin at 13000rpm 4°C for 15 min.
6. Transfer the upper phase to a new tube.
7. Proceed to ethanol precipitation.
8. After drying the pellet, resuspend in 28 µL H_2O.

A-tailing (PolyA kit, Ambion AM1350)

1. Denature RNA at 85°C for 2 min or 65°C for 10 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
   a. 5X PolyA buffer 10 µL
   b. 25 mM $MnCl_2$ 5 µL
   c. BSA 1mg/mL 2.5 µL
   d. 100mM ATP (Roche) 1 µL
   e. Anti- RNase 1 µL
   f. E_PAP Poly A polymerase 2.5 µL
   g. RNA 28 µL

3. Incubate at 37°C for 30 min.
4. Proceed to phenol-chloroform extraction.
5. Proceed to ethanol precipitation.
6. After drying the pellet, resuspend in 11 µL H_2O. Keep 1 µL for running a small RNA Bioanalyzer chip.

Ligation

1. Denature RNA at 85°C for 2 min or 65°C for 10 min. Cool on ice for 1 min.
2. Set up the reaction by adding :
   a. Anti-RNase 0.5 µL
   b. 10X T4 ligase buffer 1.5 µL
   c. BSA 0.25 µL
   d. 5'SBS 3 adapter (100 µM) 2 µL
   e. T4 RNA ligase (Ambion 5U/ µL) 0.75 µL
   f. RNA 10 µL
3. Incubate at 4 °C overnight.
4. Proceed to phenol-chloroform extraction.
5. Proceed to ethanol precipitation.
6. After drying the pellet, resuspend in 16.5 µL H_2O.

First Strand cDNA Synthesis

1. To 16.5 µL RNA, add 2 µL A-Tail RT primer.
2. Incubate at 65 °C for 5 min. Leave on ice for 5 min.
3. Then add the following :
   a. 10mM dNTPs 1.5 µL
   b. 5X first strand cDNA buffer 6 µL
   c. 0.1M DTT 1.5 µL
   d. Anti-RNase 1 µL
   e. Superscript RT III 1.5 µL

PCR

1. Set up the reaction by adding :
   a. First strand cDNA 5 µL
   b. PE 5' PCR primer 100 µM 0.5 µL
   c. PE 3' PCR primer 100 µM 0.5 µL
   d. 2X Phusion mix (NEB F-531L) 50 µL
   e. H_2O 44 µL

2. Program the thermal cycler as follows:

   1. 94°C 2 min.
   2. 94°C 15 s.
   3. 54 °C 30 s.
   4. 72 °C 20 s.
   5. Go back to step 2 and repeat 4 more times.
   6. 94°C 15 s.
   7. 60 °C 30 s.
   8. 72 °C 20 s.
   9. Go back to step 6 and repeat 12 more times.
   10. 4 °C forever

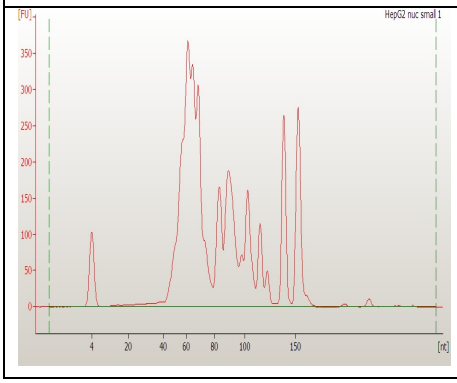
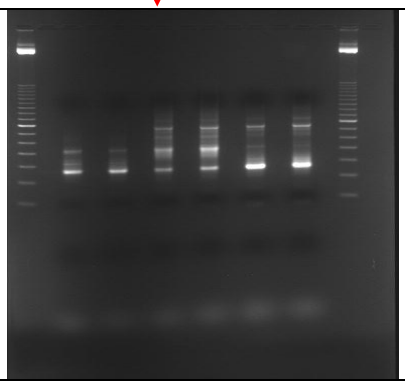
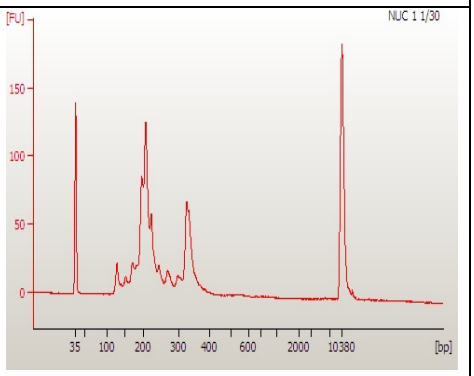
3. After PCR, clean up the reaction by putting through a Minelute column as follows:
   a. Add 5 volumes of PB buffer to the reaction.
   b. To bind DNA, apply the sample to a Minelute column. Spin at 13000rpm for 1 min. Discard the flow-through.
   c. Wash with 750 µL PE buffer. Spin at 13000rpm for 1 min. Discard the flow-through.
   d. Spin at 13000rpm for 1 min to dry the column.
   e. Add 10 µL EB buffer. Spin at 13000rpm for 1 min to elute DNA.
   f. Repeat the elution one more time.
   g. Use 1 µL of the elute for running on a High sensitivity DNA Bioanalyzer chip.

Gel extraction

1. Run the rest of the sample in a 2% agarose gel.
2. Excise the DNA from >128bp to 350bp.
3. Weigh the gel slice and add 3 volumes of QG buffer.
4. Incubate at 50°C for 10 min or until the gel slice has completely dissolved.
5. After the gel slice has dissolved completely, check that the color of the mixture is yellow. **Note** : If the color of the mixture is orange or violet, add 10 µL of 3M NaOAc pH 5.0 and mix. The color of the mixture will turn to yellow.
6. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.
7. Place a Minelute column in a provided 2 mL collection tube.
8. To bind DNA, apply the sample to the Minelute column and spin for 1 min. The maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, simply load and spin again.

9. Discard the flow-through and place the Minelute column back in the same collection tube.
10. Add 500 µL QG buffer to the spin column and spin for 1 min.
11. Discard the flow-through and place the Minelute column back in the same collection tube.
12. To wash, add 750 µL PE buffer to the Minelute column and spin for 1 min.
13. Discard the flow-through and spin the column for an additional 1 min at >10,000g.
14. Place the Minelute column into a clean 1.5 mL tube.
15. To elute DNA, add 10 µL EB buffer to the center of the membrane, let the column stand for 1 min. and spin for 1 min.
16. Repeat the elution to get a higher yield of DNA.
17. Proceed to ethanol precipitation.

Quantification

1. After drying the pellet, resuspend in 20 µL H_2O.
2. Measure the concentration on the Nanodrop.
3. Run 1 uL on the High sensitivity DNA Bioanalyzer chip.

Input RNA	Gel Purification	Final Library Image