Gingeras Lab RNA-Seq Library Production Document

ENCODE Transcriptome

Sample Description: Human Renal Epithelial Cell RAMPAGE

Cat#4125Lot#13744

RNA ID: 298WC

Library ID: 295491

Composite Library ID : 295493

Cold Spring Harbor Laboratory

Genome Center

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Total RNA was purchased from ScienCell Research Labs.

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.

Long RNA Purification

1. Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix well.
2. Add 250 µl ethanol (96–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
4. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
Reuse the collection tube in step 5.
Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).
5. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.
6. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 5.
7. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
8. Repeat step 7 using another 30–50 µl RNasefree water.
Reuse the collection tube from step 7.
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°C for 2hrs.
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 500 µL 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 50 µl RNase-free water.

Ribo Zero Magnetic Separation Protocol (r-RNA Removal)

(Epicentre Ribo-Zero rRNA Removal Kit- Human/Mouse/Rat- Cat. No. MRZH11124)

A. Individual Washing Procedure Note:

1. Mix the Magnetic Beads well by pipetting or gentle vortexing.

2. For each reaction, pipet 225 µl of Magnetic Beads into a 1.5-ml RNase-free microcentrifuge tube. Pipet the Magnetic Bead suspension slowly to avoid air bubbles and to ensure pipetting of the correct volume. Store unused Magnetic Beads at 4°C.

3. Place each 1.5-ml microcentrifuge tube on the magnetic stand for at least 1 minute (until the solution appears clear).

4. With the 1.5-ml microcentrifuge tube still on the stand, remove and discard the supernatant. ***Caution:*** *The supernatant contains 0.1% sodium azide.*

5. Remove the 1.5-ml microcentrifuge tube from the magnetic stand and add 225 µl of RNase-Free Water to each tube. Mix well by repeated pipetting or vortexing at medium speed.

6. Repeat steps 3, 4 and 5 (i.e. wash the beads a total of 2 times with RNase-Free water).

7. Remove the 1.5-ml microcentrifuge tube from the magnetic stand. Add 65 µl of Magnetic Bead Resuspension Solution to each tube. Mix well by repeated pipetting or vortexing at medium speed.

8. Optional: Add 1 µl of RiboGuard RNase Inhibitor to each tube of resuspended Magnetic Beads, and mix briefly by vortexing.

9. Store the microcentrifuge tubes at room temperature until required in Part C.

B. Treatment of the Total RNA Sample with Ribo-Zero rRNA Removal Solution

1. In a 0.2-ml or 0.5-ml RNase-free microcentrifuge tube, combine in the order given:

RNase-Free Water	x µl
Ribo-Zero Reaction Buffer	4 µl
Total RNA sample	1-5 µg
Ribo-Zero rRNA Removal Solution	10 µl
Total volume	40 µl

2. Gently mix the reaction(s) by pipetting and incubate at 68°C for 10 minutes. Store the remaining Ribo-Zero rRNA Removal Solution and Ribo-Zero Reaction Buffer at –70°C to –80°C.

3. Remove the reaction tube(s) and incubate each at room temperature for 5 minutes.

C. Magnetic Bead Reaction and rRNA Removal

Required in Part C: 50°C water bath or heating block for 2.0-ml tubes.

1. Using a pipette, add the treated RNA from Part B to the 1.5-ml microcentrifuge tube containing the washed Magnetic Beads and, without changing the pipet tip, ***immediately and thoroughly mix the contents of the tube by pipetting at least 10 times. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.*** Repeat this process for each sample.***Important!*** *Always add the treated RNA sample to the washed Magnetic Beads and immediately mix by pipetting. Never add the Magnetic Beads to the treated RNA sample. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact the efficiency of the rRNA removal.*

2. Incubate the 1.5 ml microcentrifuge tube at room temperature for 5 minutes.

3. Following incubation, mix the reactions by vortexing at medium speed for 10 seconds and then place at 50°C for 5 minutes in an appropriate water bath or heating block. Avoid any significant condensation during this incubation step.

4. After the 5-minute incubation at 50°C, remove the microcentrifuge tubes and immediately place them on a magnetic stand for at least 1 minute (until the solution appears clear).

5. Carefully remove each supernatant (85-90 µl) containing the RNA and transfer to a labeled 1.5-ml RNase-free microcentrifuge tube. ***Important!*** *The supernatant contains rRNA-depleted RNA*

.***Optional:*** *If a small amount of Magnetic Beads are still visible in the supernatant, place the collected supernatant onto the magnetic stand for 1 minute. Remove the supernatant containing the rRNA-depleted RNA and transfer to a new 1.5-ml RNase-free microcentrifuge tube.*

6. Ethanol precipitate and resuspend in 10 µl for library construction.
7. Use 1.5 µl for running on a Pico-chip on the Bioanalyzer.

Preparation of 5'-complete cDNAs for paired-end sequencing

Reagents & Consumables

Reverse-transcription

- SuperScript III Reverse-transcriptase (Invitrogen, 4x10,000U, Cat # 18080-085)
- First strand buffer (comes with SuperScript III)
- DTT 100mM (comes with SuperScript III)
- 10mM dNTP mix (Invitrogen, 100µl, Cat # 18427-013)
- Betaine 5M Sigma-Aldrich, Cat # B0300-1VL)
- D(-)-Sorbitol (Wako Pure Chemical Industries, 25g, Cat # 194-03752)
- D(+)-Trehalose dihydrate (Sigma-Aldrich, 25g, T9531-25G)
- Sorbitol/Trehalose solution must be prepared as described in Recipe 1.
- 96-well 200µl reaction plates

Agencourt RNAClean XP purification:

- Agencourt RNAClean XP Kit (Beckman Coulter, 40 mL, Cat # A63987)

Agencourt AMPure XP purification:

- Agencourt AMPure XP Kit (Beckman Coulter Inc., 60 mL, Cat # A63881)

Diol Oxidization:

- Sodium periodate ($NaIO_4$) ≥99.8% (Sigma-Aldrich, 5g, Cat # 311448-5G)
- NaOAc 3M, pH 5.5 (Ambion, 100 mL, Cat # AM9740)
- Tris-HCl 1M, pH7.4 (Sigma-Aldrich, 100 mL, Cat # T2194-100ML)

Biotinylation:

- Biotin hydrazide long arm (Vector Labs, 50mg, Cat # SP-1100)
- Sodium Citrate (Sigma-Aldrich, 500g, Cat # S1804-500G)

RNAse I digestion:

- RNAse I (5-10 U/µl) (Promega, 1,000U, Cat # M4261)
- EDTA 0.5M, pH8.0 (Ambion, 100 mL, Cat # AM9260G)

Cap-trapping:

- MPG Streptavidin beads (PureBiotech LLC, 2mL (20mg), Cat # MSTR0502)
- *E. coli* tRNA Type XX (Sigma-Aldrich, 500U, Cat # R1753-500UN). Must be DNAse-treated as described in Support Protocol 1.
- NaCl 5M (Ambion, 100mL, Cat # AM9760G)
- NaOH Solution, 10M in H_2O (Sigma-Aldrich, 100mL, Cat # 72068-100ML)

PCR amplification:

- Phusion High-Fidelity PCR Master Mix with HF Buffer (Cat # M0531L)

Miscellaneous:

- H_2O Molecular biology grade (Sigma-Aldrich, 100ml, Cat # 95284-100ML)
- HCl 36.5-38%, Molecular Biol. grade (Sigma-Aldrich, 100ml, Cat # H1758-100ML)
- Glycerol (Sigma-Aldrich, 100ml, Cat # G5516-100ML)
- SDS (Sigma-Aldrich, 500ml, Cat # G05030-500ML-F)
- High-recovery tubes 1.7 mL (Axygen, Case of 10 x 250, Cat # MCT-175-L-C)
- High-recovery tubes 0.2 mL (Axygen, Pack of 1,000, Cat # PCR-02-L-C)
- RNAse-free pipette tips (1,000, 200, 20µl)

Synthetic oligonucleotides:
All ordered from IDT. Purification procedure: standard desalting. Synthesis scale 100 nmol, unless otherwise specified.

Template switching oligos:
TSCAGE_6N_**
5'- TAGTCGAACTGAAGGTCTCCAGCA***NNNNNN***rGrGrG
The 6 N's of the sequence correspond to the library identification barcodes.
The "**" part in the name is reserved for barcode numbers.
(!) The last 3 residues must be riboguanosines.
Order as 250 nmol RNA oligo batches.
See "Sequence barcodes" section below for a list of barcodes and technical details.

RT primer:
CAGEscan_RT_15N
5'-TAGTCGAACGAAGGTCTCCGAACCGCTCTTCCGATCT$(N)_{15}$

Final PCR primers:
rampage_F
5’- AATGATACGGCGACCACCGAGATCTACACTAGTCGAACTGAAGG
rampage_R
5’-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGC
TCTTCCGATCT

Sequencing primers:
Custom Read 1 sequencing primer (rampage_r1):
5’- TAGTCGAACTGAAGGTCTCCAGCA
Illumina Read 2 sequencing primer (SBS8):
5’- CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Sequence barcodes

A set of 40 6-base barcodes were designed, with the requirements of a GC content between 20 and 80% and a minimum Hamming distance (i.e., number of differing positions) of 3 between any 2 barcodes in the set. This second requirement ensures that even barcodes with one sequencing error can still be unambiguously identified, thus maximizing the proportion of barcodes recovered while minimizing the risk of barcode misassignment. The sequences of these 40 barcoded oligos are listed below:

Set1: index1GGATAC-index2TTAACG-index3GAGTGC-index4AAGGAC-index5CGCGTT
Set2: index1ATGCGT-index2ATAAGC-index3ATCTCC-index4TAACTC-index5ATAGAG
Set3: index1AGCCTA-index2TGTAGT-index3AAACGG-index4CCTACG-index5ACTAGA
Set4: index1CCCTCT-index2GGTATA-index3GATCCC-index4CAATGT-index5GCGTTG
Set5: : index1AACTGA-index2CCAATA-index3GCGACT-index4GGGGAT-index5TCTTCC

Equipment

Thermal cycler (Biorad)
Magnet (microcentrifuge tubes)
Bioanalyzer (Agilent)
Bioanalyzer RNA Pico, Nano and DNA High Sensitivity chip kits (Agilent)

Reverse-transcription

1. Prepare the reverse-transcription (RT) mix:

RNA	7.5µl
CAGEscan_RT_15N oligo (400µM)	1µl
TSCAGE_6N_** oligo, set 4(4mM)	1µl

Set3: index1AGCCTA-index2TGTAGT-index3AAACGG-index4CCTACG-index5ACTAGA

2. Denature for 10 min at 65ºC. Immediately place on an ice-cold metal block for 2 min.

Proper denaturation is important for reverse-transcription efficiency, as secondary structures can diminish the processivity of the enzyme.

3. Add the RT reaction mix:

Invitrogen 1st strand buffer	7.5µl
dNTPs mix	1.9µl
Sorbitol/Trehalose mix	7.5µl
DTT (100mM)	1.9µl
Betaine (5M)	5.6µl
SuperScript III RT (200U/µl)	4µl

4. Incubate in a thermal cycler with the following program:

4ºC	10''
22ºC	1 min
42ºC	30 min
75ºC	15 min
4ºC	Hold

5. Purify the digested RNA sample by RNACleanXP cleanup, roughly as per manufacturer's protocol. Briefly:

- Add 65µl of RNACleanXP and mix thoroughly by vortexing or pipetting.
- Precipitate for 5 min at room temperature.
- Place on a magnet for 3 min and carefully remove the supernatant.
- Wash twice with 150µl freshly prepared ethanol 70%.
- Air-dry for 5 min (but without completely drying out the beads)
- Elute with 42µl H_2O, resuspend beads well by pipetting, incubate 3 min at room temperature.
- Place on the magnet for 3-5 min (until the beads are well separated) and recover 40µl of supernatant.

Samples can be stored at -20ºC at this point. Carefully prevent degradation, though: RNA integrity still matters at this point.

5’ Cap oxidization

In this step, periodate is used to oxidize ribose residues that bear free 2’ and 3’ hydroxyl groups. All riboses in 3’ terminal nucledotides, as well as those in 5’ cap structures, are affected.

6. Prepare 250mM NaIO4 solution: dissolve 26.7mg $NaIO_4$ in 500µl H_2O.
This solution should always be prepared fresh. In addition it is light-sensitive, and should be kept covered in aluminum foil, on ice.

7. Add 2 µl of 1M NaOAc, pH 4.5 to RNA/cDNA solution.
The pH of this solution is critical.

8. Add 2 µl of 250mM $NaIO_4$ and mix well. Incubate on ice in the dark (or in foil) for 45 min.

9. Stop the reaction by adding 2µl of 40% glycerol. Mix well by pipetting. Add 14µl of 1M Tris-HCl, pH 8.5. Mix well by pipetting.

10. RNACleanXP cleanup: Add 105µl beads suspension, wash 2x with 200µl EtOH 70%, elute with 40µl H_2O.

5’ Cap biotinylation

Ribose residues whose 2’ and 3’ hydroxyls have been oxidized to aldehydes in the previous step can be biotinylated by reaction with biotin hydrazide.

11. Prepare 15mM biotin solution by dissolving 4.2mg biotin hydrazide in 750µl H2O.
Biotin does not dissolve well in water: vortex for 20-30 min at room temperature while covered in aluminum foil.
Always prepare fresh. Solution should be kept at all times on ice, covered in foil.

12. Add 4µl of 1M NaCitrate, pH 6.0. Add 13.5µl of 15mM biotin solution and mix well by pipetting.

13. Incubate at room temperature for 14-15 hours in the dark (cover with foil).
No cleanup after this step: directly proceed to RNAseI digest.

RNAseI digest

14. Prepare RNaseI mix. Per reaction:

1M Tris-HCl, pH 8.5	6 µl
0.5M EDTA, pH 8.0	1 µl
RNaseI (10U/µl)	5 µl

15. Add RNaseI mix (12µl) and mix well by pipetting. Incubate 30 min at 37°C.
When pooling many libraries you can extend the incubation time to 60 min,

16. Incubate 5 min at 65°C and immediately place on ice for 2 minutes.

17. RNACleanXP cleanup: Add 125µl beads suspension, wash 2x with 200µl EtOH 70%, elute with 40µl H_2O.

Streptavidin pulldown ("Cap-trapping")

18. During RNaseI digest and cleanup, prepare magnetic streptavidin beads as follows:
- Resuspend beads suspension by vortexing vigorously
- Transfer 100µl of suspension to a new 1.7ml tube
- Add 1.5µl of 20 µg/µl *E. coli* tRNA and mix well
- Incubate 30 min at RT (vortex every 3 min to resuspend the beads)

19. Prepare wash buffers:
Buffers can be stored at RT for several months.

Wash Buffer 1

Reagents	Volume	Final concentration
5 M NaCl	45 ml	4.5 M
0.5 M EDTA, pH 8.0	5 ml	50 mM
Total	50 ml	

Wash Buffer 2

Reagents	Volume	Final concentration
5 M NaCl	3 ml	0.3 M
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
H_2O	46.9 ml	
Total	50 ml	

Wash Buffer 3

Reagents	Volume	Final concentration
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
10% SDS	2 ml	0.4%
1 M NaOAc, pH6.1	25 ml	0.5 M
1 M Tris-HCl, pH 8.5	1 ml	20 mM
H_2O	21.9 ml	
Total	50 ml	

Wash Buffer 4

Reagents	Volume	Final concentration
0.5 M EDTA, pH 8.0	0.1 ml	1 mM
1 M NaOAc, pH6.1	25 ml	0.5 M
1 M Tris-HCl, pH 8.5	0.5 ml	10 mM
H_2O	24.4 ml	
Total	50 ml	

20. Finish preparing the beads:
    - Place streptavidin beads on magnetic stand for 3 min and remove supernatant
    - Add 50µl Buffer 1, resuspend beads well by pipetting, separate on magnetic stand, remove supernatant. Repeat a 2^{nd} time.
    - Resuspend beads in 80µl Buffer 1

21. Add 80µl of beads suspension to the RNAseI-treated sample. Incubate 30 min at room temperature (mix by gentle vortexing every 3 min).

22. Place on magnetic stand for 5 min and discard supernatant.

23. Wash with buffer 1: add 150µl buffer 1, resuspend beads well by pipetting, place on magnetic stand for 3 min, discard supernatant.

24. Wash with buffer 2 (same procedure).

25. Wash twice with buffer 3.

26. Wash twice with buffer 4. Make sure the supernatant is completely removed.

27. Elution from the beads:
    - Dispense 12µl of 1M Tris-HCl, pH 7.0 to a new tube and keep on ice
    - Add 65µl of 50mM NaOH to the RNA/cDNA-bound beads. Mix well by pipetting.
    - Incubate for 10 min at room temperature. Vortex gently every 2-3 min.
    - Place on magnetic stand for 3 min.
    - Transfer supernatant to the tube containing Tris-HCl pH 7.0.

28. AMPureXP cleanup: add 130µl beads suspension, precipitate 5 min, wash 2x with 200µl EtOH 70%, air-dry for 2 min, elute with 43µl H_2O.

PCR amplification

29. Prepare the PCR reaction mix as follows:

Template	42µl	
2X HF Phusion Master mix	50µl	
CAGEscan er-F (10µM)		4µl
CAGEscan er-R (10µM)		4µl

PCR program:

95ºC	1 min 15''
55ºC	10''
68ºC	2 min
95ºC	*15''*
65ºC	*10''*
68ºC	*2 min*

Repeat the *last 3 steps* another 16 times (18 cycles total)

68ºC	5 min

AMPure XP PCR Cleanup Protocol

30. AMPureXP cleanup: add 80µl beads suspension, precipitate 5 min, wash 2x with 200µl EtOH 70%, air-dry for 5 min, elute with 30µl H_2O.

Quality control & Quantification:

31. Run the final library on a Bioanalyzer High Sensitivity DNA chip for quality control and preliminary quantification. Follow the manufacturer's instructions. Expected size range: ~ 300-1000bp.

32. Adjust the concentration of the library to 10nM.

33. Sequence on an Illumina platform (GA_{II}, HiSeq, MiSeq). Paired-end run, read length as desired. Loading concentration as recommended by the manufacturer for the platform of choice.

Sequencing primers:

Read 1 primer CAGEscan_r1Seq 5'- TAGTCGAACTGAAGGTCTCCAGCA
Read2 Primer: SBS8 5' CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT (Illumina)

(!) IMPORTANT: This type of library has poor sequence heterogeneity early in read 1. To avoid failure due to this, please spike in phiX at 10% and use a separate control lane. Thanks!

Recipe 1: Sorbitol/Trehalose (3.3M /0.66 M) stock solution preparation

Materials

- D(-)-Sorbitol (Wako Pure Chemical Industries, 25g, Cat # 194-03752)
- D(+)-Trehalose dihydrate (Sigma-Aldrich, 25g, T9531-25G)
- RNAse-free water (molecular biology grade)

Equipment

- 50 ml centrifuge tubes
- RNAse-free, autoclavable glass bottle (100 ml)
- Autoclave

1. Add 2ml of RNAse-free water in a 50ml tube.

2. Weigh 8.02g of trehalose directly into the tube.

3. Add 3ml of water and mix.

4. Weigh 17.8g of sorbitol directly into the tube.

5. Add 5.5ml of water and mix.

6. Add water to a total volume of solution of 30ml and mix well.

7. Transfer to an RNAse-free glass bottle and autoclave at 121ºC for 30 min.

8. Store at room temperature in 1.5ml aliquots protected from light with aluminum foil. This solution can be kept for up to 6 months.

Support Protocol 1: tRNA stock solution preparation

This RNA will be used to saturate non-specific interactions of RNA molecules with the streptavidin-coated beads. It needs to be carefully DNAse-treated, protease-treated, and purified prior to this. Aliquots of purified tRNA can be stored for later use.

Materials

- *E. coli* tRNA Type XX (Sigma-Aldrich, 500U, Cat # R1753-500UN).
- DNAse RQ1 (Promega, 1,000U, Cat # M6101)
- Proteinase K (NEB, 60 mg, Cat # P8102S)
- RNACleanXP (Agencourt)
- Ethanol 70%

Equipment

- Microcentrifuge
- Heating block (for 1.5 mL tubes)
- 1.5 mL tubes

1. In a 2ml microcentrifuge tube, dissolve the tRNA in water and prepare the DNAse digestion mix:

tRNA	30mg
H_2O	400µl
RQ1 DNAse buffer	45µl
DNAse RQ1 (1U/µl)	30µl

2. Incubate at 37ºC for 2 hours.

3. Add to the mix:

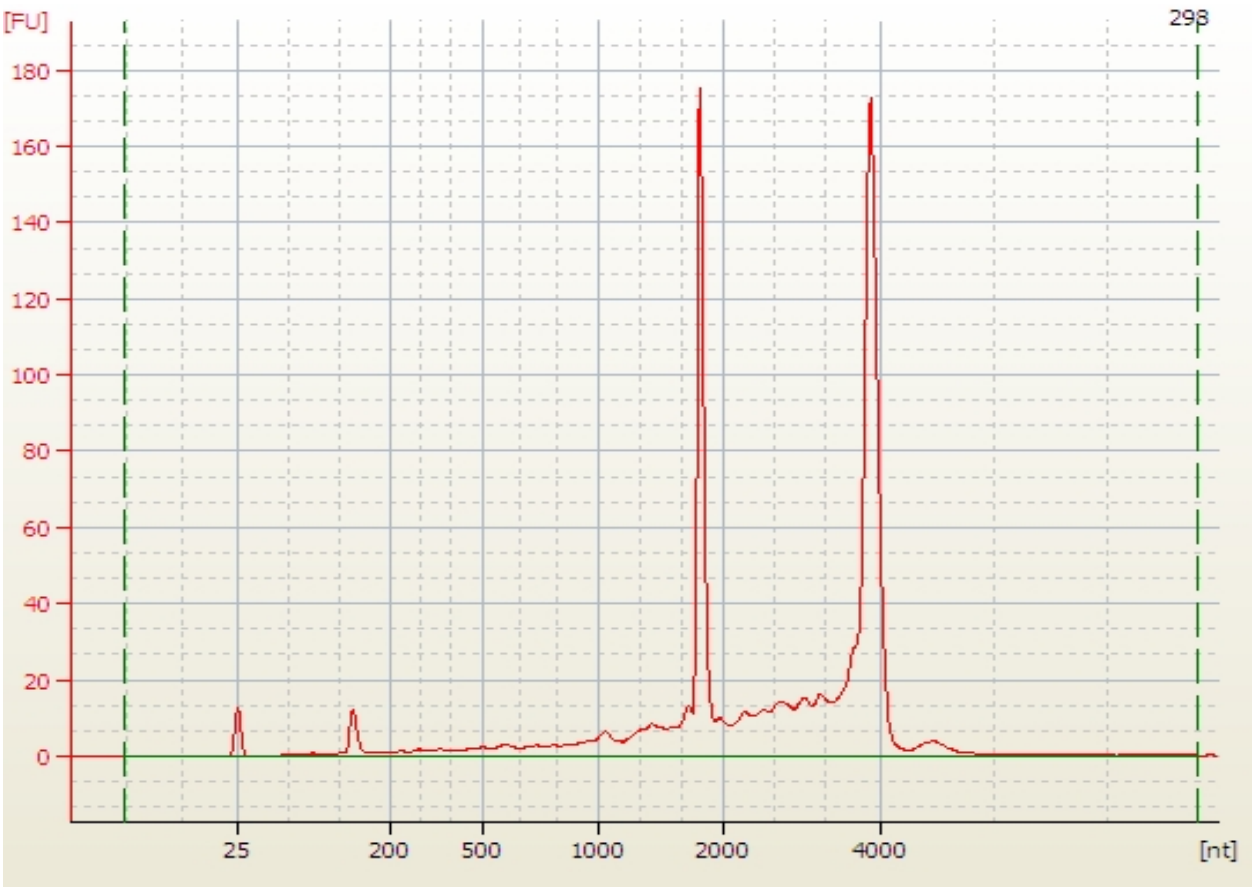
0.5M EDTA	10µl
10% SDS	10µl
Proteinase K (10mg/ml)	10µl

4. Incubate at 45ºC for 30 min.

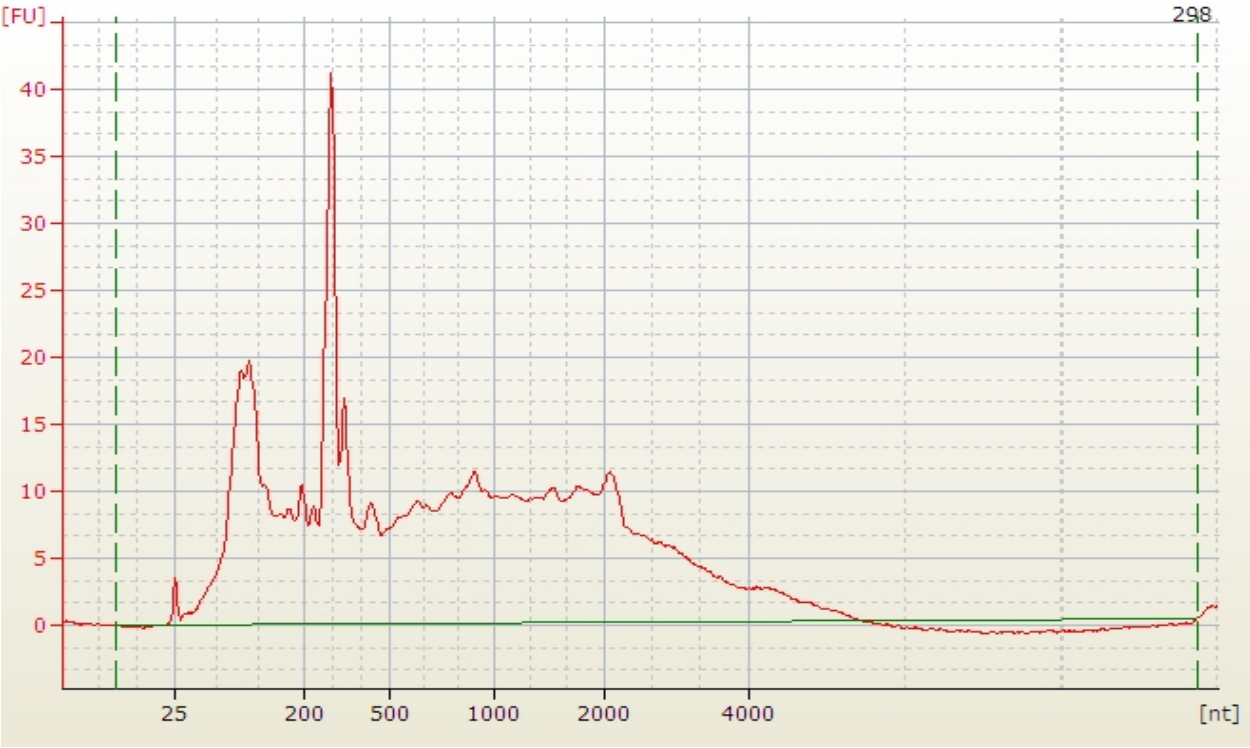
5. RNACleanXP cleanup:
    - Add 900µl of RNACleanXP suspension and mix well.
    - Precipitate for 5 mins at room temperature.
    - Place on magnet for 5-10 mins (until the solution is clear).
    - Discard the supernatant and wash 3 times with 1.8ml of 70% ethanol. Remove the ethanol.
    - Centrifuge for a few seconds to bring all remaining ethanol to the bottom of the tube and place back on magnet for 1 min.
    - Remove any remaining ethanol.
    - Air-dry for 3 mins.
    - Elute with 1.5ml water, mix well by pipetting, incubate at room temperature for 5 mins, place on magnet for 5-10 mins (until the solution is clear).
    - Recover the supernatant.

6. Store in small aliquots (e.g., 100µl) at -20ºC for up to a year.

BioAnalyzer: Total RNA



BioAnalyzer: ribo Zero RNA:



BioAnalyzer: Final Library: