csRNA-seq_V2

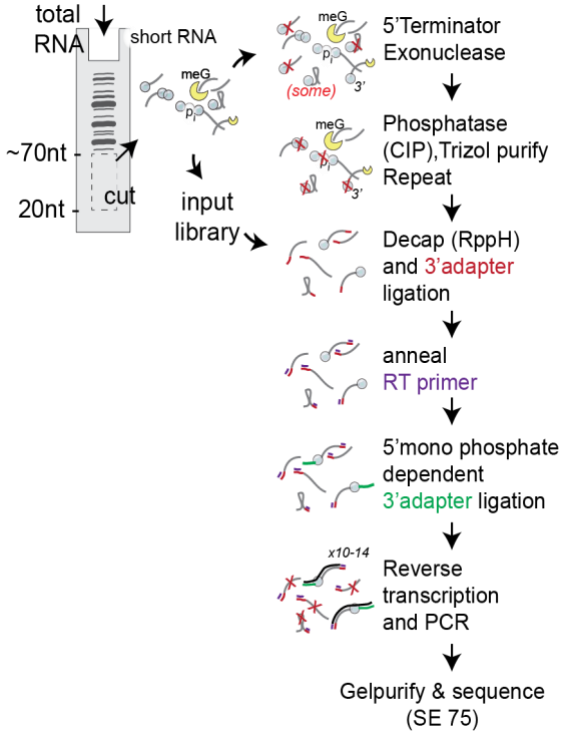
Version Patricia Montilla Pérez 05/04/2021

Use total or (if easy to obtain) nuclear RNA. The input requirement for total RNA is about ~3 ug but we have successfully generated libraries from as little as 500 ng. But: more is better ☺. The maximum a gel can take ~25ug/well. If possible, run all samples of interest in parallel to avoid issues with batch effects.

Few notes:

- Watch the video online before starting: https://www.youtube.com/watch?v=tFSEX7W4EEM&t=5s&ab_channel=csRNA-seqMethod

- Extract your RNA with TRIZOL-chloroform. You don't need to perform a DNAse treatment.
- Use shield to protect the RNA.
- When working with the 8-strip tube, do not add sample in the extreme tubes, use them for handling.
- Try to optimize your sample handling to allow fast temperature transitions: i.e. move samples from ice to 37C or 75 directly on wet ice to reduce secondary structures in your RNA and optimal efficiency of your enzymes.
- On day 2 and 3, on the reaction chain, 10 minutes before the reaction is done, we can prepare the master mix of the next reaction and add it on a new set of lids. When the reaction is done, we just need to quick spin, change lids, mix by inversion very well and incubate the next reaction.
- If working with hundreds of samples, size selection can also be done using SPRI beads. However, this requires very careful optimization. Hence in general, it is much better to use a gel for size selection.
- Do not vortex enzymes. Instead, invert the samples vigorously so the liquid moves from top to bottom.
- Mix and quick spin <u>all buffers</u>, primers etc..
- When working with PEG (i.e. RppH & 3'Adapter Ligation), mix samples reaaallly well by vigorously inverting them.
- Keep stock enzymes in a -20C cooler, not on ice. (2x PCR MM is ok).

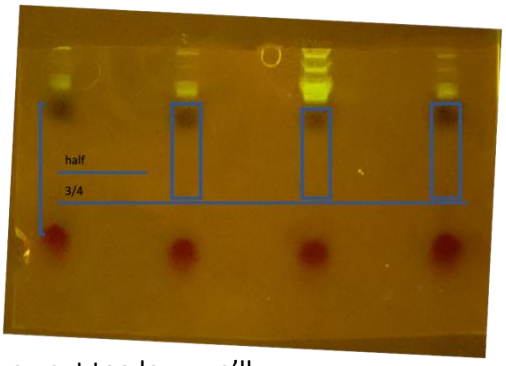


Summary of the method (see also end of file)

Day1 - csRNA-seq: Size selection (critical step)

Gel purification:

1. Pre-run UREA TBE gel (EC68852BOX) in 1x TBE at 200V for 20 minutes. *Make sure to clean the wells with a 200ul pipette. You can add 1-2ul of FLB buffer to the first lane in gel one, second lane in gel two, etc to make sure that its running.*
2. Mix 1:1 RNA with 2xFLB [see Buffers]. Denaturate RNA - heat at 75°C for 3 minutes for 1.5ml tubes and 90 seconds if PCR tubes are used. ***Quickly* chill on ice and keep them on ice.**
3. Load RNA on the gel with generous spacing *(2 empty spaces between samples)*. Optional: Load 5 µl of ssRNA ladder (NEB #0364) *in the first lane and 1ul in the last lane to give the gel direction*. Run gel ~40' at 200V until bromphenol blue is ¾ down.
4. In the meantime, prepare one set of safe-lock tubes with the Gelbreaker Tubes (Cat# 3388-100) inside and label both.
5. Prepare to stain the gel in 0.5 µg/mL GelGreen® Nucleic Acid Gel Stain [Biotium #41005] in 1xTBS for ~2-5 mins *on square petri dish. Use 1.5 ul of the marker on 10ml of TBE from the same running buffer.*
6. Cut gel to size select RNA:



- Open the cassette, the gel should have a direction, cut the wells and the lower end of the gel. With a <u>wet</u> spatula, lift the gel and transfer it to the square petri dish. Go to the dark room with the set of tubes previously prepared. Transfer the gel to the inside of the lid of the petri dish and take a picture for record.

- Cut the gel and transfer it to the prelabeled set of tubes. It is critical to cut <u>below</u> (**not touching!**) any strong band that appears on the gel. (~55 nt)

- lower end usually ~20 nt (or: 1/3rd down between xylene and bromphenol blue works well ~12 nt, you'll refine selection later). It's OK if you cut too low, we'll adjust this in the DNA gel again.

-You can line up the razor blade perpendicular to the xylene blye and bromophonol blue to cut the sides

*Position the cut gel in a way that the xylene blue will enter first thetube.

7. Shred gel slices by spinning the cut gel through a Gelbreaker Tubes [IST Engineering Inc 3388-100] (or through a 0.5 ml PCR perforated 3-4 times with 22 g needle) placed into a LowBind tube for 3 minutes at max g at RT. Here it's best to use the "**<u>slow" acceleration</u>** setting on the centrifuge to avoid lids flying off. *If there is any piecse of gel leftover on the gelbraker tube, you can spin again or you can inverse the tube and flick it to release the gel into the safe-lock tube.*
8. Add 350 µl csEB [400 mM NaOAc, 0.05% Tween, 1 mM EDTA, 10 mM Tris pH7.5] and elute sRNA under agitation in the dark for 2-3h at RT (Vortex setting 0). In the meantime, label another set of 1.5ml tubes.
9. Quick spin. Use the wide orifice p200 pipet tip (VWR46620-642) to pipette the slurry up and down 3-4 times and transfer to a spin column (UltraFree MC, 0.45 µm, Millipore UFC30HVNB). <u>Do NOT spin</u>.
10. Cut off the spin column with your slurry and move it onto a new 1.5ml LowBind tube containing 1.5µl GlycoBlue™ Coprecipitant (ThermoFisher AM9516, 15 mg/mL). *Process 6 tubes at the time otherwise, they will start to flow through.* Once you have transfer all of them, spin at 1000g for 1 minute at RT.

Note: mix glycoblue thoroughly before use to ensure it maintains its concentration

11. Discard the spin column, add >3x Vol of 100% EtOH, <u>mix well</u> and precipitate sRNA at -20 °C O/N.

<u>Day2:</u>

12. Let the tubes rest for 5' at RT, then mix well and pellet RNA by spinning at ~20k g at 4°C for 30 minutes.

*** If you do not see nice blue pellets, vortex the sample and spin again for 10 minutes ***

13. Remove all supernatant. Quick spin and use a filter gel loading tip to remove all access residue. *Close the tubes when processing all the samples, the alcohol can evaporate.*

14. Wash pellet with 400 µL 75% EtOH, *(make sure the pellet moves around*), then again quick spin and remove all residue.
15. Dry pellet for 3-5 minutes at RT. *Very important completely dry.* **SAFE STOP POINT. Freeze pellets at -80**

5'-Cap Enrichment (in 1.5 ml tubes)

1. Add 6µL TET [0.05% Tween, 1 mM EDTA, 10 mM Tris pH 7.5] to a new set of lids, close them and shake them, let them incubate few minutes at RT until the pellets dissolve, you can also vortex them. Spin them down and heat to 75°C for 3', chill on ice.

	TermMM (for 20 µl final)	**1x**	
1	ddH_2O+0.05% Tween	11.25	
2	10x TermA Buffer	2 µl	
3	Superase	0.25 µl	
Vortex well the master mix and spin down			
	TER51020	1 µl	
Invert vigorously and spin down			
	Sample 5.5µl +14.5µl MM = 20µl	= 14.5 µl	

1. **Important: take 0.5 µL INPUT control.** In a PCR 8 strip, add 1 µL TE'T and then add 0.5 µL of input per sample (each).
2. Add 14.5 µL TermMM, mix well and incubate at 30°C for 1hour. *When you add the MM, do not pipette up and down in the sample, just add the master mix, close the tubes and then mix well adding another rack on top of the rack where you have your samples and move up and down few times to mix well. Quick spin and incubate for an hour.*

	Cip1MM (for 50 µl final)	**1x**	
	ddH_2O+0.05% Tween	24 µl	
	10x Cutsmart Buffer	5 µl	
Vortex well the master mix and spin down			
	quickCIP (NEB M0525L)	0.75 µl	
Invert vigorously and spin down			
	Sample 20µl + 30µl MM = 50µl	= 30µl	

1. Quick spin, add 30 µL CIP1 MM, mix well and incubate at 37°C for 45 minutes. *You can add this MM at RT.*
2. Heat sample to 75°C for 90 seconds, then quick chill on wet ice.
3. Add 10 µL of CIP2 MM, mix well and incubate at 37°C for 30 minutes.

	Cip2MM (for 20 µl final)	**1x**	
	ddH_2O+0.05% Tween	8.5	
	10x Cutsmart Buffer	1 µl	
Vortex well the master mix and spin down			
	quickCIP	0.5 µl	
	Invert vigorously and spin down		
	Sample 50µl + 10µl MM =60µl	= 10µl	

1. **Trizol LS purify RNA**:
   - add 500 Trizol LS, 5' vortex, then 150 µl TE'T & 150 µl $CHCl_3$ +IAA, spin 10 minutes at 14K @ RT!).
   - carefully take of supernatant *(with a P1000)* without disturbing the interphase and pipette it to a new tube containing 60 µL 3M NaOAc and optional: 0,5 µL Glycoblue (IF the pellet was not strong enough last precipitation. Otherwise, no need to add more). Mix well and quick spin.
   - Add 1 total volume of Isopropanol (usually ~650 µL), Mix well and quick spin.
2. Precipitate in isopropanol (Never place in -80°C!) for 20 minutes on ice or -20°C O/N.
3. Pellet RNA by spinning at >20k g (or more) at 4°C for 30 minutes.
4. Remove all supernatant.
5. Quick spin and use a filter gel loading tip to remove all access residue.
6. Add 400 µL 75% EtOH, pipette up and down to loosen the pellet and transfer it with a P1000 in in the 75% EtOH to a PCR strip tube or 96 well plate for the library prep.
7. Quick spin and remove all remaining EtOH with a gel loading tip.
8. Dry pellet for 3-5 minutes. **SAFE STOP POINT. Freeze pellets at -80**

Day3: Library Prep

I like the NEB sRNA kit E7330, **E7560S** or similar as instructed within. Use ~0,2 µl of adapter; otherwise for input, you may use half volumes. This protocol uses half of volume than the NEB protocol, you will get about double reactions.

5'decapping

1. Resuspend RNA pellets in 3 µl TE'T 2' @75°C, then place immediately on ice and keep them on ice.
2. Optional: Take Cap- control (10%)
3. To PCR strip lid: add 5 µl MM1 to samples and 2.5 µl for inputs, flick to mix & incubate for **1-2 h** at 37°C, lid 42°C.

	MM1	**1x**	
1	10x T4RL buffer	0.8 µl	
2	SUPERase-IN (20 U/µl)	0.3 µl	
3	50% PEG8000	3 µl	
	Vortex well the master mix and spin down		
4	RppH	1 µl	
	Invert vigorously and spin down		
	3µl RNA + 5µl MM1= 8µl	= 5 µl	

3' adapter ligation

- Add 4 µl MM2 to a new set of lids, 5 minutes before the 5'decapping ends, then change the lids, flick and spin. Incubate at **22°C** for 1-2h or 16°C for longer, lid at 40°C. [RppH is inactive at 20°C and hence does not destroy adapter]

	MM2 (Green)	**1x**	
1	2x NEB 3'Adapter buffer	2 µl	
2	9 µM 3' Adapter	0.3 µl	
	Vortex well the master mix and spin down		
3	NEB 3'Enzyme MM	1.7 µl	
	Invert vigorously and spin down		
	8µl reaction + 4µl MM2= 12µl	= 4 µl	

RT primer hybridization

- On ice, add 1 µlof 5 µM RT primer with the multichannel, previously diluted 1 to 1. Incubate at 75°C for 2', then 37°C for 30', then 25°C for 15'.

5' adapter ligation

SR5' Adapter is stored at -80. Before use, heat to 70 ˚C foe 2 minutes and immediately place the tube on ice. Use the denatured adapter within 30 minutes of denaturation.

- Add 4 µl MM3 and incubate at 25 °C for ~1 ½ h.

	MM3 (Yellow)	**1x**	
1	ddH2O+0.1% Tween	1.95 µl	
2	NEB 5'Buffer	0.5 µl	
3	sRNA5' 10 µM hAdapter (-80)	0.3 µl	
	Vortex well the master mix and spin down		
4	NEB 5'Enzyme	1.25 µl	
	Invert vigorously and spin down		
	13µl reaction + 4µl MM3= 17µl	= 4 µl	

Reverse Transcription

- Add 5.25 µl of MM4 to ligation reaction. Incubate at 50°C for ~1h, then ice. Alternatively, you can incubate overnight with the following program:
1h 50°C, 5' 45°C, 55' 50°C, 5' 80°C and 12°C ∞

	MM4 (Red)	**1x**	
1	NEB First Strand B.	4.5 µl	
2	RT (i.e. Protoscript 2)	0.75µl	
	17µl reaction + 5.25µl MM4=22.25µl	=5.25 µl	

PCR: amplify to wanted quantity. From 11 to 14 cycles. (usually ~11)

1. Add 25.5 µl of the PCR mix to a new set of lids for the samples and 12.25 µl for the inputs.
2. Add 2 µl of the Barcode specifically to each sample.

Program: 94˚C for 3' then 11 cycles at 94˚C 45'' / 63˚C 30' / 70˚C 15'', Hold 5' at 70˚C, 4˚C ∞.

SAFE STOP POINT. Freeze samples at -20.

PCR (Blue)	**1x**	
100 µM 5' Primer	0.2 µl	
10 µM Barcode	2 µl	
Betaine	0.3 µl	
2x LongAmp PCR MasterMix	25 µl	

Size Selection and Gel Purification

1. Add 75 µl Beads (3 µl XP beads, 34 µl 5M NaCl, 37.5 µl 40% PEG) to csRNA PCR reaction and 37.5 µl to inputs samples. Vortex, spin and incubate 10 minutes at room temperature.
2. Place the samples in the magnetic rack, incubate until the sample is clear.
3. Pipette off the supernatant (contains the PEG)
4. Then wash twice with 200 µl of 80% EtOH, moving the samples from one side to the other side of the magnet about 5x-6x. Aspirate the supernatant. Spin one more time to remove all residual EtOH and dry very well. This step is critical for accurate size selection. Do not skip!
5. Elute in 15 µl 1x Loading buffer.
6. Run half of the elute on 12-well 10% TBE gel with 0.5 µl 25 bp ladder. Run input and & csRNA-seq sample next to each other, this is critical to size select the gel identically. Run the gel as long as possible, until the Xylene Cyanole is 5mm from the bottom edge. Adapter migrates with the lower edge of Xylene Cyanole. Conditions: 20 min at 80V, 75 mins at 180 V.

   While the gel run, prepare another set of LoBind 1.5ml tubes and label them.

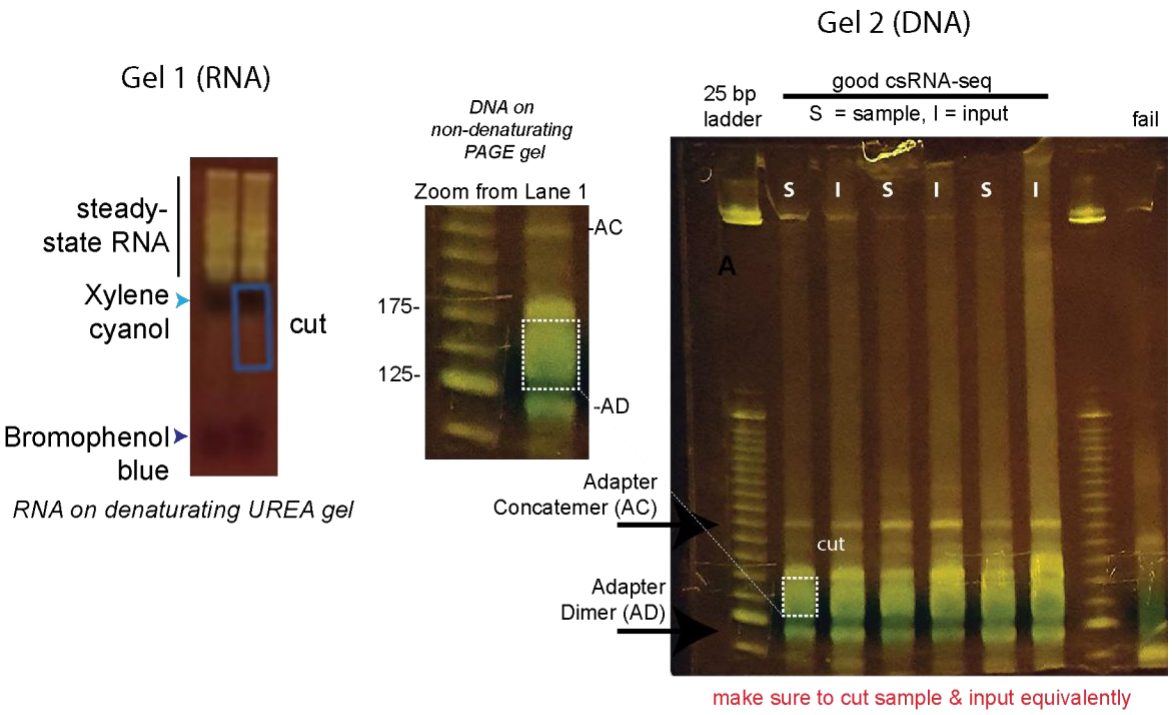
   Open the gel and place it on a square petri dish containing about 10 ml of 1xTBE and 1 µl of Syber Gold. Incubate few minutes and you are ready to cut.

   *Adapters are 124 bp. Cut 140-175bp (or a little higher but avoid the first steady state RNA seen on the RNA gel).
7. Add 150 µl Gel Elution Buffer and elute for a few hours or O/N on shaker. Cut the gel in 2 with a 20 µl pipette and ensure the gel is completely submerged on the buffer.
8. Add 750 µl ChIP DNA-binding buffer and pipette up and down and transfer the buffer into Zymogen Minelute columns.
9. Wash the column with 200 µl Wash Buffer, spin 20' at 4000g. Wash again with 200 µl WB, spin 60' at 14000g.
10. Elute in 20 µl warm *sequencing* TET [sTET: 10 mM Tris 8.0 0.1 mM EDTA 0.05% Tween].
11. Qubit for samples and prepare the document for sequencing.

Notes on csRNA-seq

- csRNA-seq/START-seq is a non-quantitative method as TSS strength depends on nuclei cleanliness.
- csRNA-seq also maps "degraded" RNA as CIP can do 3' repair. Thus TSS of stable and/or highly expressed mRNA's are enriched. 3' repair ("dephosphorylation) can be minimized by omitting CIP and using RNA polyphosphate & Terminator. However, note that there are still RNases & spontaneous 3' dephoshorylation events that contribute to "non-nascent" transcript substrate for the library prep.
- csRNA-seq, unlike 5'GRO can be utilized on fixed or frozen tissue
- csRNA-seq requires a higher input as 5'GRO and particularly 5'GRO-click.

Good luck ☺. Feedback/improvements welcome!



Gel1: Example gel of RNA size selection - 10 µg RNA were loaded on a 15% acrylamide 1xTBE 7M Urea gel and stained with CybrGreen for 5 minutes.

Gel2: Example gel of DNA size selection on 10% acrylamide 1xTBE with a 25bp ladder (prominent band = 125 bp).

Primer (from NEB sRNA kit – any other Illumina compatible primer would work, too!)

SR primer = i5
5´-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG-s-A-3´

Index1 (RPI RNA PCR Index primers) = i7
5´-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´

RT
5´-AGACGTGTGCTCTTCCGATCT-3´
5′ hAdapter
5´- GTTCAGAGTTCTACArGUrCrCrGrArCrGrAUrC-3´
3′Adapter
5´-rAppAGATCGGAAGAGCACACGTCT-NH2-3´ (or biotin)

Alignment:

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                                                    IndexPrimer
                                                    TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG<Index>TAGAGCATACGGCAGAAGACGAAC
                                                 <-TCTAGCCTTCTCGTGTGCAGA  RT
                                  GTTCAGAGTTCTACAGTCCGACGATC<insert>AGATCGGAAGAGCACACGTCT-NH2-3´
5´-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3´(SR primer)
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Library DNA (Adapters: 121bp)

AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC
TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG<Index>TAGAGCATACGGCAGAAGACGAAC

Solexa 1 GA (i5 or 5′ end)
5′-AATGATACGGCGACCACCGA
Solexa 1 GB (i7 or 3′/indexed end)
5′-CAAGCAGAAGACGGCATACGA

Buffers

2xFLB

	Stock	50ml
5 mM EDTA	0.5M	500 µl
95% Formamide		
0.1% Bromophenol		
0.1% Xelene cyanole		
H20		

csElution Buffer

	Stock	50ml
400mM NaOAc	3M	6.66 ml
0.05% Tween-20	10%	250 µl
1mM EDTA	0.5M	100 µl
10mM Tris pH 7.5	1M	500 µl
H20		42.5 ml

TET

	Stock	50ml
10mM **Tris pH 7.5**	1M	500 µl
1mM EDTA	0.5M	100 µl
0.05% Tween	10%	250 µl
H20		49.150

TE'T

	Stock	50ml
10mM **Tris pH 7.5**	1M	500 µl
0.1mM EDTA	0.5M	10 µl
0.05% Tween	10%	250 µl
H20		49.240 ml

Sequencing TET Buffer

	Stock	50ml
10mM Tris **pH 8**	1M	500 µl
0.1mM EDTA	0.5M	10 µl
0.05% Tween	10%	250 µl
H20		49.240

ddH_2O+0.05% Tween

	Stock	50ml
0.05% Tween	10%	250 µl
H20		49,750 ml

Gel Elution Buffer

	Stock	50ml
0.5M LiCl	8M	3.125 ml
0.1% SDS	20%	250 µl
5mM EDTA	0.5M	500 µl
10mM Tris pH 7.5	1M	500 µl
H20		45.625 ml

Elution buffer

Beads for size selection 75 µl Beads (3 µl XP beads, 34 µl 5M NaCl, 37.5 µl 40% PEG)

	Stock	1x = 75 µl
Beads XP	--	3 µl
NaCL	5M	34 µl
40%PEG	0.5M	37.5 µl

1xTBE

Dilute stock solution to 1x with milliQ water.

List of reagents:

Buffer reagents:

- UltaPure™ Destilled Water (Thermo Fisher Scientific, Cat. no. 10977015)
- Formamide (Millipore Sigma, Cat. no. F9037-100ML)
- Bromophenol Blue (Millipore Sigma, Cat. no. B0126-25G)
- Tris (1 M), pH 8.0, RNase-free, (Thermo Fisher Scientific, Cat. no. AM9855G)
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- Tris (1M), pH 7.5, RNase-free (thermo Fisher Scientific, Cat. no. AM9851)
- Xylene Cyanole FF (Bluish-Green Powder/Electrophoresis), (Fisher Scientific, Cat. no. BP565-10)
- 10x TBE 4L (BioPioneer, Cat. no. MB1044-4)
- Sodium Dodecyl Sulfate (SDS), White Powder, Electrophoresis, Fisher BioReagents™(Fisher Scientific, Cat. no. BP166-500)
- Lithium Chloride (Millipore Sigma, Cat. no. L4408-500G)
- Sodium Acetate (3 M), pH 5.5, RNase-free (Thermo Fisher Scientific, Cat. no. AM9740)
- Tween-20 viscous liquid (Sigma Millipore, Cat. no. P1379-100ML)
- NaCl (5 M), RNase-free (Thermo Fisher Scientific, Cat. no. AM9759)
- UltraPure™ 0.5M EDTA, pH 8.0 (Thermo Fisher Scientific, Cat. no. 15575020)
- UltraPure™ 1 M Tris-HCI Buffer, pH 7.5 (Thermo Fisher Scientific, Cat. no. 15567027)
- TRIzol™ Reagent (Invitrogen, Cat. No. 15596026)
- Chloroform:Isoamyl alcohol 24:1(Millipore Sigma, Cat. No. C0549-1QT)
- GlycoBlue™ Coprecipitant (15 mg/mL) (Thermofisher Scientific, Cat. No. AM9515)
- 1.7 tubes Safeseal Microcentrifuge Tubes, low binding, Soreson, Cat. No. 39640T
- 0.2ml PCR 8-tubes tubes and dome strip caps, USA scientific, Cat. No. 1402-2700
- Novex™ TBE-Urea Gels, 15%, 12 well, Invitrogen, Cat. No. #EC68852BOX
- Low Range ssRNA Ladder, (NEB, Cat. No. N0364S)
- GelGreen® Nucleic Acid Gel Stain, Biotium, Cat. No. 41005]

- Petri Dish square with Grid 100mm x 100mm, Fisherbrand, Cat. No. FB0875711A
- Gelbreaker Tubes, IST Engineering Inc, Cat. No. 3388-100
- Wide orifice p200 pipet tip (Fisherbrand, Cat. no. 02-707-465)
- UltraFree MC, 0.45 µm pore size, hydrophilic PVDF, 0.5ml volume, Millipore, Cat. No. UFC30HVNB
- GlycoBlue™ Coprecipitant (ThermoFisher, Cat. No. AM9516, 15 mg/mL).
- Ethanol absolute, KOPTEC (VWR, Cat. no. 89125-186)
- Terminator 5'-Phosphate-Dependent Exonuclease, Lucigen, Cat. No. TER51020
- SUPERase•In™ RNase Inhibitor (20 U/µL) (Thermofisher Scientific, Cat. No. AM2696)
- QuickCIP (New England Biolabs, Cat. No. M0525L)
- Trizol LS (Thermofisher scientific, Cat. No. 10296010)
- 2-Propanol (Sigma, Cat. No. I9516-25ML)
- NEBNext® Multiplex Small RNA Library Prep Kit for Illumina® (Index Primers 1-48), NEB, Cat. No. E7560S
- T4 RNA Ligase Reaction Buffer (New England Biolabs, Cat. no. B0216SVIAL)
- 50% PEG 80009 (New England Biolabs, Cat. no. B1004SVIAL)
- Betain Solution (Sigma Millipore Cat. no. B0300-1Vl)
- SpeedBead Magnetic Carboxylate Modified particles (Sigma, cat. no. 65152105050250)
- Poly (ethylene glycol) (Sigma, cat. no. P2139-500G)
- Hi-Density TBE Sample Buffer (5x) (Thermo Fisher Scientific, Cat. No. LC6678)
- Novex ™ TBE gel 10% (Thermo Fisher Scientific, Cat. No. EC62752BOX)
- 25 bp ladder (Invitrogen Cat. No. 10488-022) (Alternative Cat. No. 10488023)
- SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Cat. no. S11494)
- ChIP-DNA clean and concentrator (Zymo, Cat. no. D5205)

List of equipment:

- DynaMag 96 Side skirted (Thermo Fisher Scientific, Cat. no. 12027)
- Running gel system
- Shaker
- vortex
- PCR
- Heat block
- Centrifuge with fix angle
- Qubit
- Gel visualization
- -20, - 80
- Minicentrifuge
- Multichannel