RNAtag-Seq Experimental Protocol

Simultaneous generation of many RNA-seq libraries in a single reaction

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Updated versions of this protocol can be found by visiting the Guttman lab website at www.lncRNA.caltech.edu

RNAtag-seq Protocol for batch of 32 RNA samples

Reagents:

- Turbo DNase; Ambion/Applied Biosystems, Cat.# AM2239
- FastAP Thermosensitive Alkaline Phosphatase; Thermo Scientific, Cat.# EF0651
- RLT buffer (RNeasy Lysis Buffer); Qiagen, Cat.# 79216 (220mL)
- T4 RNA Ligase 1 (Custom Order from NEB, contact Kellie Dodd); 30,000U/mL (0.5mL) - 3x concentrated, normally 10,000U/mL, Cat.# MO 204L and comes with the following:
  - ATP (100mM); alternative vendor: Roche, Cat.# 11140965001 (aliquot and store at -80°C, always use fresh aliquot)
  - PEG 8000, 50% in water
- DMSO (100%); Sigma, Cat.# D8418-50ML for Molecular Biology
- RNase Inhibitor, Murine (40U/µL); NEB, Cat.# M0314S (3,000 units)
- SUPERase-IN 20U/µL (Ambion; AM2694; 2500U)
- AffinityScript Multiple Temperature cDNA Synthesis Kit, 50 reaction; Agilent, Cat.# 200436 – includes the dNTPs, 10x RT buffer, RNase Block Ribonuclease Inhibitor (40U/µL)
- 10X AffinityScript RT buffer; Agilent, Cat.# 600100-52
- RNA Clean & Concentrator™-5 columns; Zymo Research, Cat.# R1015 (50 preps)
- RNAClean XP beads; Agencourt/Beckman, Cat.# A63987 (40mL)
- Ribo-Zero:
  - Bacteria:
    Ribo-Zero™ Magnetic Gold Kit (Bacteria); Epicentre/Illumina, Cat.# MRZMB126
  - Human:
    Ribo-Zero™ Magnetic Gold Kit (Human/Mouse/Rat); Epicentre/Illumina, Cat.# MRZG126
- Sodium hydroxide solution (5M); Sigma-Aldrich, Cat.# 656046 Fluka
- Glacial acetic acid (17.4M); Sigma-Aldrich, Cat.# ARK2183
- Nuclease free water; Ambion, Cat.# 4387936
- 1x low TE (10mM Tris pH 8.0, 0.1mM EDTA); Affymetrix/USB Cat.#75793
- 2100 Electrophoresis Bioanalyzer Instrument; Agilent, Cat.# G2939AA
- Agilent RNA 6000 Pico Kit; Agilent, Cat.# 5067-1513
- QIAvac 24 Plus Vacuum manifold (1-24 spin columns; Qiagen cat# 19413)

Plates/Tubes/Caps:

- Flat PCR 12-cap strips, Optically clear; USA Scientific, Cat.# 1400-3120
- TempPlate No-skirt PCR Plates, 96 wells, 10 plates; USA Scientific, Cat.# 1402-9608
- MaxyClear; Maxymum Recovery; 0.2mL; Flat cap (Axygen, cat#PCR-02-L-C)

Procedure:

1. Quality and quantity of RNA with Agilent Bioanalyzer

- Check RNA quality by running on the Agilent Bioanalyzer
- Calculate up to 400ng and place in a tube.
- Increase the volume to 15µL with Nuclease free water
- Add 1uL of SUPERase-IN (20U/µL)

- Final total volume = 16µL (25ng/µL)
- Continue to next step or freeze at -80 until ready to process samples

2. Fragmentation and DNase –FastAP combination step

2.1. Fragmentation using 2x FastAP buffer

- Set up the following reaction:

Reagents	1 reaction	Final conc
FastAP buffer (10x)	4 µL	2x
RNA (from step 1.)	16 µL	up to 500ng total RNA
Total vol (µL)	20 µL	

- Add 4uL of FastAP buffer to the RNA and mix well.
- Incubate on pre-heated thermal cycler for **3 min at 94°C**
    a. **If RNA is partially degraded (RIN<7), fragment 3min at 92°C – prevents over-fragmenting samples**
- Place on ice

2.2. DNase and FastAP treatment

- Set up the following reaction mix

Reagents	1 reaction	Final
fragmented RNA (from step 2.1)	20 µL	1x
RNase Inhibitor, Murine (40U/µL)	1 µL	10U
TURBO DNase (2U/µL)	4 µL	8 U
FastAP (1U/µL)	10 µL	10U
Nuclease free water	5 µL	
Total vol (µL)	40 µL	

- Make enzyme/water master mix and mix well
- Aliquot 20 µL into each tube/well with the fragmented RNA
- Mix well
- Incubate on preheated thermal cycler for 30 min at 37°C

2.3. SPRI (2x)

- Increase reaction volume to 80 µL with nuclease free water **(add 40 µL water/reaction)**
- Add 2.0x reaction volume of Agencourt RNAClean XP beads **(160 µL)** and follow manufacturer's protocol to capture RNA on beads: Briefly
    - Incubate at room temperature for 15min to bind RNA
    - Place on magnet for 5 min or until solution is clear
    - Aspirate out and discard clear solution
    - Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30sec
    - Aspirate out and discard the clear solution
    - Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30sec
    - Aspirate out and discard the clear solution and let air dry for 10min
- Elute off beads with **12 µL nuclease free water**
- Take 5 µL of each sample and proceed to 1st ligation
- **QC: Save 1.2 µL from remaining RNA before addition of SUPERase-IN**
  - Run 11 random samplings on Agilent RNA pico chip to check the fragmentation profile of each batch of 32

- Add 1uL SUPERase-IN (20U/µL) to the remaining material and store at -80°C.
  - this can be used as back up if it is necessary to repeat process

3. **FIRST LIGATION (RNA/DNA) 3' Linker Ligation**
NOTE: Set up RNA/barcoded adapter in single tube or use the TempPlate No-skirt PCR Plates for batched samples (these rigid plates are easy to handle and can mix well by flicking by hand) – with a razor, cut a column (for 8 samples) or row (for 12 samples) and use as strip and cover with the Flat PCR 12-cap strips (these strips fit tightly on these plates and will not leak)

- Add 1 µL of barcoded adapter (100 pmole*) to 5 µL of dephosphorylated RNA (*100 pmole =1 µL of 100µM; **barcoded adapters need: 5'-P and 3' ddC (or 3'-C3 spacer)**
- **Heat at 70°C for 2 min** →place **in cold block** on ice
- Set up Ligation mix below
  NOTE:
  - Make up mix at **room temp** so the reagents don't start precipitating when combined (if DMSO is added directly into cold buffer it will precipitate)
  - Pipette very slowly for accurate aspiration of PEG (very viscous)
  - When setting up mix for multiple reactions include 25% extra to account for pipetting error due to the viscosity of the PEG in the mix
  - All reagents except enzymes (-20°C) should be stored at -80°C in single use aliquots:

Ligation Mix (set up at room temp)	1 reaction	40 reaction mix (for set of 32)
10× T4 RNA Ligase Buffer	2 µL	80 µL
DMSO (100%)	1.8 µL	72 µL
ATP (100 mM)	0.2 µL	8 µL
PEG 8000 (50%)	8 µL	320 µL
20-50ng of Repaired RNA (tested up to 250ng)	5	
Barcoded Adapter, 100 pmoles (1µL of 100 µM stock)	1 (5µM final)	
RNase inhibitor, Murine (40U/µL)	0.3 µL	12 µL
T4 RNA Ligase 1 (30,000U/mL)	1.8 µL	72 µL
Total	20.1 µL	564 µL

- **Mix really well by flicking tube since the solution is very viscous,**
- **Quick spin of mastermix tube**
- Add 14.1 µL of ligation mix to each well containing 6 µL denatured RNA + adapter
- **Mix well MANY times; mix by flicking since the solution is very viscous**
  (If setting this up on a robot, the reaction should be mixed up/down for 5-10min with low retention tips to mix well!)
- Incubate at 22°C (room temp) for 1 hour 30 minutes

4. Pooling step: **RLT buffer + Zymo column**
   - Maximum binding capacity of columns is 5 µg; do not exceed when pooling samples
   - Add 60 µL of **RLT buffer** to each sample to inhibit ligase and mix well (80 µL total)
   - Pooling and clean up using Zymo Clean & Concentrator™-5 column (200nt cut off)
     - Follow manufacturer's 200nt cut off protocol
     - Add 2x reaction vol (160 µL=2x 80 µL) of 1:1 binding buffer: EtOH (100%) / reaction

NOTE: When pooling multiple samples (>700 µL) onto Zymo column use a vacuum manifold then proceed to centrifugation steps according to the manual

- Elute 2 times with 16 µL nuclease free water for a total volume of 32 µL
  NOTE: 2 elutions help improve recovery/yield of RNA
- **Optional: Save 2 µL for QC-** Run on Agilent RNA pico chip to check the fragmentation profile

5. **Quick Protocol for Ribo-Zero™**
   See Appendix (bacterial kit)

6. **First Strand cDNA**
   - Take **12 µL** rRNA depleted RNA (use all the material from above)
   - Add **2 µL** (50 pmoles) of AR2 primer (25 µM)
     - 5’ TAC ACG ACG CTC TTC CGA T 3’ — AR2 – 53% GC, 19bp,
   - Mix well
   - **Heat the mixture to 70°C for 2 min and immediately place on ice** (on cold block on ice)
   - Make RT mix below: For multiple samples, a mastermix can be prepared ahead of time and added to the RNA/AR2 tube on ice.

   Add (in order on ice):

RT Mix	1 reaction
10× AffinityScript RT Buffer	2 µL
DTT (0.1M)	2 µL
25mM dNTP Mix (25mM each)	0.8 µL
RNase inhibitor, murine (40U/µL)	0.4 µL
AffinityScript RT Enzyme	0.8 µL
rRNA depleted RNA + AR2 primer	14 µL
Total	20 µL

   - Add 6 µL of RT mix to the 14 µL rRNA depleted RNA + AR2 RT primer on ice
   - Mix well and spin for 5 sec
   - Place in **HOT** (55°C) incubator or thermocycler. **Incubate at 55°C for 55 minutes**

7. **RNA degradation after Reverse Transcription**
   NOTE: make fresh working stock solutions of NaOH and Acetic Acid

   - Add 10% reaction vol. of 1N NaOH **(2 µL)** to each reaction
   - Incubate at 70°C for 12 minutes
   - Neutralize with 4 µL of 0.5M Acetic Acid**; mix well**
   - Total volume = 26 µL

8. **Reverse Transcription primer cleanup (2x SPRI)**
   - Add 14 µL of sterile water to each reaction for a final volume of 40 µL
   - **Transfer to new tubes** (NaOH will start degrading tubes)
   - Add 2x reaction volume RNAClean XP beads (80µL) to the sample in new tubes, and mix up/down 15x

- Incubate at room temperature for 15min
- Place on magnet for 5 min or until solution is clear
- Aspirate out and discard clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30sec
- Aspirate out and discard the clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30sec
- Aspirate out and discard the clear solution and let air dry for 10min
- Elute off beads with 5 µL RNase/DNase free water **- DO NOT TRANSFER TO NEW TUBES AND KEEP BEADS**

9. Second LIGATION (ssDNA/ssDNA) 3' Linker Ligation with beads

-3Tr3 adapter: 5' AGATCGGAAGAGCACACGTCTG 3' = 55% GC, 22bp; Needs: 5'-P and 3' ddC (or 3'-C3
spacer)

- Add 2 µL of 3Tr3 adapter to cDNA
- Heat at 75°C for 3 min; Place on cold block on ice
- Make ligation reaction mix: For multiple samples, a mastermix can be prepared ahead of time and added to the cDNA/3Tr3 tube on ice.

2nd Ligation Mix	1 reaction
10× T4 Ligase Buffer	2 µL
DMSO (100%)	0.8 µL
ATP (100 mM)	0.2 µL
PEG 8000 (50%)	8.5 µL
cDNA	5 µL
3Tr3 adapter, 80 pmoles (2 µL of 40 µM stock)	2 µL (4 µM final)
T4 RNA Ligase 1 (30,000U/mL)	1.5 µL
Total	20 µL

- Swirl the cDNA/beads/water with pipet tip PRIOR to dispensing 13 µL ligation mix
- Mix well by pipetting up and down ~10x or cap tubes and flick several times; solution is viscous
- Quick spin (low speed centrifuge, to get everything to bottom of tube)
- Incubate overnight at 22°C

10. SPRI clean up (2x): Add fresh SPRI beads

- Increase to 2x reaction volume with water (to dilute out viscous ligation reaction)
- Add 2x reaction vol. (80 µL) of fresh SPRI beads
- Mix up/down 15x
- Let sit at room temperature for 15 minutes
- Place on magnet 5 min or until solution is clear and aspirate and discard clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Let air dry for 10min

- Elute off beads by pipetting 25 µL RNase/DNase free water and transfer to new tube.

11. 2nd SPRI clean up (1.5x) to remove the remaining adapters

- Add 1.5x reaction volume RNAClean XP beads
  25 µL x 1.5 = 37.5 µL SPRI /reaction
- Mix up/down 15x
- Let sit at room temperature for 15 minutes
- Place on magnet 5min (or until solution is clear). Aspirate out and discard clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Add 200 µL fresh 70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Let air dry for **2-3** min
- Elute with 25 µL RNase/DNase free water

12. PCR Enrichment-TEST to determine final cycle number

- Set up a test PCR using 5 µL of ss cDNA sample and 9-12 cycles of PCR **(based on experience with pool of 16 reactions, each starting with ~400ng total RNA)**
- Include a negative control for each primer set

  - Make a mix consisting of:

PCR Mix	1 reaction
water	15.4 µL
10x AccuPrime PCR buffer 1	2.5 µL
Primer 1 (2P_univP5, 12.5 µM)	1 µL
Primer 2 (2P_barcode, 12.5 µM)	1 µL
cDNA	5 µL
AccuPrime HiFi Taq (5U/µL)	0.1 µL
Total	25 µL

  - Mix well
  - Aliquot 18 µL / well
  - Add 2 µL total of primer to each well
  - Add 5 µL of ss cDNA or water (for negative control)
  - Mix well and aliquot 8ul into 1 well of the 384 well plate(s) x 3 plates
  - Place each place in a thermal cycler with the following conditions

    Cycling conditions:
    1 cycle: 98°C, 3min
    cycles**:** 9, 12, 15 cycles (for test PCR)
    98°C, 30sec
    55°C, 30sec
    65°C, 60sec
    1cycle: 65°C, 10min
    4°C, hold

13. SPRI clean up (1.5x):

- Increase to 25uL volume with sterile water
- Add 1.5x AMPure beads (37.5uL), and mix up/down 15x
- Incubate at RT for 15min
- Place on magnet for 5min or until solution is clear

- Aspirate out and discard clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec.
- Aspirate out and discard the clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution and let air dry for 10min
- Elute off beads with 10 µL 1x low TE (10 mM Tris, 0.1M EDTA)

14. QC test enrichments on Agilent DNA HS chip

-Based on test results change the cycle number, if necessary, and set up more reactions to provide enough material to send for sequencing

15. PCR for Sequencing library

- **Choose the optimal PCR cycle # based on Bioanalyzer QC and amplify**
- Make a PCR mix consisting of:

PCR Mix	1 reaction
water	30.8 µL
10x AccuPrime PCR buffer 1	5 µL
Primer 1 (2P_univP5, 12.5 µM)	2 µL
Primer 2 (2P_barcode, 12.5 µM)	2 µL
cDNA	10 µL
AccuPrime HiFi Taq (5U/µL)	0.2 µL
Total	50 µL

- Mix well and aliquot 8ul into 6 wells of a 384 well plate (amplification is more robust in smaller volumes)
- Place each place in a thermal cycler with the following conditions

Cycling conditions:
1 cycle: 98°C, 3min
cycles: # from test PCR
98°C, 30sec
55°C, 30sec
65°C, 60sec
1cycle: 65°C, 10min
4°C, hold

16. SPRI clean up (1.5x):

- Pool PCR reaction and increase to 2x reaction volume with sterile water
- Add 1.5x AMPure beads, and mix up/down 15x
- Incubate at RT for 15min
- Place on magnet for 5min or until solution is clear
- Aspirate out and discard clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution and let air dry for 10min
- Elute off beads with 25 µL sterile water

17. Final SPRI clean up (0.7x):

- Add 0.7x AMPure beads (17.5uL), and mix up/down 15x
- Incubate at RT for 15min

- Place on magnet for 5min or until solution is clear
- Aspirate out and discard clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution
- Add 200 µL fresh70% EtOH without removing from magnet and incubate for 30 sec
- Aspirate out and discard the clear solution and let air dry for 10min
- Elute off beads with 25 µL 1x low TE (10 mM Tris, 0.1M EDTA)

Ordering Barcoded Oligos: (regular desalting is sufficient)

- The barcoded oligos and 3Tr3 second adapter oligo require a 5' phosphate and 3' blocking group (either a 3'-spacer C3 or ddC)
  e.g., 3Tr3: 5'-/5Phos/AGA TCG GAA GAG CAC ACG TCT G/3SpC3/-3'
- No special modifications for the AR2 oligo (regular desalting):
  - 5' TAC ACG ACG CTC TTC CGA T 3'

Set of 32 DNA barcoded oligos:

barcode sequence (5'-3')	oligo	**barcode read**
ACATTATT	AACATTATTAGATCGGAAGAGCGTCGTGTA	**AATAATGT**
AAGTGTTG	AAAGTGTTGAGATCGGAAGAGCGTCGTGTA	**CAACACTT**
AGAATTAT	AAGAATTATAGATCGGAAGAGCGTCGTGTA	**ATAATTCT**
ATATGGAC	AATATGGACAGATCGGAAGAGCGTCGTGTA	**GTCCATAT**
ATCACTTG	AATCACTTGAGATCGGAAGAGCGTCGTGTA	**CAAGTGAT**
CCAAGTCG	ACCAAGTCGAGATCGGAAGAGCGTCGTGTA	**CGACTTGG**
CAACTCGC	ACAACTCGCAGATCGGAAGAGCGTCGTGTA	**GCGAGTTG**
CCCGTCTT	ACCCGTCTTAGATCGGAAGAGCGTCGTGTA	**AAGACGGG**
CCCTACAG	ACCCTACAGAGATCGGAAGAGCGTCGTGTA	**CTGTAGGG**
CCCTCGGC	ACCCTCGGCAGATCGGAAGAGCGTCGTGTA	**GCCGAGGG**
CCGGTACC	ACCGGTACCAGATCGGAAGAGCGTCGTGTA	**GGTACCGG**
CGGAGGGC	ACGGAGGGCAGATCGGAAGAGCGTCGTGTA	**GCCCTCCG**
CTCGGTAC	ACTCGGTACAGATCGGAAGAGCGTCGTGTA	**GTACCGAG**
CGGCACTT	ACGGCACTTAGATCGGAAGAGCGTCGTGTA	**AAGTGCCG**
CTCTAACT	ACTCTAACTAGATCGGAAGAGCGTCGTGTA	**AGTTAGAG**
CTGGATCG	ACTGGATCGAGATCGGAAGAGCGTCGTGTA	**CGATCCAG**
GCAGCCAC	AGCAGCCACAGATCGGAAGAGCGTCGTGTA	**GTGGCTGC**
GAGATTGT	AGAGATTGTAGATCGGAAGAGCGTCGTGTA	**ACAATCTC**
GAGCCATC	AGAGCCATCAGATCGGAAGAGCGTCGTGTA	**GATGGCTC**
GTAACTGC	AGTAACTGCAGATCGGAAGAGCGTCGTGTA	**GCAGTTAC**
GGCCCAAG	AGGCCCAAGAGATCGGAAGAGCGTCGTGTA	**CTTGGGCC**
GTCTGGCG	AGTCTGGCGAGATCGGAAGAGCGTCGTGTA	**CGCCAGAC**
GGTCCTCT	AGGTCCTCTAGATCGGAAGAGCGTCGTGTA	**AGAGGACC**

TCATCGTG	ATCATCGTGAGATCGGAAGAGCGTCGTGTA	**CACGATGA**
TACAACAT	ATACAACATAGATCGGAAGAGCGTCGTGTA	**ATGTTGTA**
TCCCGCGG	ATCCCGCGGAGATCGGAAGAGCGTCGTGTA	**CCGCGGGA**
TACAGATG	ATACAGATGAGATCGGAAGAGCGTCGTGTA	**CATCTGTA**
TACCGGCC	ATACCGGCCAGATCGGAAGAGCGTCGTGTA	**GGCCGGTA**
TTACCACG	ATTACCACGAGATCGGAAGAGCGTCGTGTA	**CGTGGTAA**
TGAACCAG	ATGAACCAGAGATCGGAAGAGCGTCGTGTA	**CTGGTTCA**
TGGGAGAC	ATGGGAGACAGATCGGAAGAGCGTCGTGTA	**GTCTCCCA**
TTTCTAAC	ATTTCTAACAGATCGGAAGAGCGTCGTGTA	GTTAGAAA

Set of 54 RNA barcoded oligos:

Barcode	Sequence	6 base Barcode Read + T
rUrU rGrCrU rU	/5Phos/rArUrU rGrCrU rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	AAGCAAT
rUrG rArArU rU	/5Phos/rArUrG rArArU rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	AATTCAT
rArC rUrUrG rU	/5Phos/rArArC rUrUrG rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ACAAGTT
rGrG rCrUrG rU	/5Phos/rArGrG rCrUrG rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ACAGCCT
rUrC rArGrG rU	/5Phos/rArUrC rArGrG rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ACCTGAT
rArU rUrArG rU	/5Phos/rArArU rUrArG rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ACTAATT
rUrU rGrArG rU	/5Phos/rArUrU rGrArG rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ACTCAAT
rUrG rGrUrC rU	/5Phos/rArUrG rGrUrC rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	AGACCAT
rGrU rUrUrA rU	/5Phos/rArGrU rUrUrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATAAACT
rArU rGrUrA rU	/5Phos/rArArU rGrUrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATACATT
rArU rUrGrA rU	/5Phos/rArArU rUrGrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATCAATT
rGrU rGrGrA rU	/5Phos/rArGrU rGrGrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATCCACT
rGrA rArGrA rU	/5Phos/rArGrA rArGrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATCTTCT
rGrG rUrCrA rU	/5Phos/rArGrG rUrCrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATGACCT
rGrA rGrCrA rU	/5Phos/rArGrA rGrCrA rUrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	ATGCTCT
rCrA rGrUrU rG	/5Phos/rArCrA rGrUrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CAACTGT
rGrG rUrGrU rG	/5Phos/rArGrG rUrGrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CACACCT
rGrA rGrGrU rG	/5Phos/rArGrA rGrGrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CACCTCT

rUrU rCrGrU rG	/5Phos/rArUrU rCrGrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CACGAAT
rUrC rArGrU rG	/5Phos/rArUrC rArGrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CACTGAT
rArA rGrCrU rG	/5Phos/rArArA rGrCrU rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CAGCTTT
rGrA rCrUrG rG	/5Phos/rArGrA rCrUrG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCAGTCT
rArU rCrGrG rG	/5Phos/rArArU rCrGrG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCCGATT
rArG rUrCrG rG	/5Phos/rArArG rUrCrG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCGACTT
rUrA rUrArG rG	/5Phos/rArUrA rUrArG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCTATAT
rCrG rGrArG rG	/5Phos/rArCrG rGrArG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCTCCGT
rCrU rArArG rG	/5Phos/rArCrU rArArG rGrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	CCTTAGT
rGrU rGrGrU rC	/5Phos/rArGrU rGrGrU rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GACCACT
rGrG rGrArU rC	/5Phos/rArGrG rGrArU rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GATCCCT
rGrA rGrUrG rC	/5Phos/rArGrA rGrUrG rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GCACTCT
rArU rGrGrG rC	/5Phos/rArArU rGrGrG rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GCCCATT
rGrU rArUrC rC	/5Phos/rArGrU rArUrC rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GGATACT
rGrU rUrGrC rC	/5Phos/rArGrU rUrGrC rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GGCAACT
rGrA rGrGrC rC	/5Phos/rArGrA rGrGrC rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GGCCTCT
rCrA rArGrC rC	/5Phos/rArCrA rArGrC rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GGCTTGT
rGrG rGrUrA rC	/5Phos/rArGrG rGrUrA rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GTACCCT
rGrC rArArA rC	/5Phos/rArGrC rArArA rCrArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	GTTTGCT
rUrG rArUrU rA	/5Phos/rArUrG rArUrU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TAATCAT
rArC rUrGrU rA	/5Phos/rArArC rUrGrU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TACAGTT
rUrA rUrGrU rA	/5Phos/rArUrA rUrGrU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TACATAT
rCrA rGrGrU rA	/5Phos/rArCrA rGrGrU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TACCTGT
rGrU rCrGrU rA	/5Phos/rArGrU rCrGrU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TACGACT
rArG rUrArU rA	/5Phos/rArArG rUrArU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TATACTT
rGrU rGrArU rA	/5Phos/rArGrU rGrArU rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TATCACT
rUrU rUrUrG rA	/5Phos/rArUrU rUrUrG rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TCAAAAT

rGrA rUrGrG rA	/5Phos/rArGrA rUrGrG rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TCCATCT
rArC rArGrG rA	/5Phos/rArArC rArGrG rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TCCTGTT
rGrG rUrUrC rA	/5Phos/rArGrG rUrUrC rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TGAACCT
rArU rGrUrC rA	/5Phos/rArArU rGrUrC rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TGACATT
rArU rArGrC rA	/5Phos/rArArU rArGrC rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TGCTATT
rGrG rArCrC rA	/5Phos/rArGrG rArCrC rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TGGTCCT
rGrU rArArC rA	/5Phos/rArGrU rArArC rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TGTTACT
rCrG rGrGrA rA	/5Phos/rArCrG rGrGrA rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TTCCCGT
rGrC rGrGrA rA	/5Phos/rArGrC rGrGrA rArArG rArUrC rGrGrA rArGrA rGrCrG rUrCrG rUrGrU rArG/3SpC3/	TTCCGCT

Library Enrichment Primers (regular desalting):

Enr_P5_RNAtag: 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

P7 Enrichment Primer Sequence (5' --> 3') with barcode	barcodes (BC)	BC READ (reverse complement)
CAAGCAGAAGACGGCATACGAGATTCGTGTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TCGTGTGC	GCACACGA
CAAGCAGAAGACGGCATACGAGATTCGCCAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TCGCCAGA	TCTGGCGA
CAAGCAGAAGACGGCATACGAGATTCGCTATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TCGCTATG	CATAGCGA
CAAGCAGAAGACGGCATACGAGATGGCTCCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GGCTCCTG	CAGGAGCC
CAAGCAGAAGACGGCATACGAGATATCCGACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ATCCGACA	TGTCGGAT
CAAGCAGAAGACGGCATACGAGATAACATAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	AACATAAT	ATTATGTT
CAAGCAGAAGACGGCATACGAGATATGGTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ATGGTAGG	CCTACCAT
CAAGCAGAAGACGGCATACGAGATGCTAAGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GCTAAGTA	TACTTAGC

APPENDIX:

A. Modifications of RNAtag-Seq protocol used in generation of mouse data

10ug of total RNA from mouse ES cells was fragmented using Ambion Fragmentation reagent (Invitrogen) for 2 min and 30 sec at 70°C and placed on ice. Fragmented RNA was repaired for 35min at 37°C in 400ul of FNK buffer (50mM Tris, 50mM KCl, 5mM $MgCl_2$, 5.5mM DTT and 0.01% Triton X-100) containing 8ul of Murine RNAse inhibitor (NEB), 18ul of T4 PNK (NEB) and 18ul of FastAP (Fermentas). RNA was cleaned using the Zymo RNA concentrator-5 kit (Zymo Research). 20ng of repaired RNA was ligated to 20 pmoles unique RNA barcodes (5'-P and 3'-SpC3 modifications) in separate wells. After ligation all RNA samples were pooled and cleaned using the Zymo RNA concentrator-5 kit (Zymo Research). cDNA was synthesized using

10 pmoles of AR23s primer from IDT: 5′ – /5SpC3/CCTACACGACGCTCTTCC – 3′ and AffinityScript Reverse Transcriptase (Agilent) at 54°C for 50min. RT primer AR23s was digested after RT using ExoSAP-IT (Affymetrix), RNA was degraded and cDNA was cleaned using Silane beads (Invitrogen). The 3'-end of cDNA was ligated with 3Tr3 adapter overnight. cDNA was amplified using the NEBNext PCR module (NEB) and indexed primers (IDT).

B. Quick Protocol for Ribo-Zero™ Magnetic Kit (Bacteria) – For pools containing 1-5µg of total RNA. For lower input, consult Epicentre for low input protocol.

For experience users only! – see manual for more detailed protocol

Step	Procedure
Prepare Magnetic Beads	Add 225 µl Magnetic Beads to RNase-free tube Magnetize for 1 min at RT Wash with 225 µl RNase-Free Water Magnetize, repeat wash step Resuspend in 65 µl Resuspension Solution Optional: Add 1 µl RiboGuard RNase Inhibitor
Treat sample with rRNA Removal Solution	Mix in 40 µl total volume: See manual 1-5 µg total RNA (1-2.5 µg use **28 µL**; >2.5-5 µg use **26 µl**) rRNA Removal Solution (1-2.5 µg use **8 µL**; >2.5-5 µg use **10 µl**) 4 µl Reaction Buffer Incubate 10 min @ 68˚C, then 5 min @ RT
Remove rRNA	Mix previously prepared Magnetic Beads Add RNA mixture, mix well by pipetting, vortex briefly Incubate 5 min @ RT, vortex Incubate 5 min @ 50˚C Magnetize, transfer supernatant (rRNA-depleted sample) to RNase-free tube (~**90 µL**)
Purify rRNA-depleted sample	**SPRI cleanup (1.8x):** • Add 160 µL of AMPure XP beads containing 85-90 µL of rRNA-depleted RNA. Pipette entire volume up/down 10x • Incubate at RT for 15min • Place tubes on magnet for at least 5min (until liquid appears clear) • Remove and discard supernatant. Take care not to disturb the beads. Keep tubes on magnet • Add 200 µL freshly prepared 80% EtOH without disturbing beads, and incubate at RT for 30sec • Remove and discard all of the supernatant from tube, take care not to disturb the beads • Repeat EtOH wash (total of 2, 80% EtOH washes) • Air dry on magnet at RT for 15min • Elute off beads by mixing **14µL** of RNase/DNase free water