RNA Tag-Seq Library Preparation

Protocol by the Broad Institute's Microbial 'Omics Core (https://www.broadinstitute.org/moc)

Version 1.3a

Process Overview:

The purpose of this document is to serve as a standard operating procedure for making RNAtag-Seq libraries. The protocol takes 2-3 days to complete.

General Safety Precautions:

This SOP does not cover detailed safety procedures for handling of specific chemical, infectious, or biological materials. It is recommended that personnel follow all appropriate institutional safety guidelines and always observe universal precautions when working with samples.

Materials:

Equipment:

Item	Vendor	Model
96-well PCR block and/or incubator	any	
384-well PCR block	any	
Pipettes and Tips (P10, P200, P1000, Multichannel)	any	
Centrifuge for 96-well plate	any	
Vacuum manifold	any	
4200 TapeStation System 2100 Bioanalyzer Instrument OR Any agarose gel electrophoresis system	Agilent	G2991AA G2939BA

Supplies:

Item	Vendor	Catalog Number
50mL Nuclease-free reservoir	any	any
Nuclease-free water	any	any
Aluminum Seal, 50 µm	Axygen	PCR-AS-200
PCR 96-well Plate	Axygen	PCR96M2
PCR 384-well Plate	Eppendorf	951020702
96-well U-bottom SPRI plates	Sigma or Takara Bio	M2186-100EA 740672

Reagents:

Item	Vendor	Catalog Number
FastAP Buffer	ThermoFisher Sci	EF0652
Turbo DNase	ThermoFisher Sci	AM2239
SUPERase-IN	ThermoFisher Sci	AM2694
RNAClean XP	Beckman Coulter	A63987
T4 RNA Ligase 1	New England Biolabs	M0204S
**RiboZero	Illumina	MRZY1306, MRZG12324, MRZB12424
SMARTscribe RT	Clontech	639538
DTT	Any	
RNA Clean & Concentrator-25	Zymo Research	R1017
Accuprime	ThermoFisher Sci	12346086
DMSO	Any	
Mol. Biol. Grade Ethanol, absolute	Any	
ATP (100mM)	Any	
Exonuclease I	New England Biolabs	M0293L
Ampure XP	Beckman Coulter	A63881
RLT Buffer	Qiagen	79216
RNA High Sensitivity Kit	Agilent	5067-5579 & 5067-5580
D1000 High Sensitivity Kit	Agilent	5067-5584 & 5067-5585

**RiboZero catalog numbers reference a discontinued product line from Illumina. See the corresponding rRNA depletion methods section below for details.

Primers:

Vendor	Name	Concentration	Sequence
Any	Inline Barcodes	100uM	/5Phos/ATGTGCTGCGAGAAGGCTGAC[8bp index]A/3SpC3/
Any	5'bioAR2	100uM	/5Biosg/TACACGACGCTCTTCCGAT
Any	3Tr3 TSO	100uM	/5Me-isodC//iisodG//iMe-isodC/CAGACGTGTGCTCTTATCTrGrGrG
Any	P7 Enrichment	12.5uM	CAAGCAGAAGACGGCATACGAGAT[8bp index]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Any	P5 Enrichment	12.5uM	AATGATACGGCGACCACCGAGATCTACAC[8bp index]ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Procedure:

1) Setup and Fragmentation

For normalization of RNA input and fragmentation of RNA to 150-350bp

Decide on a pooling strategy:

It is recommended that pools contain between 12-36 samples, and that all members of a treatment group or time series stay in the same pool to reduce batch effects. It is also recommended that replicates are placed in the same pool. However, if treatment group size exceeds the recommended pool size, split the group by replicate (e.g. Sample X replicate 1 goes to Pool 1 and Sample X replicate 2 goes to Pool 2). Once the samples are appropriately divided to pools, assign each sample a unique inline barcode (added during ligation) and ensure no duplication of inline barcodes within the same pool. Overlapping inline barcodes between pools is fine as each pool will have its own unique index added during cDNA enrichment.

Other considerations: For samples treated with compounds that inhibit transcription, it is advisable to increase the standard input from 0.5µg total RNA to 1µg total RNA.

Fragmentation of Total RNA:

1. Normalize the total RNA to 31.25 ng/µl, and place 16µL of normalized total RNA (500ng total) into a 96-well PCR plate.
2. Add 4µL of 10X FastAP buffer into each tube, mix by pipette, and seal.
3. Briefly spin the tubes/plates at 1000xg to collect any droplets.
4. Incubate tubes at 95℃ for 90 seconds and then place on ice immediately to prevent over fragmentation of the RNA.

2) End Repair

After fragmentation, not all ends are suitable for ligation. FastAP removes 3' and 5' phosphate groups, allowing for the ligation of 5' phosphorylated inline barcodes to 3' dephosphorylated RNA ends. DNase digests any contaminating DNA present in the RNA sample

Setup the End Repair Master Mix:

End Repair Master Mix	
Reagent	**Per Sample (µL)**
FastAP (1 U/µL)	10
Turbo DNase (2U/µL)	4
SUPERase-IN (20U/µL)	1
Nuclease Free Water	5
Fragmented RNA	20

Repair damaged ends:

1. Add 20µL of the end repair master mix to the fragmented RNA, mix by pipette, and seal.
2. Briefly spin the tubes/plates at 1000xg to collect any droplets.
3. Incubate the samples at 37℃ for 30 minutes.
4. Once the end repair is complete, proceed immediately to SPRI clean-up

2X SPRI Clean-up

1. Transfer samples to a new 96-well U-bottom plate.
2. Add 80µL of RNAClean XP beads to the samples and pipette to mix.
3. Let samples rest at room temperature for 15 minutes to allow the RNA to bind.
4. While samples incubate, prepare 500µL of fresh 70% ethanol per sample.
5. Place on the magnet for 5 minutes or until the solution clears.
6. Remove the supernatant.
7. Add 200µL of 70% ethanol to wells without disturbing the pellets and let stand for 30 seconds.
8. Remove the supernatant.
9. Repeat steps 7 & 8.
10. Let the beads dry for ~5 minutes or until the pellets have a dry, matte finish (**but not cracked**).
11. Add 14µL of nuclease free water and fully resuspend the dried pellets.
12. Place on the magnet until the solution is clear.
13. Transfer 10µL of the supernatant (cleaned RNA) to a new PCR plate.
14. Aliquot 5µL of the cleaned RNA to another stip-cap tube or plate and store at -80℃ as backup

3) Inline Barcoded Adapter Ligation

Phosphorylated, barcoded DNA adapters are ligated onto RNA fragments by T4 RNA Ligase 1. Unique barcodes are used for each sample within a pool. The ligation process occurs in the presence of high PEG concentrations which facilitate the RNA-oligo interaction

Prep the RNA and adapters for ligation:

1. Review the inline barcoding and pooling plan from Section 1.
2. Transfer 1µL of the appropriate barcoded adapters to the corresponding wells containing 5µL of the clean, end-repaired RNA.
3. Seal the PCR plate and incubate at 70℃ and then immediately return to ice

Setup the End Repair Master Mix:

Ligation Master Mix	
Reagent	**Per Sample (µL)**
10x NEB buffer	2
DMSO (100%)	1.8
ATP (100 mM) *thaw at RT	0.2
PEG 8000 (50%)	8
SUPERase-IN (20U/µL)	0.3
T4 RNA ligase 1	1.8
RNA + Inline BC	6

1. Add the first four reagents in order. Mix by pipetting after each addition.
2. Add each enzyme to the wall of the tube and let it slowly drip into the solution to reduce precipitation. Mix by flicking the tube wall.
3. The mastermix may become cloudy but should not have any “flakes” or “chunks”. Remake if large precipitates form.

Ligate the barcoded adapters:

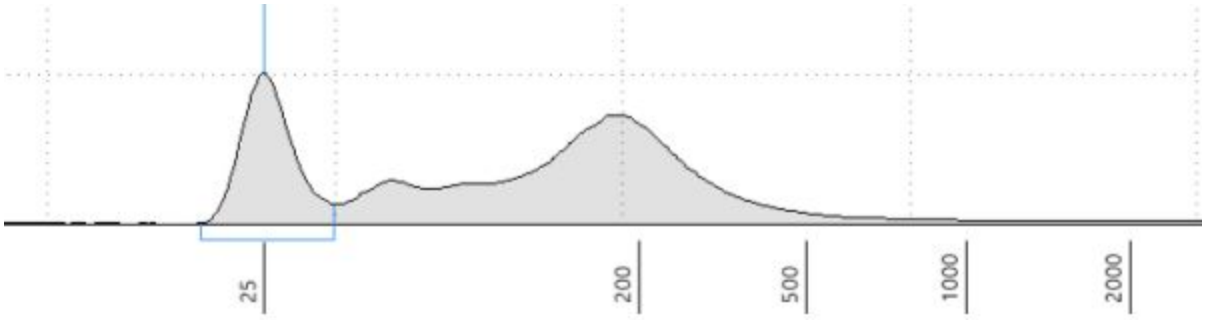
1. Due to the high viscosity of the ligation master mix, very slowly pipette 14.1µL of the mastermix and transfer to each sample.
2. Cap each well with stip-cap tubes and press in FIRMLY.
4. Place a second empty PCR plate on top of the first (flat, well-opening side down).
5. Mix samples by dragging the bottom of the sample plate on the benchtop edge for 1 minute.
6. Briefly spin the plates at 1000xg for 1 minute to collect any droplets.
7. Remove strip caps slowly and re-seal the plate.
8. Incubate at 22℃ for 90 minutes.

4) Pooling and Zymo Clean and Concentrator

Samples are pooled according to the previously set guidelines. Column-based cleanup is used due to the presence of PEG in the Ligation mix, which would interfere with SPRI cleanup.

Sample pooling and clean-up:

1. Make 240µL fresh 70% ethanol for each sample.
2. Add 60µL RLT to each sample and mix by pipette.
3. Based on the predetermined scheme, pool the appropriate samples into a nuclease free reagent reservoir/trough and mix by pipette.
4. Transfer each pool to a new 50mL conical tube.
5. To each tube, add 70% ethanol according to the formula below:
    a. µL 70% ethanol = 240µL * (# of samples in pool)
6. Slowly invert the tube to mix.
7. Place one labeled Zymo RNA Clean & Concentrator filter per pool onto the vacuum manifold.
8. Insert 50mL adapters into each filter and connect the manifold to the vacuum.
9. Add 1mL of the pool solution to columns slowly repeating until all the pool has passed through the column.
10. Remove the 50mL adapter from the Zymo C&C filter when the liquid level dips below the tip of the adapter to prevent carry over of PEG into the final eluent.
11. Once all pools have been suspended on the filter, remove from the manifold and place into a new clean and concentrate collection tube.
12. Add 400µL RNA Prep Buffer onto each filter and centrifuge at 12,000xg for 30 seconds.
13. Discard the flow through and add 700µL RNA Wash Buffer.
14. Centrifuge at 12,000xg for 30 seconds.
15. Discard the flow through and add 400µL RNA Wash Buffer.
16. Centrifuge at 12,000xg for 1 minute.
17. Place the filter into a new collection tube and spin dry spin at 12,000xg for 30 seconds.
18. Place the filter into a new 1.5mL microfuge tube and add 32µL nuclease free water.
19. Centrifuge at 10,000xg for 30 seconds to elute.
20. Add 1µL of SUPERase-IN to each pool and mix by pipetting.
21. Measure the fragment size distribution of each pool by capillary or agarose gel electrophoresis.
    a. Successful fragmentation will be reflected by a distribution of RNA between 100-300.



22. Store RNA pools at -80°C OR proceeding directly to rRNA depletion

*****This is a good point at which to stop and resume the next day *****

5) rRNA depletion

*Pooled samples are treated with the appropriate RiboZero kit to remove rRNA. This kit should be aliquoted upon first thaw to prevent multiple freeze thaws. RiboZero is Illumina's oligo-based capture kit, in which unwanted RNA binds to magnetic beads which are then discarded. Be sure not to carry over any beads for optimal depletion. ***NOTE: The legacy version of RiboZero that uses oligo capture for rRNA removal has been discontinued and replaced with an RNaseH-based kit. RNaseH based depletion kits have perform very poorly in the RNA TagSeq workflow because it leads to over fragmentation of the RNA. We have not vetted a suitable alternative. However, other oligo-based depletion system exist including RiboMinus (ThermoFisher), riboPool (siTOOLS Biotech), and RiboCOP (Lexogen)****

Prepare the RiboZero Streptavidin Beads:

1. For each sample pool, aliquot 225µL of RiboZero magnetic into a 1.5mL RNAse-free microfuge tube.
2. Place on a magnetic tube rack for 1 minute and remove the supernatant.
3. With tubes still on the magnetic rack, add 225µL nuclease-free water per sample to wash the pellet.
4. Incubate for 1 minute or until the solution clears.
5. Discard the supernatant.
6. Repeat steps 2-4 for a total of two washes.
7. Remove all tubes from the magnet. Resuspend the beads in 60µL of resuspension solution per sample
8. Transfer 65uL of beads from each tube into the wells of a 96-well PCR plate and seal.

rRNA Removal

1. Add 21µL of nuclease-free water, 4µL of RiboZero reaction buffer, and 10µL of rRNA removal solution to each well of a new 96-well PCR plate.
2. Add 7µL of the pooled RNA to each corresponding well and pipette well to mix.
3. Place the plate on the thermocycler run the following program:
    a. 68°C for 10 minutes
    b. 22°C for 5 minutes
4. Add the RNA mixture (~40µL) to the 65µL of beads in the prepared plate, pipette to mix, and then briefly vortex at medium speed.
5. Incubate on the bench top for 5 minutes.
6. Gently centrifuge the plate to remove liquid droplets that are adhered to the plate seal.
    a. Avoid spinning the plate with too much force causing the beads to pellet.
7. Place the plate on the thermocycler and incubate at 50°C for 5 minutes.
8. Place on magnet until solution clears and **immediately** elute to a new 96-well U-bottom plate.
    a. **If any beads are drawn up into the pipet tip**, elute into an empty row on the plate and re-aspirate the volume. Repeat until the supernatant is completely free of beads. Any beads carried into subsequent steps will result in poor rRNA depletion.
9. Check and record the volume for the subsequent SPRI clean-up.
    a. Expect ~80µL of recovered eluate containing the mRNA.

1.8x SPRI

1. Add 1.8x the recorded sample volume of RNACleanXP beads to the respective wells and mix by pipette.
2. Incubate at room temperature for 15 minutes.
3. During the incubation, prepare 500µL of 80% ethanol per sample.
4. Place the plate on the magnet and incubate for 5 minutes at room temperature.
5. Discard supernatant.
6. Add 200µL of 80% ethanol to each sample and incubate for 30 seconds.
7. Discard the supernatant .
8. Repeat steps 6 & 7 for a total of 2 washes.
9. Let the beads dry for ~5 minutes or until the pellets have a dry, matte finish (**but not cracked**).
10. Remove the plate from the magnet, add 12µL of nuclease-free water to each sample, and pipette to mix.
11. Return the plate to the magnet and allow the solution to clear.
12. Transfer the entire sample volume to a new 96-well plate and proceed directly to reverse transcription.

6) Reverse transcription and Template Switching

Two primers are added during cDNA synthesis. AR2 anneals to the previously ligated DNA adapter and primes the Reverse Transcriptase enzyme. 3Tr3 provides 3 G's complementing the 3 C's that SmartScribe Reverse Transcriptase adds at the 3' end of synthesis, continuing the synthesis through the rest of the 3Tr3 oligo.

Setup the RT master mix:

Reverse Transcription Master Mix	
Reagent	**Per Sample (µL)**
5X SMARTscribe First strand buffer	4
DTT (100 mM)	0.5
25mM dNTP Mix (25mM each)	0.8
Nuclease free water	0.2
SUPERase-IN (20U/µL)	0.5
SMARTScribe Reverse Transcriptase	2

RT & Template switching:

1. Add 1µL of the AR2 oligo and 1µL of 3Tr3 oligo per sample.
2. Seal the plate and briefly spin at 1000xg for 1 minute.
3. Place the plate on the thermocycler run the following program:
    a. 72℃ for 3 minutes
    b. 42℃ for 2 minutes
4. Remove the plate from the thermocycler immediately after the initial incubation is complete.
5. Add 8µL of reverse transcription master mix to each sample and mix by pipetting.
6. Reseal the plate, place on the thermocycler, and run the following program:
    a. 42℃ for 60 minutes
    b. 70℃ for 10 minutes
7. Remove the plate from the thermocycler once the incubation is complete.
8. Add 1µL of Exonuclease I to each sample and mix by pipetting.
9. Reseal the plate, place on the thermocycler, and incubate at 37C for 30 minutes.
10. Once the final incubation is complete, proceed to the SPRI clean-up.

1.5X SPRI Clean-up

1. Add 18µL nuclease-free water and 60µL RNAClean XP beads to each sample and mix by pipetting.
2. Incubate at room temperature for 15 minutes.

3. During the incubation, prepare 500µL of 70% ethanol per sample.
4. Transfer samples to a U-bottom plate, and place on the magnet for 5 minutes at room temperature.
5. Discard the supernatant.
6. Add 200µL of 70% ethanol to each sample and let stand for 30 seconds.
7. Discard the supernatant.
8. Repeat steps 6 & 7 for a total of two ethanol washes.
9. Let the beads dry for ~5 minutes or until the pellets have a dry, matte finish (**but not cracked**).
10. Remove the plate from the magnet, add 25µL of nuclease-free water to each sample, and mix by pipette.
11. Return to the magnet for and let stand until the solution in each well is clear.
12. Transfer the entire volume to a new PCR plate
13. Store at -20℃ or proceed to the final cDNA enrichment

*****This is a good point at which to stop and resume the next day *****

7) cDNA Enrichment

The cDNA from the sample pools are amplified with uniquely-indexed primers containing illumina adapter sequences.

Setup the enrichment PCR master mix:

Enrichment Master Mix	
Reagent	**Per Sample (µL)**
nuclease free water	15.3
10x AccuPrime PCR buffer I	2.5
AccuPrime HiFi Taq (5U/µL)	0.2

cDNA enrichment/amplification:

1. Assign a unique dual index set to each sample pool before preparing the enrichment reactions.
2. Aliquot 5µL of clean cDNA to a new PCR plate.
3. Add 18µL of master mix into each sample for a final reaction volume of 20µL.
4. Add 1µL (each) of the appropriate P5 and P7 index primers to the sample wells and mix by pipette.
5. Spin down all cDNA plates at 1000xg for 1 minute.
6. Split the reaction into 4 wells of a 384 well plate, aliquoting 6ul each time.
    a. Splitting each reaction into 4 technical replicates (and then recombining in the clean-up section) works to reduce PCR bias initiated at early amplification cycles.

7. Place the plate on the thermocycler run the following program:
    a. Step 1: **1 cycle**
        i. 98℃ for 3 minutes
    b. Step 2: **9-14 cycles**
        i. 98℃ for 30 seconds
        ii. 55℃ for 30 seconds
        iii. 65℃ for 1 minute
    c. Step 3: **1 cycle**
        i. 65℃ for 10 minutes
    d. Step 4: Hold at 4℃
8. 12 cycles is typically sufficient for the majority of sample pools.
    a. Pools with fewer than 12 samples may require more amplification cycles.
9. Once the PCR is complete store samples at 4℃ for 1-2 days or proceed directly to the final clean-up.

1.0X SPRI Clean-up

1. Repool each reaction from the 384-well plate into the wells of the U-bottom plate.
2. Add 25µL of DNA SPRI beads to each sample and mix by pipetting.
3. Incubate for 15 min at room temperature.
4. While the cDNA binds, prepare 500µL of 70% EtOH per sample.
5. Place the plate on the magnet and incubate for 5 minutes at room temperature.
6. Discard supernatant.
7. Add 200µL of 70% ethanol to each sample and let rest for Incubate for 30 seconds.
8. Discard supernatant.
9. Repeat the steps 7-8 for a total of 2 washes.
10. Let the beads dry for ~5 minutes or until the pellets have a dry, matte finish (**but not cracked**).
11. Remove the plate from the magnet, add 25µL of nuclease free water to each sample, and pipette to mix.
12. Incubate the samples for 1 minute at room temperature.
13. Return the plate to the magnet and incubate for 3 minutes.
14. Transfer all samples to a new 96-well PCR plate.

8) Library Quality Control

It is critical to make sure the final library for each pool of samples has the appropriate fragment size distribution and sufficient concentration before sequencing

1. Run all final libraries on the Tapestation (High Sensitivity D1000), BioAnalyzer (High Sensitivity DNA), or agarose gel to visualize the fragment size distribution.
    a. The median fragment size should be ~300 bps.
2. Quantify your libraries using dye- (Picogreen, Qubit, Quant-It, etc) or qPCR-based methods
    a. We find that a 1ng/µL concentration will equate to a ~4nM final library.
3. If any samples are below the required concentration for sequencing on your desired Illumina platform, repeat enrichment and final clean-up sections with a new aliquot of cDNA increasing the cycle number up to 14.
    a. If after 14 cycles sufficient final library concentrations are not achieved, repeat the library construction process from the beginning using more total RNA as input or combine a larger number of samples at the pooling stage.

Protocol Version History			
Version	**Author**	**Date**	**Summary of Changes**
1.0	JessADNA	9-1-16	Started Version 1
1.0	JessADNA	11-2-16	Continued writing Version 1
1.1	JessADNA	1-4-17	Proof-read of Version 1
1.2	JessADNA	3-3-17	Updated fragmentation time to 1.5min (from 3min)
1.3	JessADNA	7-3-17	Changed name of Sample Information Sheet to Kiddie Pool Log. Changed EMOC so SMOC
1.3a	TEC; HV, JL, TG	18Nov2020	Version 1.3 protocol was copied over to MOC's new protocol template, with minor formatting adjustments; review protocol and make publicly shareable.