RNA Tagging Sample Prep. (v. 4.3)

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Equipment

Refrigerated general purpose centrifuge
Refrigerated microcentrifuge
UV-Vis spectrophotometer (we use a NanoDrop)
Agarose gel electrophoresis setup
Magnetic stand (for 1.75 mL tubes)
Vortex
Adjustable temperature thermomixer
Thermocycler (0.2 mL tube volume)

Reagent	Manufacturer	Catalog #
425-600 µm acid-washed glass beads	Sigma-ALDRICH	G8772
Phenol:Chloroform:Isoamyl Alcohol (25:24:1) pH 6.7	ThermoFisher Scientific	BP1752
TURBO DNaseI	ThermoFisher Scientific	AM1907M
GeneJET RNA Purification kit	ThermoFisher Scientific	K0731
Dynabeads mRNA purification kit	ThermoFisher Scientific	61006
Ribo-Zero Magnetic Gold kit for yeast	Epicentre/Illumina	MRZY1324
Agencourt RNAclean XP beads	Beckman Coulter	A63987
Yeast poly(A) polymerase (PAP)	Affymetrix	74225Y
GlycoBlue	ThermoFisher Scientific	AM9515
SuperScript III reverse transcriptase	ThermoFisher Scientific	18080
RNaseOUT	ThermoFisher Scientific	10777
Ribonuclease H	ThermoFisher Scientific	18021
GeneJET PCR Purification kit	ThermoFisher Scientific	K0701
5 U/µL Exo-Klenow Fragment DNA Polymerase I	ThermoFisher Scientific	AM2008
GoTaq Green 2X Master Mix	Promega	M712
10 mM dNTPs	Promega	U1515
100 mM stock of GTP (from the AmpliScribe T7-Flash kit)	Epicentre/Illumina	ASF3507
100 mM stock of ITP	Sigma-ALDRICH	I0879
3M sodium acetate (NaOAc) pH 5.2		
100% ethanol		
Nuclease-free water		

Buffers

RNA ISO buffer:

0.2 M Tris-HCl pH 7.5
0.5 M NaCl
0.01 M EDTA
1% (v/v) SDS

Filter sterilize before first use.
Store at room temperature.

10X Klenow Buffer:

500 mM Tris–HCl pH 7.5
100 mM $MgCl_2$
10 mM DTT
0.5 mg/mL BSA

Store at –20°C.

General outline and timing

Day 1: Isolate Total RNA.
Day 2: Poly(A) selection, rRNA depletion, begin G-I–tailing.
Day 3: Finish G-I–tailing, U-select reverse transcription, 2nd strand synthesis, begin PCR amplification.
Day 4: Finish PCR amplification.

Isolate Total RNA – *S. cerevisiae*

Rationale: Isolate total RNA from yeast in completely denaturing conditions.

Timing: steps 1–15: 1.5–2 hours; steps 17–30: 3–4 hours

1. Place 25 mL of A_{660} ~0.5–0.8 cultures on ice for 5 minutes.
2. Harvest cultures by centrifugation at 3200 rpm for 5 minutes at 4 °C.
3. Wash yeast pellets once with 40 mL of ice cold water.
4. Resuspend yeast in 500 µL RNA ISO buffer.
5. Add ~200 µL of acid–washed beads (425-600 µM).
6. Add 500 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) pH 6.7 (PCA).
7. Vortex for 20 sec at room temp, then incubate for 20 sec on ice. Repeat for a total of 10 cycles.
8. Split into two tubes. (Each sample is now in 2 tubes.)
9. Add 375 µL RNA ISO buffer and 375 µL PCA.
10. Mix gently by several inversions.
11. Spin at max speed (13,200 rpm) at 4°C for 10 minutes.
12. Transfer aqueous phase to new tube.
13. Add equal volume PCA and mix gently
14. Spin at max speed at 4°C for 10 minutes.
15. Remove aqueous phase to new tube.
16. Add equal volume chloroform and mix gently.
17. Spin at max speed at 4°C for 10 minutes.
18. Remove aqueous phase to new tube.
19. Add 1 mL of 100% ethanol, mix gently. Incubate for at least 1 hr at –50°C. (overnight works well as a stopping point if needed).
20. Spin at max speed at 4°C for 20 minutes. Remove supernatant.
21. Wash pellet at least once in 80% ethanol. (Twice works well.)
22. Resuspend RNA pellets in 43 µL of nuclease-free water.
23. Combine the 2 tubes for each sample (back to 1 tube per sample), add 10 µL of 10X TURBO DNase Buffer, 4 µL (8 U) of TURBO DNase.
24. Incubate for 1 hr at 37°C. (I do this in 1.75 mL microcentrifuge tubes in a 37°C incubator).
25. Clean reactions with the GeneJET RNA Purification kit, using the “RNA cleanup protocol”.
26. Elute in 30 µL of nuclease-free water.
27. Determine RNA concentration (Nanodrop, 1:10 dilutions of samples)
28. Run agarose gel to assess RNA quality (500 ng total RNA). (Optional: analyze the samples with a BioAnalyzer).
29. Store RNA at –80°C until next step.

Poly(A) Selection

Dynabeads mRNA purification kit (ThermoFisher Scientific, cat. # 61006).
https://www.thermofisher.com/order/catalog/product/61006
The protocol is essentially as recommended by the manufacturer.

Rationale: Many of the small, non-coding RNAs (rRNAs, snRNAs, SCR1, etc.) end in several uridine residues. To decrease the number of these "background" RNAs in our libraries, we enrich for polyadenylated RNAs. This step can be omitted, but the resulting libraries will be primarily composed of the small, non-coding RNAs (90-95% of the sample).

Timing: ~ 1 hour

Before use, let the aliquots of beads and buffers warm to room temp (~15 min).
Slow thaw RNAs on ice (15–20 minutes).

Prior to starting: set a thermomixer to 65°C.

1. Use 75 µg of total RNA per sample and adjust volume to 100 µL using nuclease-free water.
2. Heat RNA solution to 65°C in the thermomixer for 2 minutes to disrupt secondary structures.
3. Place samples on ice until use in step 9.
4. Set the thermomixer to 70°C.
5. Transfer 200 µL of well resuspended Dynabeads to a microcentrifuge tube.
6. Place tube on magnet. Discard supernatant.
7. Add 100 µL of Binding Buffer to calibrate the beads. Mix well.
8. Place tube back on magnet and discard the supernatant.
9. Add 100 µL of Binding Buffer to the beads. Mix well.
10. Add the RNA (from step 3) to the bead solution. It's important to have a 1:1 ratio of RNA volume to Binding Buffer volume.
11. Mix beads thoroughly. Gently vortex samples briefly every 30 sec for 5 minutes at room temp.
12. Place tube on magnet to separate beads. Remove the supernatant.
13. Wash with 200 µL Washing Buffer B. Mix thoroughly.
14. Place tube on magnet. Remove all of the supernatant.
15. Repeat steps 13–14.
16. After removing all of the supernatant from the second wash, add 28 µL of water. Resuspend the beads well.
17. Heat the samples to 70°C in the thermomixer for 2 minutes.
18. Place the samples on the magnet ***immediately*****.**
19. Collect the supernatant. This is your poly(A)+ mRNA!

rRNA depletion

Ribo–Zero Magnetic Gold kit for yeast (Epicentre/Illumina, cat. # MRZY1324)
http://www.illumina.com/products/ribo-zero-gold-rrna-removal-yeast.html
The protocol is essentially as recommended by the manufacturer.

Rationale: The poly(A) selection efficiently removes small, non-coding RNAs (such as snRNAs and SCR1) but there is bleed through of rRNAs. Thus, we use this step to remove the remaining rRNAs. This step can be omitted, but the resulting libraries will be primarily composed of rRNAs (90-95% of the sample).

Timing: ~ 2 hours.

Before use, let the magnetic beads and associated buffers warm to room temp (~15 min). Slow thaw reagents stored at –80°C on ice (15–20 minutes).

Prior to starting: Set a thermocycler to 68°C and set a thermomixer to 50°C. Also, aliquot the needed volume of RNA Clean XP beads (see Part 4) and store them at room temp until use in Part 4.

Part 1: Bead washing

1. For each reaction, pipet 225 µL of magnetic beads into a 1.75 mL centrifuge tube. Pipet slowly to avoid air bubbles. Store the unused beads at 4°C.
2. Place tubes on magnetic stand for > 1 minute.
3. Remove and discard the supernatant.
4. Remove the tube from the stand and add 225 µL of water to each tube. Mix well by repeated pipetting or vortexing at medium speed.
5. Repeat steps 2–4. Remove the tube from the stand. Add 65 µL of Magnetic Bead Resuspension Solution to each tube. Mix well by pipetting or vortexing on medium speed.
6. Add 1 µL of RiboGuard RNase Inhibitor and mix briefly by vortexing.
7. Keep the tubes at room temp until needed in *Part 3*.

Part 2: Treatment of Total RNA with Ribo–Zero rRNA removal Solution

1. For each sample, combine the following in an RNase-free 0.2 mL PCR strip-tube:

4 µL	Ribo–Zero Reaction Buffer
26 µL	poly(A)+ RNA
10 µL	Ribo–Zero rRNA Removal Solution
40 µL total volume	

2. Gently mix the reaction by pipetting and incubate at 68°C for 10 minutes in a thermocycler. Store the rest of the unused kit at –80°C.
3. Remove the reaction tube and incubate at room temperature for 5 minutes.

Part 3: Magnetic Bead Reaction and rRNA removal

Important: Always add the treated RNA sample to the washed magnetic beads and immediately mix by pipetting. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact rRNA removal efficiency.

1. Using a pipette, add the treated RNA sample to the washed magnetic beads and *immediately* mix by pipetting at least 10 times to thoroughly mix the sample. Then, vortex the tube immediately at medium setting for 10 seconds and place at room temperature.

2. Incubate the samples at room temperature for 5 minutes.
3. Then, mix the reactions by vortexing at medium speed for 10 seconds and then place at 50°C for 5 minutes. Avoid any significant condensation during this step (e.g. make sure the cover for the thermomixer is on the instrument during the incubation to keep the lids of the tubes exposed to warm air).
4. Remove the tubes from the 50°C heat block and place on a magnetic stand for >1 minute.
5. While on the stand, carefully remove the supernatant (*this is your rRNA-free RNA!)* and place in a labeled, RNase-free tube.
6. If there are residual beads in the supernatant, repeat the magnetic separation.
7. Place the RNA on ice and immediately proceed to RNA cleanup.

Part 4: Agencourt RNAclean XP bead mediated RNA cleanup

1. Mix the AMPure RNAClean XP beads well by vortexing.
2. Add 160 µL of the mixed beads to each reaction containing 85–90 µL of rRNA-depleted sample. Mix thoroughly by pipetting > 10 times. Vortex gently.
3. Incubate at room temperature for 15 minutes. During the incubation prepare a fresh 80% (v/v) ethanol solution.
4. Place the tube on a magnetic stand for > 5 minutes.
5. Remove the supernatant without disturbing the beads.
6. With the tube still on the stand, add 400 µL of fresh 80% ethanol without disturbing the beads.
7. Incubate at room temperature for 1 minute.
8. Remove the ethanol supernatant.
9. Repeat the 80% ethanol wash for a total of two wash steps.
10. Allow the tube to air dry on the magnetic stand.

Note: The beads shouldn't over dry, but make sure there is no ethanol remaining in the tube. The elution in the next step is only 12 µL, so even very small volumes of ethanol could negatively impact the downstream enzymatic reactions. I typically add the water once the beads go from glossy to more matte–like appearance, just before they start cracking (thin white lines). It typically takes about 5 minutes. According to the manufacturer, over-drying reduces elution efficiency.

11. Add 12 µL of RNase-free water to the tube and immediately and thoroughly mix.
12. Incubate the tubes at room temperature for 2 minutes.
13. Place the tubes on the magnetic stand for at least 5 minutes. Transfer the clear supernatant to a new tube, always leaving 1–2 µL behind to prevent carryover of the beads to the next steps.

G–I tailing of RNA

Rationale: Add a known sequence to the 3' end of all RNAs that can be exploited to reverse transcribe the RNA. The G/I-tailing step can be replaced by a 3' ligation step.

Timing: steps 1-8J: ~2.5 hours; steps 8L-9: ~1 hour.

1. Assemble the following master mix (volumes reflect 1 reaction):

    4 µL 5X Reaction buffer
    2 µL nuclease-free water
    1 µL 10 mM GTP
    1 µL 3.3 mM ITP

2. For each sample, aliquot 8 µL of the master mix into nuclease-free 0.2 mL PCR strip-tubes.
3. For each sample, add 10 µL of the appropriate poly(A)+/rRNA-depleted RNA and mix.
4. Add 2 µL of 600 U/µL Yeast PAP to each reaction.
5. Incubate at 37°C for 90 minutes.
6. Add an additional 2 µL of 600 U/µL Yeast PAP to each reaction.
7. Incubate at 37°C for 30 min.
8. Extract RNA and ethanol precipitate.
    A. Add 80 µL of nuclease-free water to each reaction (volume should now be ~100 µL). Transfer reactions to 1.75 mL microcentrifuge tubes.
    B. Add 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1) pH 6.7 to each reaction.
    C. Gently vortex or mix thoroughly
    D. Spin at max speed for 5 minutes at 4°C.
    E. Collect the aqueous phase (top layer, aim for 95-100 µL) and transfer to a new tube.

Note: The aqueous phase will likely have a white, cloudy precipitate. Don't worry about it. Just collect as much of the aqueous phase as possible, even if it includes the precipitate. The precipitate will disappear once the second extraction is complete.

    F. Add an equal volume of chloroform.
    G. Gently vortex or mix thoroughly
    H. Spin at max speed for 5 minutes at 4°C
    I. Collect the aqueous phase (top layer, aim for 90 µL) and transfer to a new tube.
    J. To the aqueous phase from step I, add 10 µL of 3 M NaOAc, 1 µL of 15 mg/mL GlycoBlue, and 500 µL of 100% ethanol.
    K. Mix thoroughly.
    L. Incubate for at least 1 hr at –50°C. (I usually do an overnight incubation. Works as a good stopping point).
    M. Spin at max speed for 25 minutes at 4°C.
    N. Remove supernatant. Wash pellet with 70–80% ethanol.
    O. Spin at max speed for 25 minutes at 4°C.
    P. Remove supernatant.
    Q. Pulse spin and remove residual ethanol.
9. Resuspend pelleted RNA in 10 µL of nuclease-free water.

U-select Reverse Transcription

Rationale: Use the G-I nucleotides on the 3' end of the RNA to prime reverse transcription via the U-select oligo. The three adenosines on the 3' end of the U-select oligo preferentially anneal to RNAs that end in uridines (prior to the G/I-tailing), thus selectively enriching the U-Tagged RNAs. The U-select oligo also contains Illumina 3' adapter sequence (underlined).

Recommended: Also prepare –RT reactions for each sample as a comparison.

Timing: ~ 2.5 hours

U-select oligo: 5'– <u>GCCTTGGCACCCGAGAATTCCA</u>CCCCCCCCCCAAA –3'

1. Assemble the following in strip tubes (volumes reflect 1 reaction):

1 µL	1 µM U-select oligo
5 µL	G-I–tailed RNA
1 µL	10 mM dNTP mix
6 µL	nuclease-free water

2. Assemble a reaction Master Mix a separate strip tube (volumes reflect 1 reaction):

4 µL	5X SuperScript III Reaction Buffer
1 µL	100 mM DTT
1 µL	RNaseOUT

3. Heat the reactions and master mix to 65°C for 5 min.
4. Cool the reactions and master mix to 50°C for 5 min.

Important: Perform steps 5 and 6 while the RNA/primer mix and the master mix are in the thermomixer. It's important to keep the reactions at 50 °C to maintain the U-selection.

5. Add 6 µL of the preheated (50°C) Master Mix in the thermocycler to each reaction.
6. Add 1 µL of 200 U/µL SuperScript III reverse transcriptase to each reaction.
7. Incubate at 50°C for 60 minutes.
8. Incubate at 85°C for 5 minutes.
9. Cool reactions to 4°C.
10. Add 1 µL RNase H to each reaction.
11. Incubate at 37°C for 20 minutes.
12. Add 80 µL of water to increase reaction volume to ~100 µL.
13. Clean cDNA using the GeneJET PCR Purification kit. (I do not add isopropanol).
14. To elute cDNA:
    A. Add 32 µL nuclease-free water to the dry column.
    B. Incubate the water on the column for a least 2 minutes at room temperature.
    C. Centrifuge at max speed for 2 minutes.
    D. Repeat steps A-C.
    E. Combine elution fraction to get ~60 µL of cDNA for each reaction.

Second Strand Synthesis

Rationale: Randomly synthesize the 2nd strand of DNA that is complementary to the cDNA sequence, while at the same time adding the Illumina 5' adapter sequence (underlined).

Timing: ~ 2 hours

2nd strand synthesis oligo: 5' – GTTCAGAGTTCTACAGTCCGACGATCNNNNNN –3'

Before part 1, aliquot the required volume of Agencourt RNAclean XP beads and keep them at room temperature until use.

Part 1: 2nd strand synthesis reaction

1. Assemble Master Mix:

12 µL	Nuclease-free water
10 µL	10X Klenow Buffer
5 µL	10 mM dNTPs
10 µL	10 µM 2nd strand synthesis oligo

2. Aliquot 37 µL of Master Mix for each reaction into 0.2 mL nuclease-free PCR strip tubes.
3. Add 60 µL purified cDNA to the Klenow Master Mix for each sample.
4. Add 3 µL of 5 U/µL Exo-Klenow fragment DNA polymerase I to each reaction.
5. Incubate at 37°C for 30 minutes.
6. Cool to 4°C.
7. Warm reactions to room temperature. (I let the cooled reactions sit on my bench for 5 minutes).

Part 2: Reaction clean up

Important: Make sure the beads and 2nd strand synthesis reactions are warmed to room temperature before proceeding. Use a 1:1 bead-to-reaction ratio (by volume) to efficiently remove the 2nd strand synthesis oligo.

1. Mix the AMPure RNAClean XP beads well by vortexing.
2. Add 100 µL of the mixed beads to each 100 µL of sample.
3. Mix thoroughly by pipetting > 10 times. Vortex gently.
4. Incubate at room temperature for 15 minutes. During the incubation prepare a fresh 80% (v/v) ethanol solution.
5. Place the tube on a magnetic stand for > 5 minutes.
6. Remove the supernatant without disturbing the beads.
7. With the tube still on the stand, add 400 µL of fresh 80% ethanol without disturbing the beads.
8. Incubate at room temperature for 1 minute while still on the magnetic stand.
9. Remove the ethanol supernatant.
10. Repeat the 80% ethanol wash for a total of two wash steps.
11. Allow the tube to air dry on the magnetic stand.

Note: The beads shouldn't over dry, but make sure there is no ethanol remaining in the tube. The elution in the next step is only 12 µL, so even very small volumes of ethanol could negatively impact the downstream enzymatic reactions. I typically add the water once the beads go from glossy to more matte–like appearance, just before they start cracking (thin white lines). It typically takes about 5 minutes. According to the manufacturer, over-drying reduces elution efficiency.

12. Add 100 µL of nuclease-free water to the tube and immediately and thoroughly mix.
13. Incubate the tubes at room temperature for 2 minutes.
14. Place the tubes on the magnetic stand for 5 minutes. Transfer the clear supernatant to a new tube, always leaving 1–2 µL behind to prevent carryover of the beads to the next steps.
15. Repeat steps 1-11.
16. Add 50 µL of nuclease-free water to the tube and immediately and thoroughly mix.
17. Incubate the tubes at room temperature for 2 minutes.
18. Place the tubes on the magnetic stand for 5 minutes. Transfer the clear supernatant to a new tube, always leaving 1–2 µL behind to prevent carryover of beads to the next steps.

PCR Amplification

Rationale: Amplify the dsDNA and add the remaining 5' and 3' Illumina adapter sequences for subsequent high-throughput sequencing. The PCR cleanup step efficiently removes adapter-adapter products (5' Illumina adapter – 3' Illumina adapter with no RNA insert) that will preferentially sequence.

Timing: ~ 2.5 hours

5' PCR primer: 5'– AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA –3'

3' Barcoded PCR primer:
*5'– CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA –3'*
*(barcode sequence **NNNNNN**, use unique barcode for each sample)*

Part 1: PCR

1. Assemble 10X PCR Master Mix for each sample:

83.3 µL	2X GoTaq Master Mix
6.7 µL	10 µM 3' Barcoded PCR Primer
6.7 µL	10 µM 5' PCR primer
20 µL	nuclease-free water
50 µL	purified cDNA

2. Aliquot 20 µL of the PCR Master Mix into 8 separate 0.2 mL nuclease-free PCR tubes (This gives 1 strip tube per sample).

Note: A standard cycling protocol (94°C for 10 s; 55°C for 30 s; 72°C 1 min; repeated 25 times) would likely work fine, too.

3. Amplify via the following protocol:

1. 94°C	2 min
2. 94°C	10 sec
3. 40°C	2 min
4. 72°C	1 min
5. Go to step 2	1X (once)
6. 94°C	10 sec
7. 55°C	30 sec
8. 72°C	1 min
9. Go to step 6	7X (7 times)
10. 94°C	15 sec
11. 55°C	30 sec
12. 72°C	1min
13. Go to step 10	14X (14 times)
14. 72°C	5 min
15. 4°C	for ever

Note: I most often run this protocol overnight and do the clean up the following morning.

Part 2: PCR clean up

Important: Make sure the beads and PCR samples are warmed to room temperature before proceeding. Use a 0.8:1 bead-to-reaction ratio (by volume) to remove empty adapter-adapter PCR products.

1. Combine individual PCRs for each sample into a single 1.75 mL microcentrifuge tube. (The 8 reactions per sample into a single tube. Keep each of the samples in separate tubes. There should be 140-150 µL per sample)
2. Measure the volume of each sample using a pipette.
3. Mix the AMPure RNAClean XP beads well by vortexing.
4. Add 0.8 volumes (relative to sample volume) of the pre-warmed, mixed beads to each sample. (e.g. add 120 µL of beads to 150 µL of sample)
5. Mix thoroughly by pipetting > 10 times. Vortex gently.
6. Incubate at room temperature for 15 minutes. During the incubation prepare a fresh 80% (v/v) ethanol solution.
7. Place the tube on a magnetic stand for > 5 minutes.
8. Remove the supernatant without disturbing the beads.
9. With the tube still on the stand, add 400 µL of fresh 80% ethanol without disturbing the beads.
10. Incubate at room temperature for 1 minute while still on the magnetic stand.
11. Remove the ethanol supernatant.
12. Repeat the 80% ethanol wash for a total of two wash steps.
13. Allow the tube to air dry on the magnetic stand.

Note: The beads shouldn't over dry, but make sure there is no ethanol remaining in the tube. The elution in the next step is only 12 µL, so even very small volumes of ethanol could negatively impact the downstream enzymatic reactions. I typically add the water once the beads go from glossy to more matte–like appearance, just before they start cracking (thin white lines). It typically takes about 5 minutes. According to the manufacturer, over-drying reduces elution efficiency.

14. Add 100 µL of nuclease-free water to the tube and immediately and thoroughly mix.
15. Incubate the tubes at room temperature for 2 minutes.
16. Place the tubes on the magnetic stand for 5 minutes. Transfer the clear supernatant to a new tube, always leaving 1–2 µL behind to prevent carryover of the beads to the next steps.
17. Repeat steps 3-13.
18. Add 15 µL of nuclease-free water to the dried beads and immediately and thoroughly mix.
19. Incubate the tubes at room temperature for 2 minutes.
20. Place the tubes on the magnetic stand for 5 minutes. Transfer the clear supernatant to a new tube, always leaving 1–2 µL behind to prevent carryover of the beads to the next steps.
21. Store reactions at –80°C until submission for high-throughput sequencing.
22. If desired, run 2-4 µL of the libraries on a 1% agarose gel to check library quality. Topo–Cloning of initial library preps is recommended to ensure libraries are constructed correctly.

Libraries are ready for sequencing.