Degradome protocol

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5′ adapter (equimolar mix of BA5-UMI-a and BA5-UMI-b, 41-nt custom RNA oligos, PAGE purified):
5′-GUUCAGAGUUCUACAGUCCGACGAUCNNNCGANNNUACNNN-3′
5′-GUUCAGAGUUCUACAGUCCGACGAUCNNNAUCNNNAGUNNN-3′

DEG-RT (same as CAGE RT primer)
5′–GCACCCGAGAATTCCANNNNNNNN–3′

DEG-PCR-1L (same as CAGE PCR primer)
5′–CTACACGTTCAGAGTTCTACAGTCCGA-3′

DEG-PCR-1R

5′–GCCTTGGCACCCGAGAATTCCA–3′

PCR primer1/BPCRP1 (same as small RNA-Seq library PCR primer)
5′–AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA–3′

PCR primer2/BPCRIdX**:**

PCRId1: 5′–CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId2: 5′–CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId3: 5′–CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId4: 5′–CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId5: 5′–CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId6: 5′–CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId7: 5′–CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId8: 5′–CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId9: 5′–CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId10: 5′–CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId11: 5′–CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId12: 5′–CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId13: 5′–CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId14: 5′–CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId15: 5′–CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId16: 5′–CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId17: 5′–CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′
PCRId18: 5′–CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA–3′

PROCEDURE

I. Ribosomal RNA depletion (also see rRNA depletion folder in Common Folder) & DNase treatment

Mouse: Pooled 186 rRNA antisense oligos (100 µM/each) that tile 18S, 28S, 5S, 5.8S, mt 12S, and mt 16S rRNAs. Final working concentration: 0.5 µM for each oligo.

Fly: Pooled 160 rRNA antisense oligos (100 µM/each) that tile 18S, 28S, 5.8S, mt L, and mt S rRNAs. Final working concentration: 0.5 µM for each oligo.

- Use 500 ng – 2 ug high quality total RNA (strongly recommended) as starting material. Suggested kit for RNA extraction: mirVana by Ambion.
- Add 2 µL of pooled rRNA AS oligos for every 1 µg of total RNA (ratio: 1 µL oligos for 1 µg RNA).
- Make final volume 14 µL with 1X rRNA oligo hybridization buffer (aliquots in the –20ºC radioactivity common freezer).
- Heat the mixture to 95°C, then slowly cool it down (–0.1°C/sec) to 22°C in a PCR machine. Incubate at 22°C for additional 5 minutes. Place on ice.
- Add the following to RNA (final volume: 20 µL):
  - 2 µL 10X RNase H digestion buffer
  - 2 µL water
  - 2 µL Thermostable RNase H (10 units; Hybridase from Epicentre)
- Incubate at 45°C for 30 minutes. Place on ice.
- *DNase treatment*: Add the following to each tube (final volume 50 µL):
  - 23 µL water
  - 5 µL Turbo DNase 10X buffer
  - 2 µL Turbo DNase
- Then incubate at 37ºC for 20 minutes.
- Purify RNA using RNA Clean & Concentrator-5 (Zymo Research: Cat R1015), enriching RNA> 200nt and removing 5S rRNA and tRNAs. Note that table top centrifuge can be used for steps 3,4,5 and 7.
  1. Adjust *RNA Binding Buffer*: combine 50 µL RNA binding buffer and 50 µL 100% ethanol
  2. Add 100 µL of adjusted *RNA Binding Buffer* to RNA samples. Mix well.
  3. Transfer the mixture to the Zymo-Spin column. Spin @ 17,000Xg for 30 seconds (RNA> 200nt retained in the column). Discard flow-through.
  4. Add 400 µL *RNA Prep Buffer*. Spin @ 17,000Xg for 30 seconds. Discard flow-through.
  5. Add 700 µL *RNA Wash Buffer*. Spin @ 17,000Xg for 30 seconds. Discard flow-through.
  6. Add 400 µL *RNA Wash Buffer*. Spin @ 17,000Xg for 2 minutes. Discard flow-through
  7. Spin @ 17,000Xg for 30 seconds to remove any ethanol residue
  8. Move the column to a clean 1.7mL tube.
  9. Add 7 µL water to the column. Incubate @ room temp for 1 minute. Spin @ 17,000Xg for 30 seconds to elute RNA. Transfer 6.5 µL to a new tube.

II. Library preparation

Note: This protocol is written for using 2 µg mouse testis/Hi5 total RNA as a starting point. If you work with a different organism, tissue, and/or amount of starting total RNA, it's important to optimize the PCR cycle numbers for both PCR steps.

1. 5′ adapter ligation

- Add to the 6.5 µL RNA from the previous step:

10x 5′ RNA ligase buffer	2ul
50% PEG (20% final)	8ul
25uM BA5-UMI adapter	1ul
RNAsein(40U/ul) (N2615)	0.5ul
T4 RNA Ligase High Conc(M0437M)	2ul

- Incubate at 16ºC for 16 hours.
- *Optional:* Add 30 µL water to the ligation reaction for a larger volume that′s easier to handle.
- Purify RNA with RNA Clean & Concentrator-5 (Zymo Research: Cat R1015), which enriches for ≥200nt RNAs. Elute with 11 µl water. Transfer 10 µL RNA to a 200 µL PCR tube.

2. First strand cDNA synthesis

- Add to the RNA from the previous step:

5X FS buffer	5 µL
Deg-RT primer (50 µM)	1 µL

  - 65˚C for 3 min for 4ºC forever. Place on ice.
- Add the following 9 µL mixture:

Water	5.25 µL
dNTP mixure (10 mM)	1.5 µL
DTT (100 mM)	1.25 µL
SuperScript III	1 µL

  - Incubate in a thermocycler:
    - 25°C for 5 min
    - 50°C for 1 hr
    - 70°C for 15 min
    - 4°C forever
- Purify cDNA with 45ul Agencourt AMpure XP beads. Elute with 25 µL water. Transfer 23 µL to a new tube.

3. First PCR

- Add the following to the 23 µL cDNA from the previous step:

NEBNext (Q5) 2X master mix	25 µL
Deg-PCR-1L primer (10 µM)	1 µL
Deg-PCR-1R primer (10 µM)	1 µL

- Run the following program in a PCR machine with a heated-lid:
  1. 98°C for 30 sec
  2. 98°C for 10 sec
  3. 63°C for 10 sec
  4. 72°C for 12 sec
  5. Go to 2., 4× cycles total
  6. 98°C for 10 sec
  7. 70°C for 30 sec
  8. 72°C for 12 sec
  9. Go to 6., 6× cycles total
  10. 72ºC for 3 min
  11. 11. 4ºC forever

- Purify PCR products on 1% agarose gel. Run until 200- and 400bp ladder is separated well. Generally takes 45-60 mins when run at 100-120V. Use 100 bp DNA ladder to precisely select for 200–400 bp PCR products.
- It is important to cut just above 200nt ladder. To improve the visualization gel can be stained with EtBr wash.
- Run different genotypes on different gels to prevent cross contamination.
  - *It's normal that the PCR products are not visible on the gel at this point. Do not panic.*
- Extract PCR products with Qiagen gel extraction kit. Elute DNA with 24 µL water. Transfer 23 µL DNA to a new PCR tube.

4. Second PCR

- Add the following to the 23 µL DNA from the last step:

NEBNext (Q5) 2X master mix	25 µL
BPCRP1 primer (10 µM)	1 µL
BPCRPIdX primer (10 µM)	1 µL

- Run the following program in a PCR machine with a heated-lid:
  1. 98°C for 30 sec
  2. 98°C for 10 sec
  3. 70°C for 30 sec
  4. 72°C for 14 sec
  5. Go to 2., 4× cycles total
  6. 98°C for 10 sec
  7. 72°C for 34 sec
  8. Go to 6., 8× cycles total
  9. 72ºC for 3 min
  10. 4ºC forever

- Purify the final library with 50 µL Ampure XP beads. Elute with 27 µL EB and transfer 25 µL supernatant to a new tube.

5. Purification with non-denaturing PAGE:

- Prepare a 10% non-denaturing polyacrylamide gel (20 cm × 16 cm × 1.5 mm, length × width × thickness), with 20 small wells (each well: 17 mm × 5 mm × 1.5 mm, depth × width × thickness). The gel recipe is as follows:

water	32 mL
5x TBE	5 mL
40% Acrylamide/Bis-acrylamide (Sigma-Aldrich # A7802)	12.5
10% ammonium persulfate	500 µL
TEMED	50 µL

- Pre-run the gel in 0.5× TBE buffer at the constant power of 35 W for 30 min (the surface temperature of the gel reaches 45–55°C).
- To 25µL of the library from the previous step add 5 µL of 6x Loading Dye (e.g., Tritrack, Thermofisher #R1161) and mix thoroughly.
- Wash wells with the running buffer. Load 30 µl of libraries into wells keeping at least one well empty between samples. Remember to load 100bp DNA Ladder (e.g., NEB #N3231S) into the rightmost and the leftmost wells.
- Run the gel in 0.5× TBE at the constant power of 20 W for 75 min or until the xylene cyanol bands are just above the bottom of the gel.
- Open the glass plates and cut off one bottom corner of the gel to mark the loading sequence. Gently peel the gel off the glass plate and transfer it into the glass tray containing 1× SYBR Gold in 100 ml 0.5× TBE buffer. Stain the gel for 30 sec.
- Cut off margins of an Avery diamond clear standard weight sheet protector, separate the two pieces, and lay one piece on the bench with the inside facing up. Gently transfer the gel on the sheet protector. Always keep the gel on the sheet protector to avoid contamination.
- Scan the gel with Typhoon FLA 9000 or similar scanner.
- Transfer the gel with sheet protector on the UV box. Switch on the long-wave UV light and cut the gel between the **250bp and 500bp** with a new razor blade (REMEMBER TO PROTECT YOUR EYES FROM UV LIGHT!!!). Do not use the

same blade to cut different samples. Transfer the gel slice into a 2.0 ml tube and crush it with either a single-use plastic pestel or by centrifuging it through a pierced 0.5ml tube as described in the Illumina Truseq Small RNA protocol. Add 0.7 ml of 0.3 M NaCl to each tube and rotate in cold room overnight to elute libraries.

- The following day, use a P1000 tip with the end cut off to transfer most of the gel elution mix from each 2.0 ml tube to a Spin-X column (Sigma #CLS8162), and spin at 10,000 × *g* for 5 min to filter out gel pieces.
- Split elution from each 1.7 ml tube into 2 fresh tubes, ~350 µl each, add 1 µl (15 µg) of GlycoBlue (Thermofisher # AM9515) to each tube and vortex briefly to mix.
- Add 3 volumes (1050 µl) of 100% ethanol to each tube and vortex briefly to mix. Incubate on ice for 1 hr.
- Spin at 20,000 × *g* for 30 min at 4°C.
- Wash the pellet with 900 µl of 75% ethanol. Spin at 20,000 × *g* for 5 min at 4°C and gently remove supernatant without touching the pellet.
- Spin at 20,000 × *g* for 1 min at 4°C and gently remove the residual ethanol with a P10 tip.
- Air dry for 2 min and dissolve the pellet in 5–10 µl water in each tube and combine the library from the two tubes.
- Quantify libraries with Kapa

Recipes(Morlan et al. *PLOS ONE* 2012 & Adiconis et al. *Nature Methods* 2013)

rRNA oligo Hybridization buffer
100 mM Tris-Cl (pH 7.4)
200 mM NaCl

10X RNase H digestion buffer
500 mM Tris-Cl (pH 7.4)
1M NaCl
200 mM $MgCl_2$

10× 5′ Ligation Buffer: 500 mM Tris-HCl (pH 7.8), 100 mM MgCl2, 100 mM DTT, and 10 mM ATP. Use freshly prepared buffer every prep as DTT and ATP degrade over time.

Water	10 µl
1 M Tris-HCl (pH 7.8)	50 µl
500 mM MgCl2	20 µl
1 M DTT	10 µl
100mM rATP	10 µl
Final volume	100 µl