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Drosophila small RNA isolation

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ABSTRACT

Drosophila small RNA sequencing samples are often overwhelmed by the abundant 30-nucleotide 2S rRNA fragment. Small RNA of interest can be isolated from total RNA without this fragment using gel size separation and a block oligo with sequence complementarity to the 2S rRNA fragment. In this protocol we detail the equipment, reagents and method for recovering small RNA (size range ~17-29 nucleotides) from total RNA using a urea-PAGE gel and ethanol precipitation, in preparation for deep sequencing. We draw on previous publications and available protocols to present step-by-step instructions with tips and tricks for successful small RNA isolation.

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Equipment Needed

1 Gel:
- Gibco-BRL V16-2 Polyacrylamide Gel Electrophoresis System (Catolog No. V162)

- Two Gibco-BRL 10 lane combs, 1.50 mm thick (Catalog No. CGV10-150)
- gel loading pipette tips
- 10 ml pipette
- 1000 ml pipette (for loading the gel)
- plastic trays or tubs for staining (one for each gel)
- plastic sheet protectors (one for each gel)
- binder clips (6 for each gel, 3 per side)
- gel tape
- glass cover slips
- PPE (gloves and eye protection, important when working with acrylamide)

Reagents Needed

2 Cleaning:

- RNase Away spray
- 3% hydrogen peroxide
- 70% ethanol

Gel:

- nuclease-free (NF) water
- agar
- ultrapure urea
- 40% polyacrylamide (19:1 acrylamide:bis-acrylamide) (Bio-Rad #1610144)
- TEMED
- 10% ammonium persulfate (APS)
- 10X TBE buffer
- Zymo small RNA ladder (17, 21, 25, 29 nt bands) (Zymo #R1090
- SYBR Gold stain (Thermofisher #S11494)
- 0.3 M NaCl (can make from 5M, Thermofisher #AM9760G)

Gel elution:

- Glycogen (RNA grade, 20 mg/mL) (Thermofisher #R0551)
- 100% ethanol
- 75% ethanol

For measuring small RNA yield:

- Qubit microRNA Assay kit (Thermofisher #Q32880)

  Note: This kit can only be used with Qubit model 2.0/3.0.

DNA oligo to block 2S Drosophila rRNA (order from IDT, HPLC purification):

- 5’ - TAC AAC CCT CAA CCA TAT GTA GTC CAA GCA/**3SpC3**/ - 3’
- Make 100 uM stock (use info on spec sheet), then dilute 1:10 to make 10 uM stock.
- Reference: Wickersheim, Michelle L., and Justin P. Blumenstiel. "Terminator oligo blocking efficiently eliminates rRNA from Drosophila small RNA sequencing libraries."*Biotechniques*55.5 (2013): 269-272.

Day 1: Cleaning

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3 Spray a shallow tub with RNase Away, rinse with DI water, spray with 70% ethanol, and wipe dry with a Kim wipe.

4 Soak gel combs in 3% hydrogen peroxide for 15 minutes on each side.
Rinse combs and spray with 70% ethanol.

5 Spray everything else with RNase Away, rinse with DI water, and spray with 70% ethanol.

6 Set all equipment to dry on the benchtop (spread with fresh benchtop paper) overnight.
Note: It's best to begin in the late afternoon or evening so that the equipment isn't sitting out to dry when people are running around doing things in the lab.

Day 2: Running the gel

7 Turn on heat block to 65 degrees C and start thawing (on ice) 10% APS, RNA sample buffer, ladder aliquots, and RNA samples.

8 Assemble gel cassette(s):
- Place spacers, combs, and plates in position.
- Bottom spacer should stick out a bit so that it can be easily removed (with tweezers or forceps) before running the gel.
- Tape sides with gel tape.
- Use binder clips to clamp sides of cassettes. Make sure clips extend just past the bottom of the glass plates. (If they do not extend past the bottom of the glass plates, the bottom spacer will get pushed in too far. If they extend too far, the agar solution used to seal the bottom won't reach far enough up the plates to seal it properly.)
- Remove the comb from the cassette once the rest is assembled (before setting in agar). If you wait until the agar is set to remove the combs, the agar seal around the bottom of the gel cassette is likely to be disturbed.

9 Make 1% agar solution for sealing the gel:
- Add 4 g agar to 400 ml DI water.
- Microwave, swirling occasionally, until agar has dissolved.
- Let cool slightly, to around 70 degrees C, to avoid warping the bottom gel spacer.
- If you let it cool a bit too long, you can re-microwave it to re-dissolve the agar.

10 Seal the bottom of the gel cassettes with agar to avoid gel leaking out:
- Place the gel cassettes into a shallow tub.
- It is easier to pipette the gel into the cassettes if you set it up so that the front plate of each

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cassette is facing outward, and the back plates face each other.
- Pour cooled agar around the base of the cassettes.
- Wash the flask used to dissolve agar (it is hard to wash it out once it sets).
- Let agar solution around the gel cassette set until fairly firm, about 20-30 minutes.

11 Prepare a 15% denaturing urea polyacrylamide gel:

A	B	C
Reagent	**For 1 gel (50 ml)**	**For 2 gels (100 ml)**
Urea	18 g	36 g
40% polyacrylamide	18.8 ml	37.5 ml
10X TBE	2.5 ml	5 ml
NF water	Bring to 50 ml total volume	Bring to 100 ml total volume
Dissolve the above on stir plate. Assemble gel cassette(s), with comb in, and set in agar to seal bottom. Remove combs. When ready to cast gel, add:		
10% APS	500 ul	1 ml
Put on stir plate briefly to mix.		
TEMED	50 ul	100 ul
Put on stir plate briefly to mix, then promptly use 10 milliliter pipette to load. Carefully set combs, making sure there are no bubbles in the gel, especially where the wells will be formed. Get takes 5-15 minutes to polymerize.		

Important Notes!
- The gel will start to set as soon as the TEMED is added! Work quickly to load the cassette.
- There may be excess solution left after loading the gel cassette. Leave it in the beaker, and use it to check when the gel is completely set.

12 Assemble the gel apparatus:
- Set up the gel power supply.
- Assemble gel apparatus reservoirs, orienting the apparatus with the blue part of the bottom reservoir facing you. This orients the bottom reservoirs so they are facing to your right and left, and makes for for easy access to both sides for sample loading.
- Fill gel apparatus bottom with 0.5X TBE.
- Remove comb gently from gel.
- Remove cassette from agar.
- Remove binder clips and gel tape.
- Remove bottom spacer from cassette.
- Clean gel pieces from near comb and agar from near bottom.
- Load cassettes (make sure no bubbles are introduced into the bottom gap by placing cassette in at a sliding angle) and clamp to gel rig. If bubbles are introduced, you can remove them using a bent syringe.
- Make sure TBE in bottom reservoir is at least 5 mm above bottom edge of cassette.
- Fill the top of the aparatus with 0.5X TBE, covering top edge of cassette by at least 5 mm.

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13 Pre-run at a constant current of 40 mA for 30 minutes. Gel temperature should reach ~30 degrees C.

14 While gel is pre-running, prepare samples:
- Vortex and spin down samples and RNA ladder.
- Mix 30 ul sample buffer with each 30 ul sample of total RNA (total sample loading volume = 60 ul per well).
- For each ladder well used, mix 10 ul sample buffer with 10 ul aliquote of ladder (total ladder loading volume = 20 ul per well). Pipette to mix.
- Heat samples and ladder at 65 C for 10 minutes.
- Put on ice for 5 minutes.
- Vortex and spin down briefly.

15 Load samples:
- Flush urea out of all wells thoroughly.
- Flush urea out of first well (second flush). Load ladder/sample. Repeat for each well in turn.

16 Run at a constant current of 30 mA for ~1 hour, until the lower bromophenol blue bands are near the middle of the gel.

17 Once gel has finished running:
- For each gel, mix 30 ul SYBR Gold with ~300 ml 0.5X TBE in a plastic tray for staining.
- Label trays if running more than one gel.
- Place one sheet protector in each tray.

18 Remove gels from cassettes for staining:
- Stop gel power, remove top, and remove gel cassettes from apparatus.
- To remove gel from cassette, pull out spacers (can use tweezers/forceps), pry plates apart, and loosen gel with fingers.
- Be very careful not to tear the gel or to touch the lanes.
- Place quickly and gently on top of the sheet protector in plastic tub of stain.

19 Stain gel(s) on a shaker at 115 RPM, covered, for 10-15 minutes.

20 Visualize gels with a UV illuminator:
- Lift gel out of tub using the sheet protector, draining the buffer by tilting slightly.
- Lay the gel, with sheet protector underneath, on the UV light box.
- Manipulate to exclude air bubbles.
- Take a picture.

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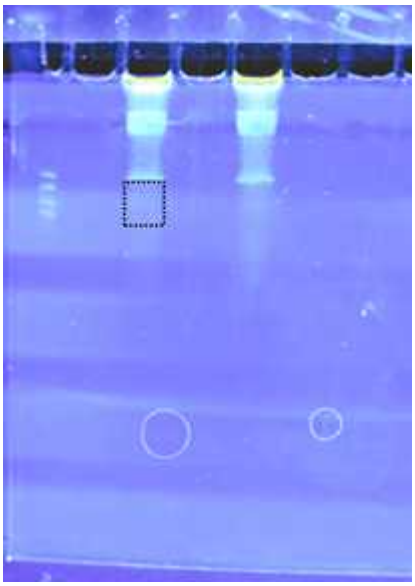
21 Excising the small RNA:

- For each lane, cut bands carefully between the 2S rRNA band and the lowest band on the ladder, using a new glass cover slip for each sample.
- Avoid collecting more gel than is absolutely necessary (excess gel will decrease total RNA yield).
- Cut up each slice into tiny pieces.
- Use the cover slip to scoop the pieces into 2 ml tubes.

Example gel photo: dotted line shows where to cut slice.



Example gel photo: dotted line shows where to cut slice.

22 Add 1.2 ml of 0.3 NaCl to each tube. Make sure slices can slosh around in the tube.

23 Rotate at room temperature overnight to elute small RNA from the gel slices.

Day 3: Precipitate small RNA

24 Transfer gel elution to fresh 1.7 ml tubes.

25 Spin at 17,000 G for 1 minute, to collect tiny gel bits at the bottom.

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26 Split each gel elution into 3 fresh tubes, 340 ul each. Some liquid will be left over; avoid taking the liquid at the bottom of spun tubes, as trying to get it results in gel fragments in your eluate.

27 Add 1 ul (20 ug) glycogen to each tube. Vortex and spin briefly.

28 Add 3 volumes of 100% ethanol. Vortex and spin briefly.

29 Incubate on ice for 1 hour.

30 Spin at 17,000 G at 4 degrees C for 15 minutes.

31 Vortex for 10 seconds to strip precipitates from the wall.

32 Spin again at 17,000 G at 4 degrees C for 15 minutes.

33 Carefully remove supernatant without touching pellet. We used a 200 uL pipette.

34 Add 900 ul of 75% ethanol, and vortex briefly.

35 Spin at 17,000 G at 4 degrees C for 5 minutes.

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36 Carefully remove almost all supernatant without touching pellet. We used a 200 uL pipette set at 200 uL.

37 Spin at 17,000 G at 4 degrees C for 1 minute.

38 Carefully remove residual ethanol. We used a 20 uL pipette.

39 Air dry (~2 minutes) and dissolve pellets sequentially in the 3 tubes of each sample with the same 7 ul of water:
- Each sample is split into 3 tubes (A, B, and C).
- Add 7 ul NF water to tube C. Let pellet dissolve (~2 minutes).
- Transfer contents of tube C to tube B. Let pellet in tube B dissolve (~2 minutes).
- Transfer contents of tube B to tube A. Let pellet in tube A dissolve (~2 minutes).

40 Final volume of each sample will be 7 ul. Store at -80 degrees C.

41 When ready, submit all 7 ul for sequencing and quality assessment. (1-2 ul for quality assessment on the Qubit, and 5 ul for library prep and sequencing)

Appendix 1: Library prep information

42 Prepare an aliquot of 2S block oligo stock at 10 uM. Use 1-2 ul per sample during library prep.

43 For the Illumina TruSeq library prep protocol, add the block oligo to the completed 3' ligation reaction, before 5' ligation. Example:
- Add block to completed 3' ligation reactions
- Incubate at 90 degrees C for 30 seconds
- Incubate at 65 degrees C for 10 minutes
- Incubate at 4 degrees C for at least 30 seconds
- Proceed to ligation of 5' adapters

Appendix 2: Recipes and dilutions

44 Stocks needed for RNA sample buffer:

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A	B	C	D	E
Reagent	**grams**	**NF water (ml)**	**This makes stock concentration:**	**Notes**
SDS	1	10	10%	None
Bromophenol Blue	0.25	10	2.5%	Pass through a 0.2 um syringe filter after dissolving
Xylene cyanol	0.25	10	2.5%	Pass through a 0.2 um syringe filter after dissolving

45 RNA denaturing sample buffer (10 ml):

A	B	C
Recipe calls for:	Stock concentration:	Use this much of stock:
47.5% formamide	ultrapure (100%)	4.75 ml
0.01% SDS	10%	10 ul
0.01% Bromophenol Blue	2.5%	40 ul
0.005% xylene cyanol	2.5%	20 ul
0.5 mM EDTA	0.5 M	10 ul

Bring volume to 10 ml with NF water.
Make 1 ml aliquots and store at -20 degrees C.

46 **10% APS (10 ml):**

- Dissolve 1 g APS in 10 ml dH20
- Make 1 ml aliquots and store at -20 degrees C.
- Use *fresh* APS or the gel will not polymerize!

47 **10X TBE (1L):**

- 108 g Tris Base
- 55 g boric acid
- 40 ml 0.5 M EDTA (pH 8.0)

Ensure an RNA-free buffer by cleaning beakers, bottles, stir bars, etc. as described in Section "Day 1: Cleaning".

Add the above to 800 ml dH20. Add after stir bar is already stirring, to avoid clumps.
Stir until dissolved (this may take a while).
Adjust to 1L with additional dH20.
Store at room temperature.

Note: for 1L of 0.5X TBE use 50 ml of 10X TBE.

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48 **0.5 M EDTA (pH 8.0) (1 L):**

- 186 g disodium EDTA (Na_2EDTA)
- 800 ml dH20

Adjust the pH to 8.0 with NaOH (~50 ml). The disodium salt of EDTA will not dissolve until the pH of the solution is adjusted to 8.0 with NaOH.

Sterilize by autoclaving. Store at room temperature.

49 **0.3 M NaCl (10 ml):**

Add 600 ul of 5M NaCl to 9.4 ml NF water.

References

50 Zamore Lab Illumina TruSeq Small RNA Cloning Protocol https://www.dropbox.com/s/r5d7aj3hhyaborq/2018%20Zamore%20Lab%20Illumina%20TruSeq%20Small%20RNA%20Library%20Protocol%20with%20UMI%20adapters.pdf?dl=0

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