S1 Methods - Small RNA library protocol with blocking oligos for *Drosophila melanogaster*

(i) Using the blocking oligos, and ligating 3’ adapter

- Use at least 1 µg of RNA sample, in no more than 10 µl.
- Use 10 pmol of blocker oligo (so it is the equivalent amount to 3’ adapter), and add this before 3’ adapter ligation.

*Note: if 3’ adapter stock is not already adenylated, refer to section xii*

### Add blocker. Mix in 0.2 ml PCR tube:

| Component                                           | Volume  |
|-----------------------------------------------------|---------|
| RNA sample                                          | 10 µl   |
| Blocker oligo (at 10 µM concentration)              | 1 µl    |
| NEB T4 RNA ligase buffer                            | 2 µl    |
| Riboguard RNase inhibitor                           | 0.75 µl |
| PEG8000 (warm to 37°C first to get rid of cloudiness, then keep at room temperature) | 4 µl |

- Incubate in PCR machine: 70°C for 2 mins, 60°C for 5 mins

### Ligate 3’ adapter. To the above mix, add:

| Component                                           | Volume                                      |
|-----------------------------------------------------|---------------------------------------------|
| pre-adenylated 3’ adapter *(see section xii details)* | 10 pmol (volume depends on concentration)   |
| NEB truncated T4 RNA ligase                         | 1 µl                                        |

- Incubate in the PCR machine: 26°C for 2 hours

(ii) Clean up using Zymo kit

Use RNA cleaning and concentrating kit (Zymo, R1018 – Cambridge Bioscience) to clean up reaction.

### In 0.5 ml tube:

| Step                                                   | Action                                                                 |
|--------------------------------------------------------|------------------------------------------------------------------------|
| Make volume up to 50 µl total with H₂O                |                                                                        |
| Add 100 µl RNA binding buffer                         |                                                                        |
| Add 150 µl 100% ethanol                               |                                                                        |
| Transfer to spin column and spin at 12,000 rpm for 1 minute |                                                              |
| Discard flow-through and add 400 µl RNA prep buffer. Spin at 12,000 rpm for 1 minute |
Discard flow-through and add 800 µl RNA wash buffer and spin at 12,000 rpm for 30 seconds

Repeat this wash step with 400 µl wash buffer

Discard flow-through and spin at 12,000 rpm for 2 minutes

Transfer column to clean tube and elute with 13 µl H₂O by spinning at 12,000 rpm for 1 minute.

(iii) Removing the 3’ adapter

Add the following to the 12.1 µl eluted RNA in a 0.2 ml PCR tube:

| Mix in a 0.2 ml tube:              |         |
|-----------------------------------|---------|
| Eluted RNA                        | 12.1 µl |
| 10x deadenylase buffer            | 1.6 µl  |
| (available from Cambio, DA11101K) |         |
| 100 mM DTT                        | 0.8 µl  |
| Riboguard RNase inhibitor         | 0.5 µl  |
| (available from Cambio, RG90925)  |         |
| Scriptminer finishing enzyme      | 1 µl    |
| Incubate at 30°C for 30 minutes   |         |
| Add 4 µl Scriptminer stop solution|         |

Now mix the above reaction with the following:

| 3’ adapter degradation:          |         |
|----------------------------------|---------|
| Scriptminer degradase buffer     | 2 µl    |
| (OR 500 mM Tris-HCl pH9.0)       |         |
| Scriptminer MgCl₂                | 7 µl    |
| Scriptminer degradase enzyme     | 1 µl    |
| Incubate at 37°C for 30 minutes  |         |

The entire product of this reaction is to be used in the next 5’ adapter ligation step.
(iv) 5’ adapter ligation

Use a total of 20 pmol of 5’ adapter per reaction.

| Denature the 5’ adapter by heating at 70°C for 2 minutes, then place on ice. |
|---------------------------------|------------------|
| Entire RNA sample following the previous degradase reaction | 30 µl |
| Scriptminer 5’-RNA ligation buffer | 1 µl |
| 10 mM ATP | 1 µl |
| Denatured 5’ adapter (10 µM) | 2 µl |
| Scriptminer 5’ RNA ligase | 1 µl |
| 50% PEG | 7 µl |

**Incubate reaction at 26°C for 2 hours**

**Add 8 µl H₂O to make up the total volume to 50 µl**
(v) **Clean up using Zymo kit**

| Step                                                                 | Description |
|----------------------------------------------------------------------|-------------|
| Transfer the 50 µl of ligated sample to a 0.5 ml tube               |             |
| Add 100 µl RNA binding buffer                                       |             |
| Add 150 µl 100% ethanol                                             |             |
| Transfer to Zymo spin column and spin at 12,000 rpm for 1 min       |             |
| Discard flowthrough                                                 |             |
| Add 400 µl RNA prep buffer and spin at 12,000 rpm for 1 min         |             |
| Discard flowthrough                                                 |             |
| Add 800 µl RNA wash buffer and spin at 12,000 rpm for 30 secs       |             |
| Repeat wash step with 400 µl RNA wash buffer                       |             |
| Discard flowthrough and spin at 12,000 rpm for 2 minutes           |             |
| Transfer column to an RNase free tube                              |             |
| **Elute the samples TWICE using 15 µl H₂O each time (to end up with ~30 µl sample)** |             |

(vi) **cDNA synthesis**

The RNA is now tagged with 3’ and 5’ adapters. To convert to cDNA mix together the following:

| Component                                      | Volume |
|------------------------------------------------|--------|
| **di-tagged RNA sample**                       | 30 µl  |
| MMLV reverse transcription buffer              | 4 µl   |
| dNTP PreMix                                    | 2 µl   |
| DTT                                            | 2 µl   |
| RTP primer                                     | 1 µl   |
| Scriptminer MMLV reverse transcriptase         | 1 µl   |

**Incubate at 37°C for 20 minutes**

**Terminate by incubating at 85°C for 15 minutes, then keep on ice.**
(vii) PCR Amplification, round 1

Use 4 µl of the cDNA to run a 20 µl PCR reaction.

Note: for each different sample, use a unique index primer

For each sample, run 3 different PCR cycle numbers in order to optimise the reaction. As a guide, use 5, 7 and 9 cycles for the first attempt (if there is no contaminating 30mer band, the cycle number can be increased).

| 1 reaction                           |
|-------------------------------------|
| H₂O                                 | 9.3 µl |
| 10 mM dNTPs                         | 0.5 µl |
| 5x high fidelity Phusion buffer     | 4 µl   |
| Illumina RP1 primer (10 µM)         | 1 µl   |
| Illumina index primer (10 µM)       | 1 µl   |
| cDNA                                | 4 µl   |
| Phusion high fidelity DNA polymerase (NEB biolabs #M0530S) | 0.2 µl |

(viii) Run PCR reaction on 8% PAGE gel

| 8% PAGE gel                        | 2 gels | 4 gels | 6 gels |
|------------------------------------|--------|--------|--------|
| H₂O                                | 10 ml  | 20 ml  | 30 ml  |
| 40% (19:1) acrylamide/bis solution | 3 ml   | 6 ml   | 9 ml   |
| 5x TBE                             | 1.5 ml | 3 ml   | 4.5 ml |
| 10% ammonium persulphate           | 150 µl | 300 µl | 450 µl |
| TEMED                              | 7.5 µl | 15 µl  | 22.5 µl |

- Use one gel per sample.
- Mix 20 µl PCR product with 5 µl 5x Novex loading dye.
• Load a total of 20 µl in each well, and then mix what is left over from each tube and load that in a fourth lane.
• Load 10 µl of 20 bp ladder (Jena Bioscience) either side of the sample lanes.
• Run the gel in 0.5x TBE buffer for 2-2.5 hours at 120V
• Stain the gel with SYBR gold (5 µl in ~50 ml of 0.5x TBE) and scan.
• Print the gel images off in real-size.

(ix) Gel Extraction

• Prepare 0.5 ml tubes by punching 4 holes in the bottom of each with a 21 gauge needle. Put each 0.5 ml tube inside a 2 ml “collection” tube. Use one tube for each gel (or sample).
• Lay the gels over the real-size print-outs and use a razor blade to cut out the area containing the band of interest (re-scan the gels afterwards to check the correct area has been excised).
• Put the slice from each gel into a prepared 0.5 ml tube. Spin the tubes at max speed for 3 minutes to shred the gel slice.
• Discard the 0.5 ml tube. Add 400 µl NEB2 buffer to the broken gel and incubate overnight, shaking at 4°C.
• Following the overnight incubation, transfer the gel mixture to a Spin-X column (0.45 µm, ThermoFisher), and spin at 2800 RPM for 3 mins, to remove gel debris.
• To 400 µl eluate, add 2 µl glycogen, 40 µl of 3M sodium acetate, and 1200 µl 100% ethanol
• Incubate at -80°C for 20-30 minutes
• Spin at 4°C, 20000 RPM for 20 minutes
• Remove supernatant, and wash the pellet in 500 µl 70% ethanol
• Spin at room temp, 13000 RPM for 2 mins
• Remove supernatant and re-suspend pellet in 13 µl H₂O
(x) PCR amplification, round 2

Take 1 µl of the gel extraction to run another PCR, again with the 3 different cycle numbers:

|                         | 1 reaction |
|-------------------------|------------|
| H₂O                     | 12.3 µl    |
| 10 mM dNTPs             | 0.5 µl     |
| 5x high fidelity Phusion buffer | 4 µl       |
| Illumina RP1 primer (10 µM) | 1 µl       |
| Illumina index primer (10 µM) | 1 µl       |
| cDNA                    | 1 µl       |
| Phusion DNA polymerase   | 0.2 µl     |

- Following this 2nd round of PCR, decide which cycle number gives you the best band. This cycle number needs to be used in the final amplification.
- Repeat the gel extraction as detailed above.

(xii) Final PCR

Set up the final PCRs, using the chosen cycle number for each sample, and set up 7 identical reactions per sample:

|                         | 1 reaction |
|-------------------------|------------|
| H₂O                     | 12.3 µl    |
| 10 mM dNTPs             | 0.5 µl     |
| 5x high fidelity Phusion buffer | 4 µl       |
| Illumina RP1 primer (10 µM) | 1 µl       |
| Illumina index primer (10 µM) | 1 µl       |
| cDNA                    | 1 µl       |
| Phusion DNA polymerase   | 0.2 µl     |

- Run all 7 of the reactions from each sample on one gel, loading the spare 8th lane with the leftovers.
• Once the gel has run, gel extract across all 8 lanes using the protocol above, and re-suspend the pellet in final volume of ~10-12 µl

(xii) Adenylation of the 3’ HD adapter (if necessary)

*Note: Also check that the 3’ HD adapter stock is already phosphorylated.*

Use 200 pmol of 3’ adapter per 40 µl reaction (this should give enough 3’ adapter to make about 20 libraries)

### Adenylate 3’ adapter. Mix in a tube:

| Component                                      | Volume |
|------------------------------------------------|--------|
| 3’ adapter                                     | 2 µl   |
| 10x 5’ DNA adenylation reaction buffer (NEB #B2610S)* | 4 µl   |
| 1 mM ATP (NEB #N0757A)*                        | 4 µl   |
| Mth RNA ligase (NEB #M2611A)*                  | 4 µl   |
| Nuclease-free H₂O                              | 26 µl  |

- Incubate for 1 hour at 65°C, then 5 mins at 85°C

### Phenol chloroform extract the adapter:

| Component                   | Volume |
|-----------------------------|--------|
| Adenylated adapter          | 40 µl  |
| H₂O                         | 60 µl  |
| Phenol chloroform           | 100 µl |

- Vortex and spin at 13000 rpm for 15 minutes
- Transfer aqueous layer to new tube

### Ethanol precipitation:

| Component        | Volume |
|------------------|--------|
| Aqueous phase    | Approx. 75 µl |
| H₂O              | 25 µl  |
| Glycogen         | 2 µl   |
| 3M sodium acetate| 10 µl  |
| 100% ethanol     | 250 µl |

- Incubate overnight at -20°C

### Obtain pellet and wash:

- Spin reaction at 13000 rpm for 20 minutes @ 4°C
- Remove supernatant
Wash pellet with 500 µl 80% ethanol

Spin at 13000 rpm for 5 minutes

Remove ALL supernatant (allow to air for a bit if necessary, but be careful to not let pellet completely dry)

Resuspend pellet in 12 µl of Ambion RNA storage solution.

*ORDERED AS A KIT FROM NEW ENGLAND BIOLABS CAT# E2610S

Running 3’ adapter on PAGE gel

| 16% PAGE urea gel | For 1 gel: | For 2 gels: |
|-------------------|-----------|-------------|
|                   | 10 ml     | 15 ml       |
| Urea*             | 4.2 g     | 6.3 g       |
| H₂O*              | 2.5 ml    | 3.5 ml      |
| 40% (19:1) acrylamide/bis solution | 4 ml | 6 ml |
| 5x TBE            | 1 ml      | 1.5 ml      |
| 10% ammonium persulfate (APS) | 100 µl | 150 µl |
| TEMED             | 5 µl      | 7.5 µl      |

*Dissolve the urea in the H₂O first by heating in a falcon tube in the microwave for 10 second bursts at a time, then add remaining ingredients to this solution.

- To check for successful 3’ adenylation, run adenylated 3’ adapter on a PAGE gel (16% urea) against non-adenylated 3’ adapter (the stock) and a Scriptminer control.
- Use ~1 pmol of adenylated and non-adenylated adapter. Also mix 1 pmol each of adenylated and non-adenylated together and run this between the other two samples.
- Run 0.5 µl of a 1:10 dilution of Scriptminer 3’ adapter as a control.
- Load a total volume of 10 µl. Use 2x denaturing (formaldehyde) loading dye.
- Run at ~120V in 0.5x TBE for 2.5-3 hours.
- Stain the gel with SYBR gold and view using a scanner.
- The adenylated adapter should be slightly larger than the non-adenylated. You should see two distinct bands in the mixed sample. The Scriptminer band should be smaller than the others.
• Note: You might see a smaller faint band in the 'adenylated' adapter sample, indicating some non-adenylated adapter remaining. This doesn't matter, the adapter is still OK to use as long as most of it is adenylated.