Chapter 18

Immunoprecipitation of piRNPs and Directional, Next Generation Sequencing of piRNAs

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Abstract

Piwi interacting RNAs (piRNAs) are small (~25 to ~30 nucleotide) and are expressed in the germline. piRNAs bind to the Piwi subclade of Argonaute proteins and form the core ribonucleoproteins (RNPs) of piRNPs. We describe a method for the massive identification of piRNAs from immunopurified piRNPs. This strategy may also be used for immunopurification and directional sequencing of RNAs from other RNPs that contain small RNAs.

Key words: piRNA, piRNP, Argonaute, Piwi, Xili, Xiwi, Mili, Miwi, Miwi2, Aub, Ago3, Y12, Next gen sequencing, Illumina, cDNA, Immunoprecipitation, RNA-Immunoprecipitation, RNA-IP, T4 RNA ligase, Reverse Transcriptase, Polymerase Chain reaction, PCR, RT-PCR, Posttranscriptional RNA processing, Gene silencing

1. Introduction

Piwi interacting RNAs (piRNAs) comprise a class of ~25 to ~30 nucleotide (nt) RNAs, which that are expressed in germline cells and bind to the Piwi subclade of Argonaute proteins. Mice express three Piwi proteins termed Mili (1), Miwi (2), and Miwi2 (3). *Drosophila melanogaster* expresses three Piwi proteins termed Aubergine (Aub) (4), Piwi (5), and Ago3 (6–8). *Xenopus tropicalis* and *X. laevis* express three Piwi proteins termed Xili, Xiwi, and Xiwi2 (9, 10). The sequence diversity of piRNAs is tremendous and hundreds of thousands of unique piRNAs have been described in diverse species (11). Many piRNAs are derived from transposable or repetitive elements and also target transposons by antisense complementarity (12). Many genic and intergenic regions

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also give rise to piRNAs but the significance of these piRNAs remains to be determined.

Here, we describe a method for the immunoprecipitation of piRNPs and the isolation and directional sequencing of associated small RNAs. This approach combines techniques developed for the immunoprecipitation of RNPs (13, 14) with techniques developed for the directional adapter ligation to small RNAs (15) and their identification by Illumina next generation sequencing. In principle, this methodology may be used for the immunopurification and directional sequencing of RNAs from any RNP that contains small RNAs.

2. Materials

1. Germline tissue (such as mouse testis; *X. laevis* oocytes or testis; *D. melanogaster* ovaries or testis).
2. PBS (Fisher).
3. Lysis buffer: 20 mM Tris–HCl, pH 7.4, 200 mM sodium chloride, 2.5 mM magnesium chloride, 0.5% NP-40, 0.1% Triton X-100, one tablet of Complete Protease Inhibitor EDTA-free (Roche) per 50 ml of lysis buffer.
4. RNasin (Promega).
5. Recombinant Protein G Agarose beads (Invitrogen).
6. Sonicator (Sonics Vibra-Cell or equivalent).
7. dNTPs (Roche).
8. ATP (Roche).
9. Millipore (MilliQ) water.
10. T4 RNA Ligase (New England Biolabs-NEB).
11. pBR322 DNA-Msp I Digest (DNA markers; NEB).
12. DNA polymerase I, large (Klenow) fragment (NEB).
13. T4 polynucleotide kinase (T4 PNK; NEB).
14. Calf intestinal alkaline phosphatase (CIP; NEB).
15. Complete protease inhibitor EDTA-free (Roche).
16. Nonimmune mouse serum or mouse IgG.
17. Anti-piRNP antibodies. For example, Y12 monoclonal antibody (Abcam, ab3138); see Note 1.
18. RNA loading buffer 1–2× (95% formamide, 18 mM EDTA, Xylene cyanol, bromophenol blue; Ambion).
19. Glycogen (at 5 mg/ml from Ambion).
20. Ethanol, 100%.

21. 3 M sodium acetate, pH 5.2.
22. 10× TBE (Ambion).
23. Acrylamide/Bis 19:1 40% (w/v) solution (Ambion).
24. Urea (Ambion).
25. 10% (w/v) ammonium persulfate (APS; dissolved in water).
26. TEMED (Biorad).
27. 10% Urea/PAGE solution (1 l): combine in a glass beaker: 480 g urea, 250 ml of 40% acrylamide/Bis (19:1), 100 ml 10× TBE, and water up to 1 l. Stir until completely dissolved, filter-sterilize, and store up to a year at RT in an aluminum-covered bottle (to protect from light).
28. 20% Urea/PAGE (1 l): combine in a glass beaker: 480 g urea, 500 ml of 40% acrylamide/Bis (19:1), 100 ml 10× TBE, and water up to 1 l. Stir until completely dissolved, filter-sterilize, and store as described above.
29. SE 400 Sturdier Gel electrophoresis apparatus with 18 × 24 cm glass plates (Amersham).
30. [γ-^{32}P] ATP at 3,000 Ci/mmol, 10 mCi/ml (NEN).
31. [α-^{32}P] dCTP, at 3,000 Ci/mmol, 10 mCi/ml (NEN).
32. Phenol/chloroform/isoamyl alcohol (25:24:1); pH 7.9 (Fisher).
33. Elution buffer (0.1% SDS, 0.3 M NaOAC, 100 μM EDTA).
34. Ethidium bromide (10 mg/ml).
35. Superscript II reverse transcriptase (RT; Invitrogen).
36. Trizol (Invitrogen).
37. Isopropanol.
38. Chloroform.
39. OR2– buffer (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM Na_2HPO_4, 5.0 mM HEPES (pH 7.8)) (1 l). Mix 20.6 ml of 4 M NaCl, 1.25 ml of 2 M KCl, 10 ml of 100 mM Na_2HPO_4, 10 ml of HEPES, 500 mM, pH 8.3, and MilliQ water to 1,000 ml.
40. OR2+ buffer (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM Na_2HPO_4, 5.0 mM HEPES (pH 7.8), 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$) (1 l). Mix 20.6 ml of 4 M NaCl, 1.25 ml of 2 M KCl, 10 ml of 100 mM Na_2HPO_4, 10 ml of HEPES, 500 mM, pH 8.3 1 ml $MgCl_2$, 10 ml of 100 mM $CaCl_2$, and MilliQ water to 1,000 ml.
41. BSA (Sigma A9418).
42. Soybean trypsin inhibitor type II-S (Sigma T9128).
43. Collagenase type IA (Sigma C9891).

44. Collagenase stock solution (0.25% BSA, 0.25% soybean trypsin inhibitor type II-S, 5% collagenase type IA in OR2–) (20 ml): Dissolve 1 g of collagenase powder, 200 mg BSA powder, 200 mg soybean trypsin inhibitor type II-S powder in 20 ml OR2–. Mix well and store at –20°C in 1.2 ml aliquots.
45. Collagenase working solution: To 1.2 ml of collagenase stock add 1.8 ml of OR2–; total volume is 3 ml. Final concentration of collagenase in working solution is 2 mg/ml.
46. Small RNA Sample Prep Kit, version 1.5 (Illumina, catalog # FC-102-1009; v1.5). This kit contains the 5′- and 3′-adapters, polymerase chain reaction (PCR) primers (sequences are indicated below), the enzymes, and other consumables. The oligonucleotide sequences are protected by copyright, which is owned by Illumina. Oligonucleotide sequences © 2006 and 2008 Illumina, Inc.

5′-RNA adapter (SRA 5): 5′ GUUCAGAGUUCUACAGUC CGACGAUC.

3′-RNA adapter (SRA 3): 5′ P-UCGUAUGCCGUCUUCU GCUUGU.

RT Primer: 5′ CAAGCAGAAGACGGCATACGA.

Small RNA PCR Primer 1 (GX1): 5′ CAAGCAGAAGACG GCATACGA.

Small RNA PCR Primer 2 (GX2): 5′ AATGATACGGCGA CCACCGACAGGTTCAGAGTTCTACAGTCCGA.

Sequencing Primer: 5′ CGACAGGTTCAGAGTTCTACAGT CCGACGATC.

3. Methods

The outline of the procedures and representative gels are shown in Fig. 1. All procedures and centrifugations are performed on ice or at 4°C unless otherwise indicated. Use RNase-free solutions, tubes, and pipettes (see Note 2).

3.1. piRNP Immunoprecipitation

3.1.1. Binding of Antibodies to Protein-G Agarose Beads

1. Bind the Y12 monoclonal antibody or nonimmune mouse serum (IgG; serves as negative control) on protein G agarose beads. For this, use 30 µl of bed volume of protein G agarose beads per immunoprecipitate. Resuspend, and wash the beads three times with 1 ml of lysis buffer (see Note 1).
2. Aspirate the last wash, while taking care not to dry the beads. Add 1 ml of lysis buffer and 5 µl of Y12 ascites (or 5 µl nonimmune mouse serum for the control immunoprecipitate).

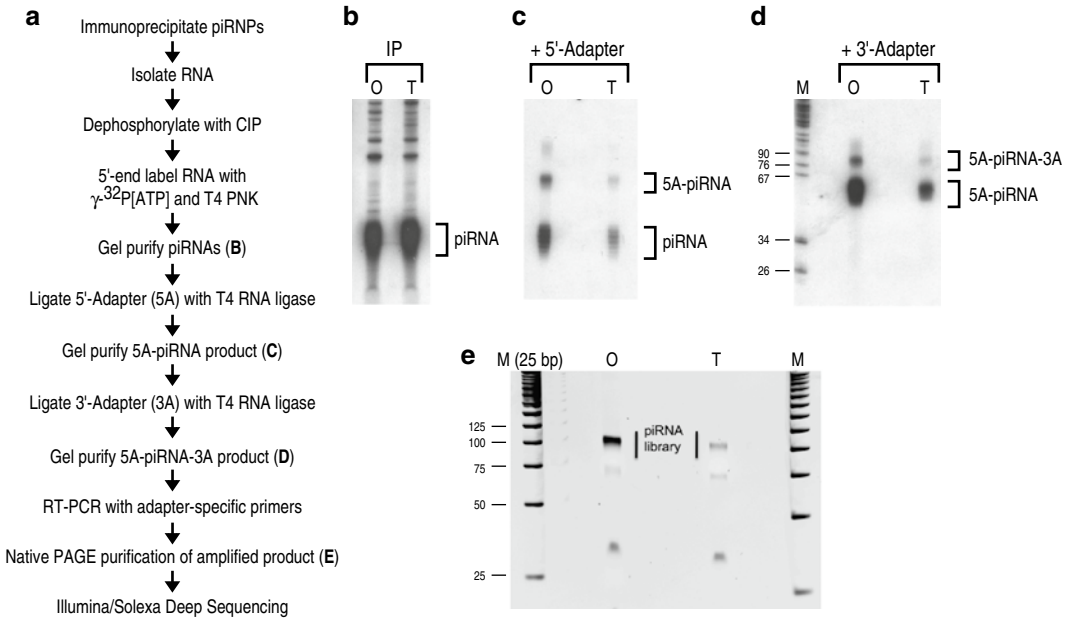

Fig. 1. piRNP immunoprecipitation, directional adapter ligation, and piRNA library preparation. (**a**) Outline of the experimental steps. (**b**) Immunoprecipitations (IP) were performed from Xenopus laevis defolliculated oocytes (O) or testis (T) cells with the Y12 monoclonal antibody that IPs X. laevis Piwi proteins. RNA was isolated, dephosphorylated with CIP, 5′-end radiolabeled with [γ-^{32}P] ATP and T4 PNK and analyzed on a 15% urea/PAGE. (**c**) A 5′-adapter (SRA-5; 5A) was ligated to gel-purified piRNAs with the T4 RNA ligase, and the ligation reaction was resolved on 15% urea/PAGE. 5A-piRNA ligated products and unligated piRNAs are indicated. (**d**) A 3′-adapter (SRA-3; 3A) was ligated to gel-purified 5A-piRNA with T4 RNA ligase and the ligation reaction was resolved on 15% urea/PAGE. The final 5A-piRNA-3A ligation products and the unligated 5A-piRNA are indicated. The marker (M) is a radiolabeled pBR322 DNA-Msp I digest. (**e**) The gel-purified 5A-piRNA–3A RNA product was used for RT-PCR and the resulting piRNA library was analyzed by 8% native PAGE and visualized by ethidium bromide staining. The DNA band corresponding to the piRNA library (indicated) was gel purified and used for Illumina sequencing.

3. Rotate for 45 min.
4. Wash the antibody-beads three times with 1 ml lysis buffer by centrifuging at 2,300×*g* for 5 s and by aspirating.

3.1.2. Preparation of the Tissue Lysate

1. The tissue lysate may be prepared while the antibodies are binding to the protein G agarose beads. Wash the tissue with PBS. Resuspend the tissue (such as defolliculated *X. laevis* oocytes, or minced *X. laevis*, or mouse testis) in 1 ml of lysis buffer (see Notes 3 and 4). Add RNasin to a final concentration of 0.1 U/μl.
2. Sonicate briefly (three times, 8–10 s each) using 40% output (on a Sonics Vibra-Cell sonicator or the equivalent).
3. Centrifuge the cell lysate at 20,000×*g* (16,000×*g* in an Eppendorf microcentrifuge) for 20 min.
4. Collect the supernatant and save an aliquot as a reference for total lysate proteins and discard the pellet.

3.1.3. Immunoprecipitation and RNA Isolation

1. Use 1/2 of the supernatant with your specific antibody-beads (i.e., Y12-beads from Subheading 3.1.1) and use the other 1/2 with the negative control antibody. Perform the immunoprecipitation in microcentrifuge tubes. If the volume of the immunoprecipitate is less than 1 ml, complete to 1 ml with lysis buffer. Rotate in the cold room for 1 h.
2. Wash the beads five times with 1 ml lysis buffer.
3. Add 500 µl of Trizol reagent to the washed beads. Vortex for 30 s to 1 min. Let the tube sit at room temperature (RT) for 5 min.
4. Add 150 µl of chloroform and vortex briefly. Let the tube sit at room temperature (RT) for 2 min.
5. Spin at 20,000 × *g* for 20 min at RT.
6. Collect the upper aqueous phase (avoid the interphase; the recovered volume will be approximately 300 µl). Add 3 µl of glycogen (5 mg/ml) and vortex briefly.
7. Add 350 µl of isopropanol and vortex briefly. Place the tube at −20°C for 20 min.
8. Spin at 20,000 × *g* for 30 min at 4°C.
9. Carefully aspirate the supernatant and let the pellet air dry (see Note 5).
10. Resuspend the pellet in 21.5 µl of MilliQ water. Proceed with piRNA isolation or store the RNA at −80°C (see Note 6).

3.2. piRNA Isolation

3.2.1. Dephosphorylation

1. Combine the following (total reaction volume 25 µl):

RNA	21.5 µl
10× NEB3 buffer	2.5 µl
CIP alkaline phosphatase	1 µl

2. Incubate at 37°C for 30 min.
3. Add 175 µl of water. Add an equal volume (200 µl) of phenol/chloroform/isoamyl alcohol and vortex for 30 s to 1 min.
4. Spin at 20,000 × *g* for 2 min at RT. Collect the upper (aqueous) phase and extract again by adding an equal volume of phenol/chloroform/isoamyl alcohol. Vortex for 30 s to 1 min and spin at 20,000 × *g* for 2 min at RT.
5. Collect the upper (aqueous) phase, add 2 µl glycogen (5 mg/ml), and 20 µl of 3 M sodium acetate (NaOAC), pH 5.2. Add 550 µl of ice-cold 100% ethanol, mix, and place at −80°C for 30 min.
6. Spin at 20,000 × *g* for 30 min at 4°C.

7. Carefully aspirate the supernatant and wash the pellet with 500 µl of ice-cold 70% ethanol. Spin at 20,000 × *g* for 10 min at 4°C.
8. Carefully aspirate the supernatant and air-dry the pellet.
9. Resuspend the pellet in 12 µl of MilliQ water.

3.2.2. 5′-End Labeling of RNA

1. For each labeling reaction combine (for a total reaction volume 15 µl):

RNA (CIP-treated)	10.5 µl
10× T4 PNK buffer	1.5 µl
[γ-^{32}P] ATP	2 µl
T4 PNK	1 µl

2. Incubate at 37°C for 1 h. Add 15 µl of RNA loading buffer.

3.2.3. piRNA Gel Purification

1. To prepare 15% urea/PAGE simply make a 1:1 mix of 10 and 20% urea/PAGE solutions.
2. Cast the gel apparatus using 0.75-mm combs and 18 × 24 cm glass plates. Dispense 40 ml of 15% urea/PAGE solution in a 50-ml Falcon tube. To polymerize, add 200 µl of 10% APS and 30 µl of TEMED, mix well, and immediately pour the gel.
3. Load most, or all of the labeled RNA. Also load radio-labeled DNA or RNA markers (see below for the preparation of radiolabeled DNA markers) and the RNA from the negative control antibody. There is no need to heat the RNA prior to loading. Run the gel until the bromophenol blue dye is at the bottom of the gel.
4. Disassemble the glass plates and lift the gel on a piece of old, exposed film. Cover with Saran wrap and expose the wet gel to a film by placing it in a cassette, between two intensifying screens, at –80°C. Use a radioactive pen or other means (e.g., preflashing) to align the gel with the film. Exposure time varies with the amount of RNA precipitated and loaded on gel. Usually ~5 h is sufficient.
5. Excise the gel piece corresponding to labeled piRNAs with a clean razor blade and place it in a microcentrifuge tube.
6. Add 400 µl of elution buffer and incubate at 37°C for 12–16 h.
7. Collect the elution buffer and place in the Illumina Spin-X cellulose acetate column. Centrifuge for 2 min at maximum speed in a table-top centrifuge at RT.
8. Add 4 µl of glycogen and 1 ml of ice-cold 100% ethanol. Place at –80°C for 30 min.

9. Spin at 20,000 × *g* for 30 min at 4°C.
10. Carefully aspirate the supernatant and wash the pellet with 500 µl of ice-cold 70% ethanol. Spin at 20,000 × *g* for 10 min at 4°C.
11. Carefully aspirate the supernatant and air-dry the pellet (do not over-dry).
12. Resuspend the pellet in 6 µl MilliQ of water.

3.3. Ligation of Adapters to piRNAs

The following steps (subheadings 3.3 and 3.4) are performed with adapters and reagents provided by the "Small RNA Sample Prep Kit" from Illumina. The 5′-adapter (5′-SRA) is 26 nucleotides and the 3′-adapter (3′-SRA) is 22 nucleotides. The sequence of the adapters and primers is shown in Subheading 2.

3.3.1. 5′ Adapter Ligation and Purification

1. Set up the following 10 µl reaction using the reagents provided with the Illumina kit:

RNA (gel-purified piRNA)	5.7 µl
SRA 5¢ adapter	1.3 µl
10× T4 RNA ligase buffer	1.0 µl
RNAse OUT	1.0 µl
T4 RNA ligase	1.0 µl

2. Incubate at 4°C for 16 h.
3. Add 10 µl of the SRA gel-loading dye to the ligation reaction.
4. Gel-purify the 5′-ligated piRNA product as above – the band should be approximately 56 nt long. After the gel purification, resuspend the eluted RNA in 7 µl of MilliQ water.

3.3.2. 3′ Adapter Ligation and Purification

1. Set up the following 10 µl reaction using the reagents provided with the Illumina kit:

RNA (5′-SRA-piRNA)	6.4 µl
SRA 3′ adapter	0.6 µl
10× T4 RNA ligase buffer	1.0 µl
RNAse OUT	1.0 µl
T4 RNA ligase	1.0 µl

2. Incubate at 4°C for 16 h.
3. Add 10 µl of SRA gel-loading dye to the ligation reaction.
4. Gel-purify the 5′, 3′-ligated piRNA product as above – the band should be approximately 78 nt long. After the gel purification, resuspend the eluted RNA in 5 µl of MilliQ water.

3.4. Amplification and Purification of Adapter-Ligated piRNAs

3.4.1. Reverse Transcription

1. Combine the following:

RNA	4.5 µl
SRA RT primer	0.5 µl

2. Heat the above mix at 65°C for 10 min. Place on ice and spin.
3. Add to the above, while still on ice:

5× First strand buffer	2 µl
DTT (0.1 M)	1 µl
RNase OUT	1 µl
dNTPs (12.5 mM)	0.5 µl

4. Heat the above mix at 48°C for 3 min.
5. Add 1 µl of SuperScript II reverse transcriptase and incubate at 44°C for 1 h.
6. Inactivate the enzyme by incubating at 65°C for 20 min. Chill on ice, spin down briefly, and store at –20°C or proceed with the PCR.

3.4.2. PCR

1. For a 50-µl reaction, assemble in a thin-walled PCR tube the following:

Water	28 µl
5× Phusion HB buffer	10 µl
dNTPs (25 mM)	0.5 µl
Primer GX1	0.5 µl
Primer GX2	0.5 µl
Phusion DNA polymerase	0.5 µl
Template (RT reaction)	10 µl

2. Perform the following PCR program:
   (a) 98°C for 30 s
   (b) 15 cycles of:
   98°C for 10 s
   60°C for 30 s
   72°C for 15 s
   (c) 72°C for 10 min
   (d) 4°C hold
3. Following the PCR, add 10 µl of 6× DNA loading buffer. The total volume is now 60 µl.

3.4.3. Native PAGE Purification of the PCR-Amplified piRNA Library

1. Prepare an 8% native PAGE gel as follows:

MilliQ water	17.5 ml
10× TBE	2.5 ml
40% of 19:1 polyacrylamide:bis	5 ml
20% APS	20 µl
TEMED	20 µl

2. Run the entire piRNA library sample (60 µl) on gel using the provided 25 bp marker as a reference.
3. Disassemble the gel and stain in 50 ml of 1× TBE with 5 µl of ethidium bromide (10 mg/ml) for 3 min.
4. Cut out the gel band corresponding to approximately 100–105 nucleotides (this is the piRNA library).
5. Elute the DNA from the gel fragment using 100 µl of Illumina kit 1× gel elution buffer, shaking at 30°C.
6. Collect the elution buffer and spin on provided Spin-X Illumina column for 2 min at 20,000 × *g*.
7. To the eluate, add 1 µl of glycogen, 10 µl of 3 M NaOAC, pH 5.2, 325 µl of –20°C 100% ethanol, and mix.
8. Spin immediately at 20,000 × *g* at RT for 20 min.
9. Remove the supernatant and wash the pellet with 500 µl of 70% ethanol at room temperature.
10. Air-dry the pellet and resuspend in 10 µl of Illumina resuspension buffer.

3.5. Illumina Next Generation Sequencing

Proceed with next generation sequencing on Illumina GAII analyzer as per the manufacturer's instructions (see Note 7). The directional adapter ligation allows the easy identification of the strand polarity of the sequenced piRNAs as their 5′-ends always follows the SRA 5′ adapter.

3.6. Preparation of Radiolabeled pBR322 DNA-Msp I Digest Markers

1. Combine the following (total reaction volume 20 µl):

pBR322/Msp I Digest (NEB)	1 µl (=1 µg)
10× EcoPolI (Klenow) buffer	2 µl
[α-^{32}P] dCTP	5 µl
DNA polymerase I (Klenow)	1 µl
Water	11 µl

2. Incubate at 30°C for 15 min.
3. Add 200 µl of water and 200 µl of phenol/chloroform/isoamyl alcohol, and vortex. Extract and ethanol precipitate the labeled marker as described above. After the final wash with

70% ethanol, dry the pellet and resuspend in 100 µl of RNA loading buffer.

4. Heat the labeled marker at 95°C for 3 min. Cool on ice, and spin briefly. This is the stock (very highly radioactive) marker. Make a 1:100 dilution of an aliquot from the stock in the RNA loading buffer to make working dilution of marker. Store the stock and the dilution at –20°C (see Note 8).

4. Notes

1. Piwi proteins from diverse species (such as mouse, *D. melanogaster*, *X. laevis*) contain symmetrical dimethylarginines (sDMAs) and these sDMAs are recognized by the Y12 monoclonal antibody (9, 16). Other antibodies directed against specific Piwi proteins (e.g., anti-Mili (9); anti-Miwi (17); anti-Aub (6), etc.) may also be used to immunoprecipitate the piRNAs bound to specific Piwi proteins.
2. The strategy outlined above may be adopted for immunopurification and the cloning of RNAs from any RNP that contains small RNAs (e.g., microRNAs), provided that these RNAs contain 5′ and 3′ ends that are amenable to ligation.
3. Defolliculation of *X. laevis* oocytes is performed by collagenase treatment of *X. laevis* as follows. Briefly, tease apart ovary into small clumps using forceps, and place the ovary in OR2– buffer. Wash with two changes of 100 ml OR2– buffer. Place ovary clumps in small glass Petri dish and add 3 ml of collagenase working solution. Shake gently on platform shaker for ~1 h at room temperature, checking frequently for the dissociation of the oocytes from follicles. When most of the oocytes are dissociated, stop and discard the few undissociated clumps. Rinse four times with 100 ml of OR2– buffer and wash at least five times with 100 ml of OR2+ buffer. Place the oocytes into 1.5 ml tubes, discard the buffer, and store at –80°C. To prepare oocyte lysate for immunoprecipitation, add 5 packed oocyte volume of lysis buffer into the tube, break the oocytes by pipetting, and proceed with the sonication protocol described in Subheading 3.
4. Before homogenizing the mouse testis, detunicate by gently grasping and peeling off the tunica albuginea using finely tipped forceps.
5. Do not over-dry the RNA pellets, as they can become very hard to resuspend. Resuspension is best if the pellet is damp and appears glassy.
6. The purified RNA may also be used for other applications, such as northern.

7. Quality control and quantification of the piRNA library may be performed by electrophoresis of a small aliquot of the purified PCR on an Agilent 2100 Bioanalyzer by following the manufacturer's protocol. The bionalyzer is typically an integral component of core facilities that perform Illumina sequencing.
8. The marker is good for at least 3 months, but adjustments need to be made on how much to load depending on the decay.

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