# Chapter 7

# HITS-CLIP (CLIP-Seq) for Mouse Piwi Proteins

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## Abstract

Piwi proteins, such as Aubergine in *Drosophila* and Miwi and Mili in mice, form a major subclade of the Argonaute family, which comprise a distinct class of RNA-binding proteins (RBPs) able to bind small RNAs. Small RNAs can target complementary RNAs. Piwis are essential for the animal germline and bind Piwi-interacting RNAs (piRNAs) to form pi-RiboNucleoProteins (piRNPs). Although many piRNAs target retrotransposons for safeguarding genome integrity of the germ cell, whether piRNAs can target other mRNAs for regulatory purposes is still under investigation. Here we present the technical protocol for "High Throughput Sequencing after in vivo Crosslinking and Immunoprecipitation" (HITS-CLIP, CLIP-Seq), adapted for mouse Piwi proteins Mili and Miwi. We also provide general recommendations for the application of this protocol for different RBPs and also for the bioinformatic analysis of the deep sequencing data.

**Key words** piRNA, piRNP, Argonaute, Piwi, Mili, Miwi, Miwi2, Aub, Ago3, Next gen sequencing, Illumina, cDNA, Immunoprecipitation, HITS-CLIP, CLIP-Seq, RNA-IP, T4 RNA ligase, Reverse transcriptase, Polymerase chain reaction, PCR, RT-PCR, Post-transcriptional RNA processing, Gene silencing

## 1 Introduction

Germ cells employ complex mechanisms to process, transport and localize, and regulate stability and translation of mRNAs that control their developmental program. Piwi proteins are essential for animal germline development and they bind piRNAs to silence retrotransposons in the germline [1, 2]. However, significant numbers of piRNAs are unique [3–5] and it is unknown whether piRNAs can use seed sequence complementarity to target RNAs other than repeat elements. *Mus musculus* Mili [6], Miwi [7], Tdrd's [8–11], and MVH [12, 13], which are expressed in postnatal testis, localize in RNA-rich, dense cytoplasmic foci that are collectively called germ granules—nuage [14]. The germ granules have been implicated in translational control and mRNA stability, but the underlying mechanisms are not understood. HITS-CLIP is the method of choice for the identification of the identities of the

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RNAs targeted by RNA-binding proteins (RBPs), but also the exact nucleotide sequences, at unprecedented resolution [15–17]. We recently undertook Mili and Miwi HITS-CLIP and biochemical characterization of testis RNPs [18], thus—(a) providing the first in vivo snapshots of piRNA precursor processing that lead to *a model for piRNA biogenesis*; (b) revealing a *piRNA independent formation of Miwi mRNPs* critical for spermiogenesis. Here, we describe the adaptation of the HITS-CLIP protocol [15], for the identification of the in vivo RNA bound by mouse Piwi proteins. General considerations for the use of this protocol with Piwi or other RBPs from various sources are suggested throughout the text, and also some guidelines for the bioinformatic analysis of the next generation sequencing data.

## 2 Materials

### 2.1 Tissue Harvesting and UV Crosslinking

1. Freshly harvested mouse testes.
2. Ice-cold HBSS (Life Technologies).
3. 1× PBS (no $Mg^{2+}$/$Ca^{2+}$) (Roche).
4. Stratalinker (Model 1800 or 2400; Stratagene).

### 2.2 Preparation of Cell Lysate, Immunoprecipitation, and Labeling of Crosslinked Protein–RNA Complexes

1. Dynabeads® protein A (Life Technologies).
2. 1 M Na-phosphate buffer (pH 8.0): Mix 1 volume of 1 M $NaH_2PO_4$ with 13.7 volumes of 1 M $Na_2HPO_4$; adjust to pH 8.0 by adding $NaH_2PO_4$ for increasing acidity, or $Na_2HPO_4$ for increasing alkalinity.
3. Antibody (Ab) binding buffer: 0.1 M Na-phosphate (pH 8.0), 0.1 % IGEPAL CA-630, 5 % Glycerol.
4. Rabbit anti-mouse IgG Fcγ Fragment Specific (Jackson ImmnoResearch).
5. Nonimmune mouse serum or mouse IgG.
6. Anti-Piwi protein antibodies. We have used our own anti-Mili (17–8 mouse monoclonal antibody) and anti-Miwi (rabbit polyclonal) and Cell signaling Technologies G82 anti-Miwi antibody with success.
7. 1× PMPG buffer: 1× PBS, 2 % Empigen (Sigma).
8. 5× PMPG wash buffer: 5× PBS, 2 % Empigen.
9. Lysis buffer (prepare fresh each time): 1× PMPG plus one tablet of Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche) per 10 mL and 1 U/μL rRNasin (Promega).
10. RQ1 DNase (Promega).
11. RNase T1 (Roche).
12. Polyallomer Microfuge tubes (for tabletop ultracentrifuge, Beckman, 357448).

13. 4× SDS reducing loading buffer: 1 mL of NUPAGE SDS loading buffer (Invitrogen) supplemented with 100 μL of β-mercaptoethanol (Bio-Rad).
14. MilliQ RNase-free water.
15. $^{32}$P-γ-ATP (10 μCi/μL, 3,000 Ci/mmol).
16. 1× PNK buffer: 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 % IGEPAL CA-630.
17. T4 Polynucleotide Kinase (T4 PNK; NEB).
18. Illustra Microspin G-25 columns (GE Healthcare).
19. Beckman Optima TL Ultracentrifuge with TLA 100.3 rotor.
20. Thermomixer (Eppendorf).
21. 10 mM ATP.
22. RL3(–P) RNA oligo: from IDT, at 250 nmol synthesis scale, and purified by RNase-free HPLC. The sequence is 5′-OH GUGUCAGUCACUUCCAGCGG 3′-Inverted dT. Inverted dT is blocking the 3′ end of RL3 adapters, preventing concatamerization and aberrant ligation of the RL3 adapter with extracted RNAs.

### 2.3 Ligation of the Labeled RL3 RNA Adapter (On-Beads)

1. MilliQ RNase-free water.
2. Antarctic Phosphatase (NEB).
3. 10× Antarctic Phosphatase buffer (NEB).
4. rRNasin (Promega).
5. 1× PNK+EGTA buffer: 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 0.5 % IGEPAL CA-630.
6. 0.2 μg/μL BSA.
7. T4 RNA Ligase (NEB).
8. 10× T4 RNA Ligase buffer (NEB).
9. 10 mM ATP.
10. 1× PMPG buffer: 1× PBS, 2 % Empigen (Sigma).
11. 5× PMPG wash buffer: 5× PBS, 2 % Empigen.
12. RL3(+P) RNA oligo: from IDT, at 250 nmol synthesis scale, and purified by RNase-free HPLC. The sequence is RL3(+P): 5′-p GUGUCAGUCACUUCCAGCGG 3′-Inverted dT. Inverted dT is blocking the 3′ end of RL3 adapters, preventing concatamerization and aberrant ligation of the RL3 adapter with extracted RNAs.

### 2.4 SDS-PAGE and Nitrocellulose Transfer Analysis of Crosslinked RNA–Protein Complexes

1. 1× PNK buffer: 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 % IGEPAL CA-630.
2. 4× SDS reducing loading buffer: 1 mL of NUPAGE SDS loading buffer (Invitrogen) supplemented with 100 μL of β-mercaptoethanol (Bio-Rad).

3. NuPAGE 4–12 % Bis-Tris precast gel (Invitrogen).
4. NuPAGE MOPS SDS Running Buffer (Invitrogen).
5. 10× Western Blot buffer (for semi-dry transfer): 0.625 M Tris base, 0.18 M Glycine.
6. 1× Western Blot buffer: 10 % (v/v) 10× buffer, 20 % (v/v) Methanol, 70 % (v/v) MilliQ $H_2O$.
7. SE 400 Sturdier Gel electrophoresis apparatus with 18 × 24 cm glass plates (Amersham).
8. Nitrocellulose Membrane (Invitrogen LC2001, 0.45 μm pore size).
9. Semi-dry transfer apparatus, TE-77 (Hoefer).
10. Autoradiography films: Kodak BioMax MR, 8 × 10 in.

### 2.5 RNA Extraction

1. 25 mg/mL Proteinase K (PK) solution (Roche).
2. 5× PK Buffer: 500 mM Tris–Cl pH 7.5, 250 mM NaCl, 50 mM EDTA.
3. 1× PK Buffer/7 M urea solution (prepare fresh each time).
4. Acid RNA phenol/$CHCl_3$ (Ambion).
5. Acid Phenol/$CHCl_3$/Isoamyl alcohol 25:24:1 (Sigma).
6. Chloroform:Isoamyl alcohol 24:1 (Sigma).
7. 5 mg/mL Glycogen (Ambion).
8. 3 M Sodium Acetate (pH 5.2) (Ambion).
9. 1:1 (v/v) Ethanol/isopropanol solution.

### 2.6 5′ Adapter (RL3) Ligation

1. 70 % Ethanol.
2. T4 RNA Ligase (Fermentas).
3. 10× T4 RNA Ligase buffer (Fermentas).
4. rRNasin (Promega).
5. 3 M Sodium Acetate (pH 5.2) (Ambion).
6. 0.2 μg/μL BSA.
7. RQ1 DNase.
8. 10 mM ATP.
9. Acid Phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma).
10. 1:1 (v/v) Ethanol/isopropanol solution.
11. RL5 RNA oligo: from IDT, at 250 nmol synthesis scale, and purified by RNase-free HPLC. The sequence is 5′-OH AGGGAGGACGAUGCGG 3′-OH.

### 2.7 Denaturing PAGE/7 M Urea Electrophoretic Analysis of Extracted RNA

1. 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 (Ambion), and water up to 1 L. Stir until completely dissolved, filter-sterilize, and store up to a year at RT in an aluminum foil-covered bottle (to protect from light).
2. 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.

### 2.8 cDNA Synthesis and Preparation of Deep Sequencing Libraries

1. dNTP mix.
2. 0.1 M DTT.
3. Superscript III Reverse Trascriptase (RT; Invitrogen).
4. Accuprime Pfx Supermix (Invitrogen).
5. MetaPhor Agarose (Lonza).
6. 1× TAE buffer.
7. Ethidium bromide.
8. QIAquick Gel Extraction Kit (Qiagen).
9. DNA primers: from IDT, at 250 nmol synthesis scale, and PAGE purified.

   DP5: 5′-AGGGAGGACGATGCGG-3′.

   DP3: 5′-CCGCTGGAAGTGACTGACAC-3′.

   DSFP5: 5′-AATGATACGGCGACCACCGACTATGGATACTTAGTCAGGGAGGACGATGCGG-3′.

   DSFP3: 5′-CAAGCAGAAGACGGCATACGACCGCTGGAAGTGACTGACAC-3′.

   SSP1: 5′-CTATGGATACTTAGTCAGGGAGGACGATGCGG-3′.

## 3 Methods

The outline of the procedures and representative figures from experiments 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.

### 3.1 Tissue Harvesting and UV Crosslinking (Day 1)

1. Harvest testes in batches of four and keep them covered in ice-cold HBSS until harvest is complete. Remove tunica albuginea using finely tipped forceps, and transfer testes into a glass dounce homogenizer. Triturate tissue by mild mechanical disruption (i.e., using a smaller size pestle and pipetting), with care not to lyse too many cells (*see* **Note 1**).

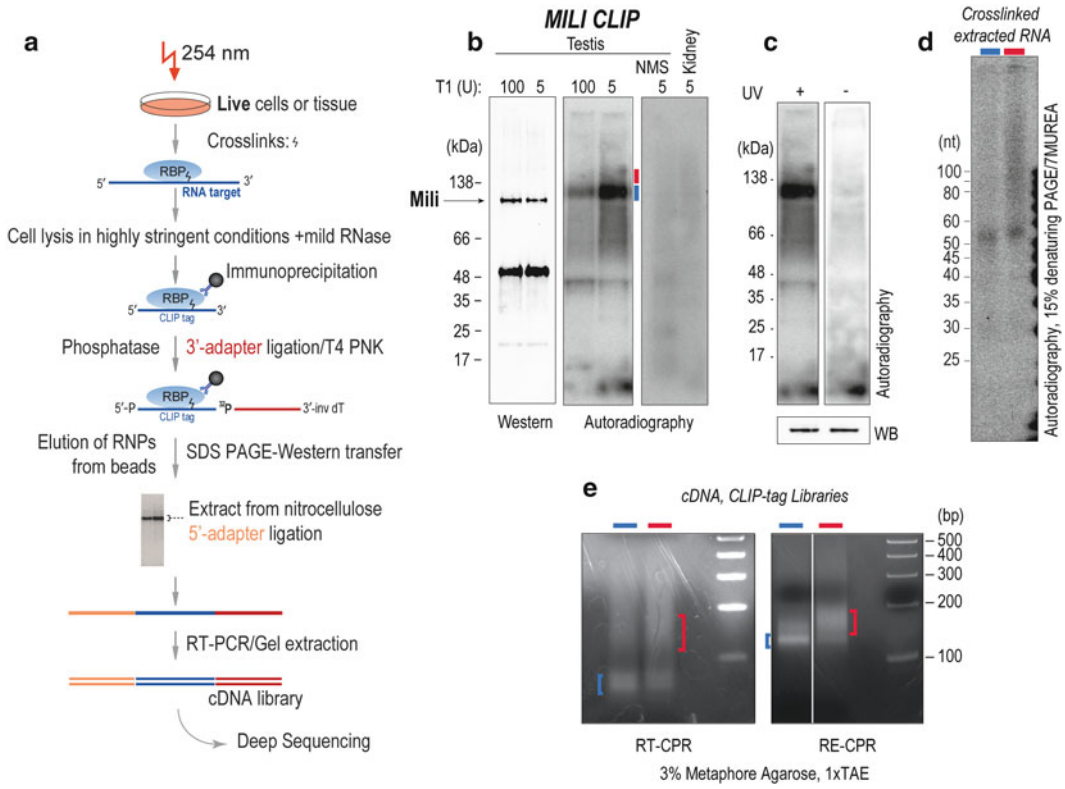


**Fig. 1** Mili HITS-CLIP. (**a**) Graphic overview of the HITS-CLIP experimental protocol. (**b**) Autoradiogram of $^{32}P$ labeled Mili protein–RNA complexes after in vivo UV-crosslinking, immunoprecipitation, labeling of the cross-linked RNA, and SDS-PAGE analysis. Western blot analysis of the same samples using the same anti-Mili antibody is also shown for comparing the difference of electrophoretic mobility between the bulk of the protein, which is not crosslinked (identified by western blot), and the crosslinked Protein–RNA complexes in the autoradiogram. CLIP using lysates subjected to mild RNase T1 treatment (5 U) and moderate RNase T1 treatment (100 U) is shown. Note the increased radioactive signal in mildly treated sample compared to moderately treated. RNA (CLIP tags) were extracted from the membranes after cutting at indicated areas: a *blue line* marks the major radioactive signal containing mainly protein–piRNA complexes; a *red line* marks larger Protein–RNA complexes which appear as a smear extending to higher molecular weights. Negative control CLIPs were performed using nonimmune mouse serum (NMS) with crosslinked testis, and anti-Mili antibodies with crosslinked kidney. (**b**) Comparison of UV-crosslinked and non-crosslinked labeled Mili Protein–RNA complexes. Absence of UV-crosslinks results in loss of the specific radioactive signal that is migrating slightly slower than the Mili western blot signal. (**c**) Denaturing PAGE electrophoretic analysis of Mili crosslinked and extracted RNA. Observe enrichment in piRNAs for the sample extracted from the main radioactive signal (*blue line*), and enrichment in large RNAs for the sample extracted from the slower migrating complexes (*red line*). (**d**) RT-PCR and RE-PCR amplification of Mili crosslinked and extracted RNA. PCR products were gel purified after Metaphor agarose gel electrophoresis. *Blue* and *red brackets* denote piRNA and large tag enriched cDNA samples, respectively

2. Transfer tissue suspension in petri dish and keep it be covered with ice-cold HBSS (200 mg of tissue—four adult testies—in a 10 cm plate, 8 mL of HBSS). Irradiate tissue suspension three times at 400 mJ/cm² in Stratalinker, with 30-s intervals for cooling. Mix suspension between each irradiation.

Always keep on ice. An important negative control is tissue that has not been exposed to UV. Other negative controls include tissues that do not normally express the target protein (in the case of Piwi any somatic tissue) and also testes from Piwi knockout mice.

3. Collect cell suspension in a 15-mL falcon tube and pellet at 1200 × *g* for 10 min at 4 °C. Resuspend the cell pellet in 1 mL of PBS and divide it in two eppendorf tubes (each aliquot is good for one CLIP). Spin at 1,000 × *g* for 30 s at 4 °C, remove supernatant, freeze pellets in liquid nitrogen and keep at −80 °C until use (*see* **Notes 2** and **3**).

## 3.2 Preparation of Cell Lysate, Immunoprecipitation, and Labeling of Crosslinked Protein–RNA Complexes (Day 2)

For bead washing steps in this and subsequent sections, use 1 mL of buffer. For each IP use 150 μL of protein A Dynabeads slurry. Normal (pre-immune) rabbit or mouse serum can be used in a negative control IP.

### 3.2.1 Preparation of Antibody-Bound Beads

1. Wash beads three times with Ab binding buffer.
2. Resuspend beads in 350 μL Ab binding buffer.
   (a) For using a *mouse monoclonal anti-Piwi Ab* for immunoprecipitation, add 3 μL rabbit anti-mouse (bridging) Ab to the beads.
   - Rotate tubes at room temperature for 45 min.
   - Wash three times with Ab binding buffer.
   - Resuspend beads in 350 μL Ab binding buffer and add 3–5 μL of ascites (or pre-immune serum) fluid or 5–10 μg of Ab in solution.
   - Rotate tubes at 4 °C for 3 h.
   - Wash one time with Ab binding buffer and two times with 1× PMPG buffer; if you are not yet ready to add crosslinked lysate, leave beads in last washing buffer.

   (b) For using a *rabbit polyclonal anti-Piwi Ab* for immunoprecipitation, add 10 μg of Ab to the beads (or 3–5 μL of pre-immune rabbit serum).
   - Rotate tubes at 4 °C for 2–3 h.
   - Wash one time with Ab binding buffer and two times with 1× PMPG buffer.

### 3.2.2 Preparation of Cell Lysate

1. Resuspend each pellet of UV treated tissue using 350 μL of Lysis buffer (*see* **Note 4**). With a 1 mL pipettor, mix until flow is unforced, with care not to foam. Let sit on ice for 10 min (*see* **Note 5**).
2. Add 10 μL of RQ1 DNase to each tube and incubate at 37 °C for 5 min on a Thermomixer at 1,000 rpm. Thoroughly mix lysates by pipetting two times.

3. Prepare dilutions of RNase T1 in 1× PMPG. The lysate is subjected to RNase treatment so that the size of long crosslinked RNAs is reduced to 50–150 nucleotides (*see* **Note 6**). The conditions of RNase treatment are critical for success and should be optimized for every different experimental setting (tissue and protein expression levels): overdigested RNA–protein complexes may not be labeled efficiently in subsequent steps (*see* **Note 7**), while undigested complexes may be too big to analyze in subsequent steps. A series of high to low RNase treatment conditions should be tested, to identify the conditions in which crosslinked RNPs give a strong and specific signal after labeling and SDS-PAGE analysis. For high RNase treatment use 1 μL of undiluted RNase T1 per 350 μL of lysate. Useful dilutions include 1/10, 1/20, 1/100, 1/1,000, and also no RNase. For mouse testis and Piwi protein CLIP, crosslinked RNAs are sufficiently cleaved by the activities of endogenous nucleases, despite the presence of rRNasin and handling at 4 °C or on ice, and therefore no exogenous RNase is required for this step.
4. For RNase treatment, add 1 μL of 1× PMPG or RNase T1 dilution per 350 μL of lysate and incubate at 37 °C for 5 min on a Thermomixer at 1,000 rpm. Thoroughly mix lysates by pipetting two times.
5. Transfer lysates to polyallomer Microspin tubes for ultracentrifugation and weigh on a precision scale, for accurate balancing. Spin lysates in prechilled ultracentrifuge at 90,000 × *g* for 30 min at 4 °C (*see* **Note 8**).
6. Carefully remove the supernatant (S90) and keep on ice. As a pre-immunoprecipitation sample save 15 μL of the S90; mix with equal volume (15 μL) of 4× SDS reducing loading buffer and incubate at 70 °C for 12 min.

### *3.2.3 Immunoprecipitation*

1. Add the S90 lysate to a tube containing Ab-bound Dynabeads from in Subheading 3.2.1 (after removing last wash buffer).
2. Rotate beads with lysate for 3 h at 4 °C (During this step, you may carry on Subheading 3.2.4).
3. Remove the supernatant (Piwi protein depleted S90, keep at −80 °C) and save 15 μL for immunoblot analysis.
4. Wash beads with ice-cold buffer:
   (a) Two times with 1× PMPG (Wash Buffer)
   (b) Two times with 5× PMPG (High-salt Wash Buffer)
   (c) Two times with 1× PNK Buffer

### *3.2.4 $^{32}$P Labeling of the 3′ RNA Adapter (RL3)*

You may perform this step during the immunoprecipitation (Subheading 3.2.3) or the previous day. Use fresh $^{32}$P-γ-ATP.

The reaction described below is for preparing enough labeled RL3 adapter for ten subsequent ligations to immunoprecipitated piRNPs.

1. Combine the following (total reaction volume 50 µL):
   (a) 6.5 µL MilliQ RNase-free $H_2O$
   (b) 2.5 µL 50 pmol/µL RL3(–P) linker
   (c) 3 µL rRNasin
   (d) 25 µL $^{32}$P-γ-ATP (10 µCi/µL, 3,000 Ci/mmol)
   (e) 5 µL 10× PNK Buffer
   (f) 8 µL T4 PNK enzyme
2. Incubate at 37 °C for 30 min.
3. Add 2 µL of 1 mM ATP, and incubate at 37 °C for 5 more min (*see* **Note 9**). Spin down and place on ice.
4. Resuspend the resin in the G-25 column by inverting several times. Pre-spin the column for 1 min at 735 × *g*, apply the sample to resin, and spin the column for 2 min at 735 × *g*. Collect the eluate (labeled RL3 adapter) in a clean tube. Probe RL3 eluate and Mini spin-column with a Geiger counter. Typically, RL3 solution accounts for more than 60–70 % of total radioactive signal.
5. Heat inactivate the residual T4 PNK in the eluted labeled RL3 at 65 °C for 20 min (*see* **Note 10**). If you are not ready to use, store RL3 adapter at –20 °C.

### *3.3 Ligation of the Labeled RL3 RNA Adapter (On-Bead)*

#### *3.3.1 Phosphatase Treatment of the Crosslinked RNAs (On-Bead)*

1. Prepare the following reaction (one per CLIP sample, total reaction volume 80 µL).
   (a) 67 µL MilliQ RNase-free $H_2O$
   (b) 8 µL 10× Antarctic phosphatase buffer
   (c) 3 µL Antarctic phosphatase
   (d) 2 µL rRNasin
2. Add 80 µL of phosphatase reaction mixture to each tube of beads from **step 4** in Subheading 3.2.3, after removing the last washing buffer.
3. Incubate at 37 °C for 20 min on a Thermomixer at 1,000 rpm (*see* **Note 11**).
4. Wash beads with ice-cold buffer:
   (a) One time with 1× PNK Buffer
   (b) One time 1× PNK + EGTA Buffer
   (c) Two times 1× PNK Buffer

### 3.3.2 3′ RNA Linker Ligation (On-Bead)

1. Prepare the following reaction (one per CLIP sample, total reaction volume 80 μL)
   (a) 47 μL water
   (b) 8 μL 10× T4 RNA ligase buffer
   (c) 8 μL 0.2 μg/μL BSA
   (d) 8 μL 10 mM ATP
   (e) 2 μL rRNasin
   (f) 2 μL T4 RNA ligase
   (g) 5 μL $^{32}$P labeled RL3 (prepared in Subheading 3.2.4)
2. Add 80 μL of ligase reaction mix to each tube of beads from previous step, after removing the washing buffer.
3. Incubate at 16 °C for 1 h on a Thermomixer at 1,000 rpm.
4. Add 4 μL of RL3(+P) (20 pmol/μL), (*see* **Note 12**), and proceed with overnight incubation at 16 °C on a Thermomixer at 1,000 rpm.

*(Day 3)*

5. Wash beads with ice-cold buffer:
   (a) One time with 1× PMPG (Wash Buffer)
   (b) One time with 5× PMPG (High-salt Wash Buffer)
   (c) Two times with 1× PNK Buffer

### 3.3.3 T4 PNK Treatment of Crosslinked RNAs (On-Bead)

This treatment restores a 5′ phosphate end of the crosslinked RNAs, which is required for ligation of the 5′ RNA adapter (Subheading 3.6).

1. Combine the following reagents for the phosphorylation reaction (80 μL total):
   (a) 65 μL water
   (b) 8 μL 10× PNK Buffer
   (c) 2 μL rRNasin
   (d) 1 μL 10 mM ATP
   (e) 4 μL T4 PNK enzyme
2. Add 80 μL of PNK mix to each tube with beads (after removing last wash) and incubate at 37 °C for 20 min on a Thermomixer at 1,000 rpm.
3. Wash beads with ice-cold buffer:
   (a) One time with 1× PMPG (Wash Buffer)
   (b) One time with 5× PMPG (High-salt Wash Buffer)
   (c) Three times with 1× PNK buffer

### 3.4 SDS-PAGE and Nitrocellulose Transfer Analysis of Crosslinked RNA–Protein Complexes

1. After removing the final wash buffer, add to the beads 15 μL of 1× PNK and 15 μL of 4× SDS reducing loading buffer (*see* **Note 13**). Resuspend by pipetting and mild vortexing, and incubate at 70 °C for 12 min on a Thermomixer at 1,000 rpm.
2. Place tubes on a magnet and separate protein eluate from beads (*see* **Note 14**). Load up to 25 μL of sample per well of a 10 well Novex NuPAGE 4–12 % Bis-Tris gel (*see* **Note 15**). Keep 5 μL of each eluate for Western blot analysis. Include pre-stained markers in the run. Run the gel using MOPS running buffer at 170 V in the cold room.
3. Stop the run when dyes reach the lower opening of the gel cassette (for consistency between different experiments, stop gel running at the same exact point each time).
4. After gel run, open the gel cassette and transfer the gel into a container with 1× western blot buffer. Incubate for 1 min.
5. Set up Western transfer to nitrocellulose using a semi-dry apparatus (*see* **Note 16**) at constant 90 mA (per gel) for 1 h 10 min.
6. After transfer, rinse the nitrocellulose membrane in MilliQ RNase-free water, and gently blot the back side of the membrane on Kimwipes (do not dry completely).
7. Heat-seal the membrane and the gel separately in hybridization plastic bags. Heat-seal one corner of the membrane to prevent it from moving inside the bag. Attach small pieces of fluorescent sticker on the bags or use fluorescent marker (the stickers/markings will be aligned with the respective signal on the film after development).
8. Expose to film (Kodak BioMax MR) (*see* **Note 17**). A single prominent band at the MW of the immunoprecipitated protein is the ideal result. A band that gives a clear signal after 1–2 h is ideal for subsequent RNA extraction step. Longer exposure (overnight) may be required for fainter signals, but in that case combine two or three samples in one to extract sufficient RNA for subsequent steps. To monitor the efficiency of RNP transfer, compare the radioactive signal on the membrane with the remaining signal on the gel. It is expected that more than 60–70 % of the total radioactive signal is transferred on the membrane.
9. On a light table, tape the film first and lay the bag with the membrane above the film. Align the markings of the fluorescent sticker pieces with their signals on the film. Correct alignment is essential (*see* **Note 18**). You should be able to see the radioactive signal on the film, through the membrane. Tape the bag in place. Open a hole in the bag without moving the membrane.

10. Two different strategies can be followed for isolating crosslinked RNAs on Piwi proteins (or Argonautes in general). (a) To isolate small RNA population and larger RNA population separately or (b) both populations in one sample. In the first strategy (followed in this protocol), for isolation of small RNAs (piRNAs), cut (using a single use surgical blade) the part of the membrane containing the main radioactive signal (Fig. 1b, marked by a blue line).
11. Put the pieces in a clean tube (cut in small pieces ~4 $mm^2$ each to facilitate RNA extraction). This sample may also contain small fragments of larger RNAs. For isolation of larger RNAs, cut a part of the membrane of equal size with the previous, above the main radioactive signal (Fig. 1b, marked by a red line) (*see* **Note 19**). This sample will also contain piRNAs (*see* **Note 20**). After analysis of the pre and post IP lysates and also CLIP eluate samples by regular Western blot, and precise alignment of pre-stained markers on western blot and on the autoradiography, note that the main radioactive band (formed by small RNAs crosslinked on the protein) migrates ≥7 kDa above the apparent molecular weight of the immunoprecipitated protein (Fig. 1b) (*see* **Note 21**).

### 3.5 RNA Extraction

1. Prepare a proteinase K solution at 4 mg/mL (the stock solution by Roche is at 25 mg/mL), in 1× PK Buffer, and preincubate this solution at 37 °C for 20 min to degrade any present nucleases.
2. Add 200 μL of proteinase K solution to each tube containing membrane fragments from previous step. Incubate at 37 °C for 20 min on a Thermomixer at 1,000 rpm.
3. Add 200 μL of 1× PK/7 M Urea solution to each tube, and incubate for 20 min at 37 °C on a Thermomixer at 1,000 rpm.
4. Add 400 μL of acid RNA phenol/$CHCl_3$, and incubate for 20 min at 37 °C on a Thermomixer at 1,000 rpm.
5. Spin tubes in a tabletop centrifuge at maximum speed (~16,000 × *g*) for 5 min at room temperature. Collect aqueous (upper) phase (contains RNA).
6. Add 400 μL of $CHCl_3$/isoamyl alcohol and vortex. Centrifuge as in previous step, collect again the aqueous (upper) phase, and add the following:
   (a) 50 μL 3 M Sodium Acetate (pH 5.2)
   (b) 0.75 μL 5 mg/mL Glycogen
   (c) 1 mL 1:1 (v/v) Ethanol/Isopropanol solution
7. Mix well and precipitate overnight at −80 °C.

### 3.6 5′ RNA Adapter (RL5) Ligation (Day 4)

1. Spin tubes in a tabletop centrifuge at maximum speed for 30 min at 4 °C to pellet the RNA (*see* **Note 22**). The pellet should be detectably radioactive, although this is not a measure of the success of the RNA extraction step for faint radioactive signals.
2. Wash pellet with 1 mL 70 % ethanol, and centrifuge again at maximum speed for 15 min at 4 °C to recover the pellet.
3. Remove ethanol and air-dry the pellet (*see* **Note 23**).
4. Resuspend in 6.5 μL MilliQ RNase-free $H_2O$. Keep 1 μL for denaturing PAGE analysis.
5. Set up the following 5′ RNA adapter (RL5) ligation:
   (a) 5.5 μL resuspended RNA
   (b) 1 μL 10× T4 RNA ligase buffer
   (c) 0.5 μL rRNasin
   (d) 1 μL 0.2 μg/μL BSA
   (e) 1 μL 10 mM ATP
   (f) 0.5 μL T4 RNA ligase
   (g) 0.5 μL 40 pmol/μL RL5 RNA adapter
6. Incubate at 16 °C for 6 h.
7. Add to the reaction:
   (a) 79 μL MilliQ RNase-free $H_2O$
   (b) 11 μL 10× DNAse I Buffer
   (c) 5 μL rRNasin
   (d) 5 μL RQ1 DNAse
8. Incubate 37 °C for 20 min.
9. Add to the above mix:
   (a) 300 μL MilliQ RNase-free $H_2O$
   (b) 400 μL Acid Phenol/$CHCl_3$/Isoamyl alcohol 25:24:1
10. Vortex for 1 min. Spin tubes in a tabletop centrifuge at maximum speed (~16,000×*g*) for 5 min at room temperature. Collect upper phase (should be approximately 400 μL).
11. Add 400 μL of $CHCl_3$/Isoamyl alcohol 24:1. Vortex, spin, and collect aqueous phase as in previous step.
12. Precipitate by adding:
   (a) 50 μL 3 M Sodium Acetate (pH 5.2)
   (b) 0.5 μL 5 mg/mL Glycogen
   (c) 1 mL 1:1 EtOH/isopropanol
13. Precipitate overnight at –80 °C.

### 3.7 Denaturing PAGE/7 M Urea Electrophoretic Analysis of Extracted RNA

1. Analyze 1 μL of RNA in 15 % Urea/PAGE [19]. Use radiolabeled small ssRNA 20–100 nts as marker.
2. Expose for 2–10 days (or even longer), depending on the amount of radioactivity of extracted RNAs. Observe enrichment in piRNAs (Fig. 1d, lane marked with blue) for the sample extracted from the main radioactive signal (Fig. 1b, blue line), and enrichment in larger RNAs (target mRNAs and piRNA precursor fragments; Fig. 1d, lane marked with red) in the sample extracted from higher molecular weights (Fig. 1b, red line).

### 3.8 cDNA Synthesis and Preparation of Deep Sequencing Libraries (Day 5)

Pellet the RL5 ligated RNA by centrifugation for 30 min at maximum speed and at 4 °C. Carefully aspirate the supernatant and wash the pellet with ice-cold 70 % ethanol. Pellet the RNA by centrifugation as before, remove the supernatant and air-dry. Resuspend the pellet in 10 μL of MilliQ RNase-free $H_2O$. The RNA now has 5′ and 3′ RNA adapters and can be reverse transcribed and amplified by PCR for the preparation of the deep sequencing library.

#### 3.8.1 Reverse Transcriptase Reaction

1. Set up the following mixture:
   (a) 10 μL ligated RNA.
   (b) 2 μL 5 pmol/μL DP3 primer.
   (c) 1 μL 10 mM dNTPs.
2. Incubate at 65 °C for 5 min. Place on ice and quick spin.
3. Add to the above:
   (a) 1 μL DTT, 0.1 M
   (b) 4 μL 5× SuperScript RT Buffer
   (c) 1 μL rRNasin
   (d) 1 μL SuperScript III
4. Incubate at 50 °C for 45 min, 55 °C for 15 min, 90 °C for 5 min, leave at 4 °C.

#### 3.8.2 Polymerase Chain Reaction

1. Set up the following PCR:
   (a) 27 μL Accuprime Pfx Supermix
   (b) 0.75 μL DP5 primer, 20 pmol/μL
   (c) 0.75 μL DP3 primer, 20 pmol/μL
   (d) 3 μL of the RT reaction
2. Perform the following PCR program:
   (a) 95°C for 2 min
   (b) 25–28 cycles of:
      - 95 °C for 20 s
      - 58 °C for 30 s
      - 68 °C for 30 s

(c) 68 °C for 5 min

(d) 4 °C hold

Perform each reaction in triplicate and combine replicate reactions before electrophoresis.

3. Speed-vac pooled samples to concentrate, and run each replicate pool on one lane on a 3 % Metaphor 1× TAE/EtBr gel at 100 V (*see* **Note 24**). Small RNAs give rise to a 60–70 bp band (piRNAs are 25–30 nucleotides plus 21 nucleotides from RL3, and 16 nucleotides from RL5) (Fig. 1e). Larger RNAs produce a smear extending from the small RNA band and upwards, usually with a more prominent population at sizes 100–200 bp (Fig. 1e) (*see* **Note 25**).
4. Cut out desired PCR products with a clean blade and transfer to a clean tube. Extract DNA with QIAquick Gel Extraction Kit (*see* **Note 26**), using 40 µL of elution buffer.

#### *3.8.3 Re-PCR with Solexa Fusion Primers*

1. Set up the following PCR reaction:
   (a) 27 µL Accuprime Pfx Supermix
   (b) 0.5 µL 20 pmol/µL DSFP5 primer
   (c) 0.5 µL 20 pmol/µL DSFP3 primer
   (d) 3 µL of gel extracted 1st PCR product.
2. Perform the following PCR program:
   (a) 95 °C for 2 min
   (b) 6–10 cycles of:
   - 95 °C for 20 s
   - 58 °C for 30 s
   - 68 °C for 30 s

   (c) 68 °C for 5 min
   (d) 4 °C hold

Again, you can perform three replicate reactions per sample and pool them before electrophoresis. Speed-vac to concentrate, and run in a single lane on 3 % Metaphor 1× TAE/EtBr gel. DSFP primers add 97 bp to the size of the CLIP tag, so piRNAs will form a band at ~130 bp, and larger RNAs a diffuse smear up to 250 bp (Fig. 1e). Cut out desired sizes, and extract DNA with QIAquick Gel Extraction kit as in previous step.

### *3.9 Illumina Next Generation Sequencing and Considerations for Bioinformatic Analysis of Sequencing Data*

1. Proceed with next generation sequencing with Illumina analyzer as per the manufacturer's instructions (*see* **Note 27**).
2. The first base of every CLIP tag sequenced corresponds to the first nucleotide of the crosslinked RNA molecule. The 3′ adapter sequence has to be "trimmed" off the CLIP tag sequence before alignment on the reference genome (*see* **Note 28**).

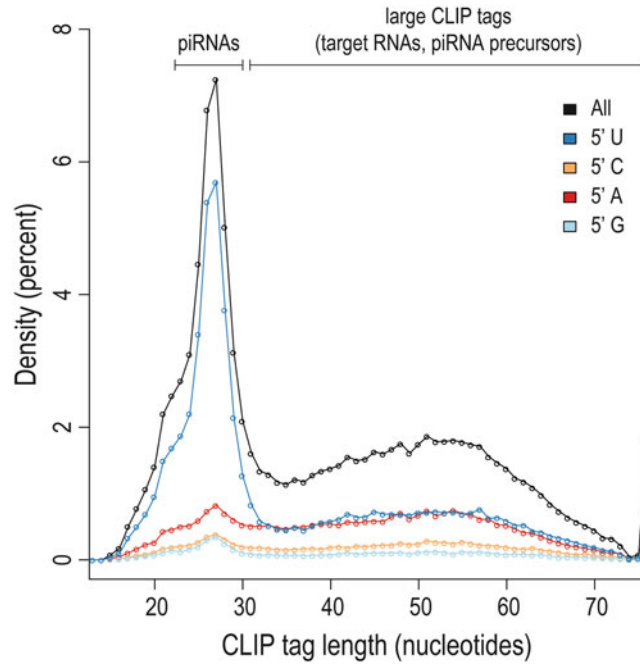


**Fig. 2** Size distribution and 5′ nucleotide preference of a Mili CLIP library enriched for large RNAs. The percentage of reads to the total number of reads of the library is plotted for every length size in *black*, and for the reads starting with any the four nucleotides separately: uridine, *blue*; cytosine, *orange*; adenine, *red*; guanine, *light blue*. Note the separation of the piRNA population by a distinct peak at 26–27 nucleotides, but also the overwhelming bias for a uridine at their 5′ends, compared to the longer RNAs. Detailed analysis revealed that a subset of the long RNAs corresponds to intermediate piRNA fragments that have mature 5′ ends with a U bias and untrimmed 3′ ends. Maximum read size of this Illumina run was 76 bases

3. The CLIP libraries prepared from RNAs extracted from the main radioactive signal and higher molecular weights should contain piRNAs, and also fragments of larger RNAs, such as piRNA precursors and target RNAs. These two populations have distinctive characteristics such as genomic origin, nucleotide biases, and size range (*see* **Note 29** and Fig. 2), and therefore downstream bioinformatic analyses should recognize these differences appropriately. Ideally these two populations should be contained in two minimally overlapping peaks in a size distribution plot of the CLIP tags (Fig. 2). The quality of a Piwi CLIP experiment depends on the use of such conditions so that these populations remain faithful to their in vivo profiles. For example, over-digestion of crosslinked RNAs will result in a shift of the average size of piRNAs. Additionally, larger RNAs might be cleaved down to piRNA size, thus "contaminating"

the piRNA population with other RNAs. Since the bioinformatic analysis of the CLIP libraries, and not their preparation and sequencing, is the slowest and most complicated step of a CLIP-Seq project, the best practice is to ensure the highest possible quality of the CLIP library during preparation.
*See* **Notes 29–31** for further useful information.

## 4 Notes

1. Preservation of cell viability and integrity is critical for success.
2. Approximately 40 % of tissue wet-weight can be lost during this step. Creating a single cell suspension of the tissue sample before crosslinking leads to further material loss and is not necessary. UV can penetrate small chunks of tissue.
3. In general, 50–60 mg of tissue is sufficient for one CLIP sample in most cases. The sufficient amount of tissue per CLIP sample depends on the expression levels of the RBP in question, and the efficiency of the immunoprecipitation step (Subheading 3.2).
4. Prepare fresh Lysis buffer each time. 1× PXL (1× PBS, 0.1 % SDS, 0.5 % deoxycholate, 0.5 % NP-40) [15] can also be used as the basis of Lysis and Immunoprecipitation buffers, with 5× PXL for subsequent washing steps (5× PBS, 0.1 % SDS, 0.5 % deoxycholate, 0.5 % NP-40). We have observed that most antibodies perform better in 1× PMPG.
5. Cells should be lysed in this step. Hard tissue may require the use of further mechanical means to lyse thoroughly, such as eppendorf pestle.
6. The size of the long crosslinked RNAs should be reduced, so that the protein–RNA complexes can be analyzed by SDS-PAGE. Large complexes will not enter the gel (*see* Subheading 3.4). Furthermore, the purpose of the HITS-CLIP protocol is to identify the binding "site", the exact area of the RNA that is bound by the protein, and therefore areas of long RNAs that are not bound have to be removed. Finally, Illumina deep sequencing is currently limited to 100–120 cycles, and therefore library preparation has to be optimized for this maximum read length. Keep in mind that certain RNases have sequence biases for the cleavage site (RNase T1 cleaves downstream of a G) and any cleaved RNAs will bear this bias.
7. Excess ribonuclease treatment may alter the native size range of the endogenous small RNA populations (piRNAs), and also may lead to the isolation of very small fragments of the long RNAs.

Fragments that are smaller than 19–20 nucleotides cannot be unambiguously mapped on the genome, and this will make bioinformatic analysis of these libraries problematic.

8. Ultracentrifugation "clears" the lysate of insoluble material and higher order complexes of target protein with other possible RBPs, ensuring the immunoprecipitations of single protein–RNA complexes, and therefore the specificity of the RNAs that are isolated at subsequent steps.
9. The addition of "cold" ATP ensures that all RNA adapter molecules have a phosphorylated 5′ end.
10. G-25 Mini spin-column will retain molecules that are smaller than the RL3 adapter (i.e., unincorporated ATP, small adapter fragments) but not the T4 PNK enzyme, which might interfere with subsequent ligation step. Therefore, it has to be heat inactivated. Alternatively, the labeled RNA adapter can be purified by phenol extraction and ethanol precipitation.
11. In order to keep Dynabeads resuspended throughout the course of on-beads enzymatic reactions, a constant mixing motion is required. For the reaction volume and amount of Dynabeads described, this is best achieved on Thermomixer (Eppendorf) set at 1,000 rpm.
12. Addition of excess cold (unlabeled) RL3 ensures that all extracted RNA molecules are ligated with a 3′ adapter and therefore can be amplified by PCR.
13. Nonreducing SDS loading buffer has been reported as an alternative for performing CLIP with proteins with molecular weight close to that of heavy antibody chain, and whose electrophoretic pattern can be affected by the excess of antibody used in the IP. Piwi Argonautes are significantly larger (around 100 kDa) than heavy Ab chains, and no such interference is observed.
14. It is recommended that fresh SDS loading-elution solution is prepared each time. Check for efficient elution by comparing radioactive counts of the eluate and remaining beads. More than 80 % of the counts should be in the eluate.
15. Depending on the size of the protein of interest, you might use different gel concentration and/or running buffer, to achieve optimal resolution at the size range of your protein. Consult the Novex gel migration chart at the Invitrogen website.
16. Wet transfer using Invitrogen's XCell II Blot module has also been used successfully. The iBlot fast transfer apparatus used under manufacturer's instructions for regular western blot is not very efficient for transfer of the crosslinked and labeled RNA–protein complexes, although regular protein is transferred efficiently.

17. Intensifying screens (Fischer) will increase the strength and improve the resolution of the radioactive signal. Keep radiographic cassette in −80 °C during exposure. Bring to room temperature before cutting the membrane.
18. Thin pieces of fluorescent sticker will give sharp signals on the film. This is critical for accurately aligning with the markings on the film after exposure. Proper alignment is a particularly important step, as slight misalignment can lead to incorrect RNA extraction, and confusing results in subsequent PCR amplification steps.
19. For RBPs other than Piwi or Argonautes in general, that may not bind more than one characteristic RNA populations (i.e., small RNAs and mRNAs), the main radioactive signal should contain fragments of all their RNA targets, and membrane fragments from higher positions will only contain larger fragments of the same RNAs.
20. Piwis (and also Agos) form complexes with two different populations of RNAs with different abundances, size and sequence characteristics: piRNAs (small RNAs) and mRNAs, hence the difference in signal intensity and the separation of these complexes by size on the membrane. In the second strategy, cut one membrane fragment containing the main signal but also higher molecular weight smear, as described above. The sample enrichment in the two RNA populations will impact the downstream bioinformatic analysis.
21. The smear extending to higher molecular weights contains longer RNAs. In conditions of high RNase treatment, and depending on the activities of endogenous nucleases during IP steps, the main radioactive signal usually appears sharper but also fainter, and the high molecular weight smear might also be fainter or absent. In conditions of mild RNase treatment (low concentration of RNase T1, or no exogenous nuclease), the signal might appear stronger, more diffuse, and more prominent in higher molecular weights (Fig. 1b). These differences in the radioactive signals correspond to the abundance and the sizes of the RNAs crosslinked with the immunoprecipitated protein, and how these are affected by RNase treatment (high RNase treatment results in smaller RNAs–smaller RNPs and vice versa). Also note that usually there is no band detected on the western blot corresponding to the signals on the autoradiography, and this is because only a small fraction of the total protein in the lysate is crosslinked with RNAs. For mouse Piwi protein CLIP, mild treatment or no exogenous RNase was used. Importantly, the specific radioactive signal is lost in non-crosslinked control samples (Fig. 1c).

22. Rarely, residual organic phase will be visible after centrifugation, and can interfere with the acquisition of the RNA pellet. Remove most of the aqueous supernatant leaving a small amount at the bottom of the tube containing the residual organic solvent and the RNA pellet. Add 400 μL of RNase-free water and vortex rigorously for 1 min. Re-extract by adding 400 μL of Acid Phenol/$CHCl_3$/Isoamyl alcohol 25:24:1, vortexing for 1 min, spinning at maximum speed (~16,000 × *g*) for 5 min at room temperature and collecting upper phase. Remove traces of phenol by extracting with 400 μL of $CHCl_3$/Isoamyl alcohol 24:1. Vortex, spin, and collect aqueous phase as in previous step. Precipitate RNA by adding to the aqueous phase: 40 μL 3 M Sodium Acetate (pH 5.2), 0.5 μL 5 mg/mL Glycogen, and 1 mL ice-cold EtOH, incubating for 30 min at −80 °C and spinning at max speed for 30 min at 4 °C.
23. At this step the RNA pellets are usually extremely small. Do not over-dry the pellets, as they can become very hard to resuspend. Air-dry or place in 37 °C heating block, while constantly monitoring the evaporation of remaining ethanol. Resuspension is best if the pellet is still damp.
24. Metaphor agarose is an intermediate melting temperature agarose with high-resolution capability. Metaphor gels are very fragile. Handle with extreme care, small cuts propagate very fast. Follow manufacturer's instruction for preparation. High temperatures during electrophoresis will render the gels even more susceptible to damage, therefore regulate voltage so that running buffer is not warm.
25. A smeary appearance of both the small RNA and larger RNA populations is preferable at this stage. If you see discrete bands, especially in the sample containing larger RNAs, it may be that only a small number of RNAs were extracted and over-amplified during PCR. Such cDNA libraries will be very poor, if not completely junk.
26. For gel extraction, follow Qiagen's instructions. Weigh each sample's gel fragment. For gel solubilization, add to each tube three times the gel weight (in mg) of QG solubilization buffer (in μL).
27. Quality control and quantification of the piRNA library may be performed by electrophoresis of a small aliquot of the purified library on an Agilent 2100 Bioanalyzer by following the manufacturer's protocol. This step is typically an integral component of the quality control that core facilities perform prior to Illumina sequencing. The protocol may also be modified to incorporate adapters with barcodes for multiplexing.

28. Depending on the length of the CLIP tag and the maximum read length, a variable part of the 3′ adapter can be present within the read, so the trimming approach has to account for this.
29. piRNAs have distinct biases in their sequence (more than 80 % start with a Uridine), their size (25–30 nucleotides long; 26–27 nucleotides for Mili bound piRNAs, and 30 nucleotides for Miwi bound piRNAs) and in their genomic origin (the vast majority cluster in characterized intergenic hotspots, forming pre-pachytene and pachytene clusters [20, 21]). Therefore, the piRNAs comprise a distinct CLIP tag population. CLIP samples enriched in piRNAs (RNA extracted from the main radioactive signal) should be highly similar to published piRNA libraries from IP experiments using standard methodology [4, 10, 18, 21, 22].
30. Recently it was shown that in vivo RNA–protein crosslinking, and subsequent immunoprecipitation, protein degradation, RNA extraction, and reverse transcription of the RNA as described in this technical protocol, introduces characteristic deletions in the deep sequencing CLIP tag reads, which are due to small protein fragments still attached to the extracted crosslinked RNA that the Reverse Transcriptase often skips without incorporating a cognate nucleotide in the cDNA chain. Hence, the *C*rosslink *I*nduced *M*utation *S*ite analysis (CIMS analysis, [16]), reveals RNAs and exact sequences on these RNAs that were bound by the "CLIPed" protein in vivo. The researcher following this protocol should verify the RNA targets of interest by identifying such deletions. We have noticed that a certain sequence bias occurs around CIMS (usually AAA, at −1 to +1 positions around CIMS, also described in ref. [23]), and therefore caution is advised for utilizing CIMS for sequence motif analysis in the protein binding sites.
31. CLIP tags representing fragments of large RNAs (33 nucleotides and up) bound on Piwi proteins should be expected to fall mainly in three categories, based on their genomic origin: piRNA precursors, retrotransposons, and mRNAs [18]. These three categories represent three separate areas of research interest. Note that these categories are not mutually exclusive, i.e., certain mRNAs are processed into piRNAs and also mRNAs contain embedded retrotransposon-derived sequences. Transcripts that are processed into piRNAs should have CLIP tags from both piRNA and large RNA populations (the latter representing unprocessed fragments or intermediate piRNA fragments) aligned into them in sense orientation. Moreover, intermediate fragments of piRNA processing should bear the hallmarks of piRNA processing, i.e., coincidence of their 5′

ends with 5′ ends of mature piRNAs, and therefore increased bias for a Uridine at the 5′ as well [18]. This analysis requires the identification of the exact genomic coordinates of each CLIP tag, and therefore can only be performed for CLIP tags that align only once in the genome (uniquely mapped tags).

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