1) Prepare lysate of cells or tissues in binding buffer.

For cells, wash 2x in PBS, resuspend in 0.5-1ml of binding buffer. Snap freeze in liquid nitrogen, thaw in 20^{o}C water bath. Repeat 1x freeze-thaw, then dounce 10-20 strokes in pre-chilled glass dounce homogenizer with B (or Type 2) pestle. Spin 15,000 G in microcentrifuge at 4^{o}C for 10 min. Save supernatant as lysate, save pellet in separate tube. Snap freeze in LN and store at -80^{o}C if doing chromatography later.

For fly tissues, add 4x times the amount of protease inhibitors to binding buffer. Add no more than 0.5ml of binding buffer to a tube. Before snap freeze, grind tissue with Kontes disposable microtube tissue grinders. Freeze-thaw once. Repeat tissue grinding one more time. Spin 15000 G in microcentrifuge at 4^{o}C for 10 min. Save supernatant as lysate. Snap freeze in LN and store at -80^{o}C if doing chromatography later.

2) Manual, small scale chromatography

1ml Hitrap FF or HP Q-columns can be purchased as a 5-pack from GE-healthcare. All liquid handling can be performed with 1ml, 5ml and 10ml syringes. Cool all solutions to 4^{o}C or on ice before procedure. Use Milli-Q purified water in all solutions.

When pushing liquid through Hitrap columns, use as light a force as possible while maintaining adequate liquid flow (i.e. 1 ml should flow through no faster than 15 seconds through the column). If you force by hand too much pressure, it will compress the column matrix (you can see by eye that the white matrix in the disposable column will get compressed), resulting in loss of material or improper binding of complexes to column.

Wash new column with 5ml water first. Next, equilibrate column with 5mlof binding buffer.

Load cell lysate onto column with 1ml syringe. Wash column with 1-4ml of binding buffer. Collect each drops coming out of column in these steps at ~1ml fractions as the flowthrough. SAVE Flowthrough! Argonautes will tend to elute in the flowthrough and not bind to the Q column in binding buffer. Most of the flowthrough will be in the first 1-2mls.

Next, wash column with 2-4ml of Elution buffer. Collect these ~1ml fractions as the eluate. SAVE Eluate. Piwis will tend to elute in this mid-salt elution buffer, but other structural RNAs (rRNAs, tRNAs) will remain bound to Q-column. Most of the Eluate will be in the first 1-2mls.

Finally, wash column with 2-4ml of HS Wash buffer. Collect these fractions for all remaining RNAs, consisting mainly of rRNA and tRNA and mRNA fragments.

These are now active protein fractions, which can be used directly in assay, or proceed to RNA extraction below.

3) Deproteinize and extract RNA if analyzing RNA.

Add 1 volume buffer-saturated phenol (pH 7.4) and 1 volume chloroform/isoamyl alcohol (24:1 ChIA). Vortex, spin to separate phases. Reextract 1x more with ChIA. Add 1ul of glycogen to aqueous phase.

Add enough NaCl to bring to 300 mM concentration to the fractions from the Flowthrough (binding buffer). No extra salt is needed for Eluate or HS Wash.

Precipitate extracted small RNAs with 2X volume 200proof ethanol. Wash pellet with 70% ethanol. Resuspend pellet in 10-20ul water.

RNAs are ready for direct ligation to 3' adaptor, no additional size selection is necessary.

4) Although 1ml Hitrap columns are priced to be almost disposable, one can regenerate and store them by immediately washing column in 5ml of 3M NaCl followed by 5ml of 0.1M NaOH followed by 5ml of water and finally with 5ml of 20% ethanol. Column can now be stored (4C to room temp) and re-used later.

5) A note on column capacity. If one is analyzing cells and tissues greater than on a micro scale (i.e. more than ~100 ul volume of tissue), you may need to scale up to a 5ml Hitrap column. Scale all solution volumes by 5x as well. Overloading a column may result in structural RNAs leaking through the column into the flowthrough.

Binding Buffer:

20mM Hepes pH 7.9 (with KOH)
10% glycerol
100 mM KOAc
0.2 mM EDTA
1.5 mM $MgCl_2$

Add the following fresh and in order to final concentration:
0.2 mM PMSF (solubilize in isopropanol)
1.0 mM DTT
1X Roche Complete EDTA-free Protease Inhibitor Cocktail (from 20x frozen stock)

Elution Buffer: same as Binding buffer, but with 400 mM KOAc

HS Wash Buffer: same as Binding buffer, but with 1M KOAc