Immunoprecipitation and High–Throughput Sequencing of ARGONAUTE–Bound Target RNAs from Plants

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Abstract

ARGONAUTE (AGO) proteins function in small RNA (sRNA)–based RNA silencing pathways to regulate gene expression and control invading nucleic acids. In post–transcriptional RNA silencing pathways, plant AGOs associate with sRNAs to interact with highly sequence–complementary target RNAs. Once the AGO–sRNA–target RNA ternary complex is formed, target RNA is typically repressed through AGO–mediated cleavage or through other cleavage–independent mechanisms. The universe of sRNAs associating with diverse plant AGOs has been determined though AGO immunoprecipitation (IP) and high–throughput sequencing of co–immunoprecipitated sRNAs. To better understand the biological functions of AGO–sRNA complexes it is crucial to identify the repertoire of target RNAs they regulate. Here I present a detailed AGO RNA IP followed by high–throughput sequencing (AGO RIP–Seq) methodology for the isolation of AGO ternary complexes from plant tissues and the high–throughput sequencing of AGO–bound target RNAs. In particular, the protocol describes the IP of slicer–deficient hemagglutinin (HA)–tagged AGO proteins expressed in plant tissues, the isolation of AGO–bound RNAs, and the generation of target RNA libraries for high–throughput sequencing.

Key words: ARGONAUTE, RNA immunoprecipitation, high–throughput sequencing, RIP–Seq, small RNA, RNA–Seq, library preparation.

1 Introduction

In plants, ARGONAUTE (AGO) proteins are the effectors of small RNA (sRNA)–based RNA silencing pathways regulating key biological processes such as development, response to stress, chromosome structure and genome integrity, and antiviral defense [1-4]. In post–transcriptional RNA silencing pathways, AGOs associate with specific sRNAs based on the identity of the sRNA 5' nucleotide and on the structure of the sRNA duplex among other factors [5-9]. AGO–loaded sRNAs serve as guides for AGO recognition and interaction with highly sequence–complementary RNAs. Once the ternary complex including the AGO, the sRNA and the target RNA is formed, target RNAs are typically repressed either through AGO–mediated endonucleolytic cleavage (or slicing) or through other cleavage–independent mechanisms such as target destabilization or translational inhibition [2].

AGO immunoprecipitation (IP) followed by high–throughput sequencing of bound sRNAs has been used to determine the universe of plant sRNAs associating with diverse AGOs in different species [5,6,8-11]. However, similar methods have not been described for the identification of AGO–bound target RNAs until recently [12]. *Arabidopsis thaliana* AGO1 target RNAs were efficiently co–immunoprecipitated and sequenced when using slicer–deficient hemagglutinin (HA)–tagged AGO1 forms but not with their wild–type catalytically active counterparts [12].

In this chapter, I present a detailed AGO RNA IP followed by high–throughput sequencing (AGO RIP–Seq) protocol for the genome–wide analysis of AGO–bound target RNAs. I describe the IP of slicer–deficient HA–tagged AGO proteins from plant tissue, the isolation of associated RNAs, and the generation of target RNA libraries for high–throughput sequencing (Fig. 1).

2 Materials

2.1 Plant material

This protocol is intended for processing plant tissue accumulating slicer–deficient HA–tagged AGO proteins (*see* **Note 1**).

2.2 Immunoprecipitation

1. IP buffer: 50 mM Tris–HCl at pH 7.4, 2.5 mM magnesium chloride ($MgCl_2$), 100 mM potassium chloride (KCl), 0.1% Nonidet P–40, 1 μg/mL leupeptin, 1 μg/mL aprotonin, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), one tablet of cOmplete™ Ethylenediaminetetraacetic acid (EDTA)–Free Protease Inhibitor Cocktail Tablets (Sigma–Aldrich) per 50 mL IP buffer, and 50 U/mL RNase inhibitor.
2. Proteinase K (PK) buffer: 0.1 M Tris–HCl pH 7.4, 10 mM EDTA, 300 mM sodium chloride (NaCl), 2% sodium dodecyl sulfate (SDS), 1 μg/μL proteinase K.
3. 2× protein dissociation buffer (PDB): 0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 10% ß–mercaptoethanol, 0.02% bromophenol blue.
4. Anti–HA (12CA5) antibody (Sigma–Aldrich).
5. Protein A agarose (Sigma–Aldrich).
6. Micrococcal nuclease (MNase, Worthington).
7. Ethylene glycol–bis (ß–aminoethyl ether)–N, N, N', N'–tetraacetic acid (EGTA) 0.5 M pH 8 sterile.
8. Sterile Miracloth paper (Merck Millipore).

2.3 RNA Extraction

1. IP buffer–equilibrated phenol: 75% Tris–saturated phenol pH 4.5, 25% IP buffer.
2. Phenol:chloroform:isoamyl alcohol, 25:24:1.
3. Choloroform.
4. Ethanol.
5. Sodium acetate (NaOAc), 3 M pH 5.2.
6. Glycoblue (Thermo Fisher Scientific).
7. Diethylpyrocarbonate (DEPC)–treated H_2O.
8. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.4 Western Blot

1. NuPAGE Novex 4–12% bis–tris Gel 1.0 mm (Thermo Fisher Scientific)
2. Protran nitrocellulose transfer membrane (VWR).
3. Ponceau S solution: 0.1% Ponceau S, 5% acetic acid.
4. Phosphate–buffered saline (PBS) pH 7.4 (10×): 1.06 mM potassium phosphate monobasic (KH_2P0_4), 155.17 mM NaCl, 2.97 mM sodium phosphate dibasic (Na_2HPO_4–$7H_2O$).
5. 1× PBST: 1× PBS, 0.1% Tween 20.
6. Blocking solution: 1× PBST, 5% milk powder.
7. Anti–HA–peroxidase high affinity clone 3F10 (Sigma–Aldrich).
8. Western Lighting Plus ECL kit (PerkinElmer).

2.5 Northern Blot

1. 0.5× Tris–borate–EDTA (TBE).

2. 17% polyacrylamide el containing 7 M urea in 0.5× TBE. Mix 17 mL of 30% polyacrylamide (acrylamide:bisacrylamide, 37.5:1), 12.6 g of urea, 1.5 mL of 10× TBE and 2 mL H_2O. Mix thoroughly by inversion, do not shake or vortex as this incorporates air bubbles to the solution which inhibit polymerization. Heat to 65°C (bath) for 10 min to dissolve the urea. Let for additional 10 min on the bench to allow final resuspension of urea. Add 25 μL TEMED and mix by inversion. Add 150 μL of 10% ammonium persulfate and mix quickly by inversion. Pour the gel and allow it to polymerize for at least 30 min.
3. Whatman papers Protean XL size (Bio–Rad).
4. Nytran SuperCharge nylon membrane (Sigma–Aldrich).
5. USB OptiKinase (Affymetrix).
6. [^{32}P] γ–ATP, 6000 ci/mmol, 10 mCi/mL (PerkinElmer).
7. P–6 spin columns (Bio–Rad).
8. PerfectHyb™Plus buffer (Sigma–Aldrich).
9. Wash buffer 1: 2× SSC, 0.2% SDS.
10. Wash buffer 2: 1× SSC 0.1% SDS.
11. 3× saline sodium citrate (SSC).
12. DEPC–treated H_2O.

2.6 RT–PCR

1. Superscript III First–Strand Synthesis System (Thermo Fisher Scientific).
2. Phusion High–Fidelity DNA Polymerase (Thermo Fisher Scientific).

2.7 RNA–Seq

1. DNA/RNA LoBind 1.5 mL tubes (Eppendorf).

2. Turbo DNA–free Kit (Thermo Fisher Scientific).
3. DEPC–treated H_2O.
4. Ribo–Zero rRNA Removal Kit Plant Leaf (Illumina).
5. Superscript II (Thermo Fisher Scientific).
6. Random primer.
7. SupeRase–In (Thermo Fisher Scientific).
8. Dithiothreitol (DTT), 0.1 M solution.
9. Agencourt RNAClean XP (Beckman Coulter).
10. 10x Blue buffer (Enzymatics).
11. Ribonuclease H (Thermo Fisher Scientific).
12. DNA polymerase I (Enzymatics).
13. Agencourt AMPure XP (Beckman Coulter).
14. EB (Qiagen).
15. 12P XP buffer: 12% PEG–8000, 2.5 M NaCl.
16. End Repair Mix LC (Enzymatics).
17. Klenow 3′–5′exo (Enzymatics).
18. 10× hybridization buffer: 1 M NaCl, 0.1 M Tris–HCl pH 7.8, 0.1 M EDTA pH 8.0.
19. TruSeq adapters (Table 1).
20. T4 DNA Ligase (Rapid) (Enzymatics).
21. Poly ethylene glycol (Sigma–Aldrich).
22. Uracil DNA glycosylase (Enzymatics).
23. Phusion Hot Start 2 high fidelity DNA polymerase (Finnzymes).
24. Paired–end (PE) primer and indexed adapters (Table 1).
25. Superscript 2 reverse transcriptase (Thermo Fisher Scientific).

26. Magnetic stands for PCR and 1.5 mL tubes.
27. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).
28. Qubit DNA HS100 assay kit (Thermo Fisher Scientific).
29. Qubit fluorometer (Thermo Fisher Scientific).
30. RNA 6000 nano kit (Agilent).
31. DNA High Sensitivity (HS) kit (Agilent).
32. Bioanalyzer instrument (Agilent).

3 Methods

3.1 Immunoprecipitation

All the steps are performed in the cold room (at 4ºC) except the nuclease and PK treatments. It is recommended to prepare some of the materials the day before (*see* **Note 2)**.

1. Mix 9 mL of IP buffer and 2 mL of protein A agarose solution (previously well resuspended by pipetting) in a 15 mL conical tube. Rotate for 1 h at 4ºC. Stop rotation, allow beads to sediment at the bottom of the tube (*see* **Note 3**) and remove supernatant. Add 1mL of IP buffer and resuspend beads by pipetting using a wide orifice tip. Rotate for 30 min at 4ºC.
2. Add 2 g of tissue to pre–cooled mortar (*see* **Note 4)**. Add a small amount of liquid nitrogen (*see* **Note 5)**, and grind vigorously with a pestle until obtaining a fine powder. Add 25 mL of IP buffer and homogenize. Transfer 12 mL of homogenate to each of two new 15 mL tubes (a total of 24 mL of homogenate are transferred). Centrifuge for 5 min at 12,000 × *g* at 4ºC to pellet cell debris. Filter the clarified homogenates with two layers of sterile Miracloth paper, and collect filtered homogenates in a single sterile flask.

3. Use 2 mL of filtered homogenate for input RNA (total RNA before IP) extraction (*see* section 3.2). Mix 50 µL of filtered homogenate and 50 µL of 2× PDB in a 1.5 mL tube for input protein analysis; incubate at 100ºC for 3 min, and store at –80ºC until Western blot analysis (*see* section 3.3).
4. Transfer 9 mL of filtered homogenate to each of two new 15 mL tubes for IP. Add 2250 U of MNase to each tube containing 9 mL of filtered homogenate (*see* **Note 6)**. Incubate 5 min at 22ºC and transfer to ice. Add 90 µL EGTA 0.5 M pH 8 to each tube and mix.
5. Transfer 1 mL of digested homogenate from each tube to a new 2 mL tube for nuclease digested (nd) input (nd–input) RNA extraction (*see* section 3.2).
6. Add 128 µg anti–HA (12CA5) antibody to each 15 mL tube containing now 8 mL of nuclease–digested homogenate. Rotate for 30 min at 4ºC. Add 0.8 mL of protein A agarose solution to each tube and rotate for 30 min at 4ºC. Centrifuge for 30 s at 2,500 × *g* at 4ºC to pellet beads. Remove the supernatant by carefully pipetting.
7. Add 3.5 mL IP buffer to each tube and mix. Rotate for 2 min. Centrifuge for 30 s at 2,500 × *g* at 4ºC to pellet beads. Remove the supernatant by carefully pipetting. Do six washes of 10 min each: for each wash add 7 mL IP buffer, rotate for 10 min, centrifuge for 30 s at 2,500 × *g* at 4ºC, and discard supernatant.
8. Pipette 20 µL of beads from each tube, and transfer to the same protein IP tube and add 40 µL of 2× PDB, mix with vortex for 10–30 s, incubate at 100ºC for 3 min, store at –80ºC for Western blot analysis (*see* section 3.3).
9. Add 800 µL of pre–warmed PK buffer containing RNase inhibitor to each tube containing the remaining beads (1,600 µL approximately) (*see* **Note 7**). Add

2,400 µg of proteinase K to each tube and mix with vortex. Incubate for 15 min at 65ºC with intermittent shaking (*see* **Note 8**).

10. Centrifuge for 1 min at 2,500 × *g* at room temperature to pellet beads. Transfer each supernatant (1 mL approximately each) to a new 2 mL tube. Proceed to IP RNA extraction (*see* section 3.2).

3.2 RNA Extraction

All centrifugations are done in a microfuge at 4ºC.

1. Mix the RNA–containing solution (non–digested filtered homogenate, digested filtered homogenate, or supernatant after PK treatment for input RNA, nd–input RNA or IP RNA samples, respectively) with equal volume of IP buffer–saturated phenol.
2. Centrifuge at 14,000 rpm for 5 min.
3. Transfer aqueous phase to fresh tube and repeat extraction with equal volume of phenol:chloroform:isoamyl alcohol.
4. Centrifuge at 14,000 rpm for 5 min.
5. Transfer aqueous phase to a new tube and repeat extraction with equal volume of chloroform.
6. Centrifuge at 14,000 rpm for 5 min at 4ºC.
7. Precipitate RNA from final aqueous phase with 0.1 volumes of NaOAc, 20 µg of glycoblue, and 2.5 volumes of 100% ethanol. Incubate overnight at –80ºC.
8. Centrifuge at 14,000 rpm for 30 min to pellet RNA.
9. Add cold 75% ethanol, centrifuge at 14,000 rpm for 10 min.
10. Remove ethanol and allow to air–dry for at least 10 min.

11. Resuspend input RNA and nd–input RNA in 20–30 µL DEPC–treated H_2O (*see* **Note 9**). Use 1 µL to measure the RNA concentration with a spectrophotometer. Verify nuclease digestion (*see* **Note 10**) (Fig. 2).
12. Resuspend IP RNA in 14 µL DEPC–treated H_2O (*see* **Note 9**). Use 12 µL for transcript library construction, and 2 µL for sRNA library construction using standard protocols [13] if needed (*see* **Note 11**).

3.3 Western Blot Analysis of HA–AGO Protein Immunoprecipitation

Confirm HA–AGO protein IP by Western blot analysis using standard protocols. Next are some specifications.

1. Load 5–10 µL of input protein and 2.5–5 µL of IP protein samples into a 4–12% Bis–Tris mini gel. For IP protein samples, be sure to pipette from the top of the tube to avoid bead pipetting. Loading beads onto the gel will lead to poor protein separation. Load a protein marker, and run the gel at 150 V for 1h 30 min or long enough to resolve the size of the HA–AGO protein (e.g. ~116 kDa for *A. thaliana* HA–AGO1).
2. Transfer proteins to the nitrocellulose membrane. Stain membrane with Ponceau S solution to check protein transfer efficiency (*see* **Note 12**) (Fig. 3a).
3. Incubate membrane in blocking solution with shaking for 30 min at room temperature. Discard blocking solution and add 1× PBS with anti–HA–Peroxidase 3F10 antibody (25 U/mL stock) at a 1:1,000 dilution. Incubate with shaking for 2 h at room temperature, and wash four times in 1× PBST (10 min/wash). Follow the manufacturer´s instructions of the Western Lighting Plus ECL kit or similar for electroluminescence–based detection of HA–AGO proteins (Fig. 3a).

3.4 Northern Blot Analysis of sRNA Co–Immunoprecipitation

Confirm the co–IP of at least one known AGO–interacting sRNA by Northern blot following standard protocols (*see* **Note 13**). Next are some specifications.

1. Prepare a 17% polyacrylamide gel containing 7 M Urea in 0.5× TBE. Pre–run the gel at 180 V in 0.5× TBE for 1 h. Rinse wells before loading samples as urea accumulates at the bottom of the gel. Heat samples for 10 min at 65ºC and immediately quench on ice briefly.
2. Load 2–10 µg of input RNA and 10–20% of IP RNA (*see* **Note 14**). Run at 180 V in 0.5× TBE until the bromophenol blue reaches the bottom of the gel (~4 h).
3. Assemble the blot sandwich with extra–thick Whatman papers (one on each side), gel and positively–charged nylon membrane in a semi–dry chamber. Transfer for 30 min at 500 mA. Auto–crosslink the membrane at 1,200 µJ × 100. Store membranes between two sheets of filter paper until use.
4. Prepare the following reaction mix (*see* **Note 15**):

DNA or LNA oligonucleotide (10 µM)	1 µL
Polynucleotide kinase 10× buffer	1 µL
DEPC–treated H_20	3 µL
[^{32}P] γ–ATP (6,000 ci/mmol; 10 mCi/mL)	4 µL
USB OptiKinase	1 µL

5. Incubate for 60 min at 37ºC. Purify probe on P–6 spin columns according to manufacturer´s instructions. Quantify counts per million (CPM)/µL.

6. Place the membrane (RNA–side–up) in a hybridization tube and pre–hybridize with rotation for at least 5 min at 38–42ºC (*see* **Note 16**) in 5 mL of PerfectHyb™Plus buffer.
7. Mix 1,000,000–2,000,000 CPM of probe with 200 µL of PerfectHyb™Plus buffer, incubate 2 min at 95ºC, and transfer immediately to ice briefly.
8. Add to hybridization tube and incubate for 12–16 h at 38–42ºC (*see* **Note 16**).
9. Remove hybridization solution and wash the membrane five times with pre–heated wash solutions as follows: wash buffer 1 thorough rinse, wash buffer 1 for 5 min with rotation at 38–42ºC, wash buffer 1 for 20 min with rotation at 55ºC, wash buffer 1 for 20 min with rotation at 55ºC, wash buffer 2 for 20 min with rotation at 55ºC, and wash buffer 2 for 30 min with rotation at 55ºC (*see* **Note 17**).
10. Rinse membrane briefly in 3× SSC, then air–dry briefly and cover in transparent plastic wrap.
11. Autoradiograph (Fig. 3b).

3.5 RT–PCR Analysis of Target RNAs

AGO–bound target RNAs can be analyzed by RT–PCR (*See* **Note 6**). In this case, cDNA is obtained from 2–4 µg of input RNA or 10–25% of IP RNA using the Superscript III system according to manufacturer´s instructions. Standard PCR reactions using Phusion high–fidelity DNA polymerase are designed to amplify fragments including the sRNA cleavage site present in the target transcript of analysis (*see* **Note 18**).

3.6 Preparation of Target RNA Libraries for High–Throughput Sequencing

In the target RNA library preparation protocol described below, nucleic acids are purified from all enzymatic reactions using SPRI magnetic beads (*see* **Note 19**). Transcript libraries are made solely from nd–input RNA and IP RNA samples as both samples have been nuclease–treated, which favors later computational analyses.

3.5.1 Preparation of Transcript Fragments

The use of DNA/RNA LoBind 1.5 mL tubes is recommended to increase the RNA recovery in steps 1 and 2.

1. Prepare the DNase I digestion reaction by treating 20 µg of nd–input RNA or 80–100% of IP RNA with TURBO DNA–free kit following the manufacturer´s instructions.
2. Measure the concentration of DNAse I–digested nd–input RNA with a Nanodrop spectrophotometer (*see* **Note 20**).
3. Use 5 µg or 100% of DNAse I–digested nd–input RNA or IP RNA for ribosomal RNA (rRNA) depletion treatment with Ribo–Zero kit following manufacturer´s instructions (*See* **Note 21**).
4. Resuspend nd–input RNA and IP RNA in 12 µL and 8 µL of DEPC–treated H_2O, respectively.
5. Check rRNA depletion (Fig. 2) (*see* **Note 22**).
6. Use 1 µL of Ribo–Zero–treated nd–input RNA to measure its concentration with a spectrophotometer.

3.5.2 cDNA Synthesis

1. Prepare a 12 µL aliquot including 100 ng or 100% of Ribo–Zero treated nd–input RNA or IP RNA, respectively, and 4 µL First Strand buffer and DEPC–treated H_2O (*see* **Note 23**).
2. Prepare the reverse transcription reaction by mixing the following components:

RNA aliquot from the previous step	12 µL
Random primer (0.5 µg/µL)	0.5 µL
SupeRase–In (20 U/µL)	0.75 µL
0.1 M DTT	1 µL

3. Heat at 65 °C for 3 min, place on ice for 2 min and add the following:

DEPC–treated H_2O	3.85 µL
0.1 M DTT	1 µL
10 mM dNTPs	0.25 µL
SupeRase–In (20 U/µL)	0.5 µL
Superscript II (200 U/µL)	0.5 µL

4. Transfer content to PCR strips, and proceed to reverse transcription reaction using the following PCR program: 10 min at 25ºC, 50 min at 42ºC, 15 min at 70ºC, and hold at 4ºC.
5. Purify the RNA/cDNA hybrid as follows: mix 21 µL of reverse transcription reaction, 38 µL of RNAClean XP beads, and 19 µL of 100% ethanol (*see* **Note 24**); pipette up and down 10 times until the solution is well mixed; place the PCR strip in a magnetic stand for PCR tubes for 5–10 min to separate beads from solution; remove the supernatant without disturbing the ring of separated magnetic beads; wash beads twice with 100 µL 75% ethanol, incubating for 30 s

at room temperature; remove PCR strip from the magnet; add 17 µL of DEPC–treated H_2O; pipette up and down 10 times until the solution is well mixed; centrifuge briefly at low speed; incubate for 2 min at room temperature; place the tubes back on the magnetic stand for 1 min; transfer the supernatant (16 µL eluate) to a new PCR strip.

6. Prepare the 2nd–strand synthesis reaction by mixing the following components (*see* **Note 25**):

RNA/cDNA hybrid	16 µL
10× Blue buffer	2 µL
dUTP mix (10 mM dA, dC, dG and 20 mM dU)	1 µL
RNAse H (2 U/µL stock)	0.5 µL
DNA polymerase I (10 U/µL)	1 µL
0.1 M DTT	0.5

7. Incubate at 16ºC for 2.5 h.
8. Purify double–stranded DNA (dsDNA) with AMPure XP beads (*see* **Note 19**) as follows: mix 21 µL of dsDNA, 38 µL of AMPure XP beads, 19 µL of 100% ethanol; mix by pipetting 10 times; let the mixed samples to incubate for 5 min at room temperature for maximum recovery; place the PCR strip onto magnetic stand for 5 min to separate beads from solution; remove the supernatant without disturbing the ring of separated magnetic beads; wash beads twice with 100 µL 75% ethanol, incubating for 30 s at room temperature; air dry for 5 min, lids opened; remove PCR strip from the magnetic stand; resuspend beads in 34 µL EB; incubate the elution suspension for 2 min at room temperature; place the

tubes back on the magnetic stand for 1 min; transfer the supernatant (32 µL of eluate) to a new tube. Save 16 µL of eluate in –80ºC freezer.

3.5.3 Library Construction

To prepare transcript libraries from nd–input RNA or IP RNA follow standard protocols [14]. Here are some specifications.

1. Prepare 12P XP beads by replacing the stock buffer of AMPure XP beads with 12P XP buffer as follows: add 1 mL of AMPure XP beads to a 1.5 mL tube; place onto a magnetic stand until the solution becomes clear; remove the supernatant; wash the beads twice with DEPC–treated H_2O; resuspend the beads in 1 mL 12P XP buffer (*see* **Note 26**).
2. Prepare the end–repair reaction on ice by mixing the following components:

dsDNA	16 µL
10× End Repair buffer	2 µL
10 mM dNTPs	1 µL
End Repair Mix LC	1 µL

3. Incubate at 20ºC for 30 min in thermocycler.
4. Purify end–repaired DNA using 28 µL of AMPure XP beads with 14 µL of 100% ethanol. Elute with 18 µL DEPC–treated H_2O. Transfer 17 µL of eluate to a new tube.
5. Prepare the dA–tailing reaction by mixing the following components:

DNA	17 µL
10× Blue buffer	2 µL
10 mM dATP	1 µL

Klenow 3’–5’ exo	0.5 µL

6. Incubate at 37ºC for 30 min.
7. Purify A–tailed DNA using 28 µL of AMPure XP beads with 14 µL of 100% ethanol. Elute with 11 µL of DEPC–treated H_2O. Transfer 10 µL of eluate to a new tube.
8. Prepare the Y–shape adapter ligation by mixing the following components (*see* **Note 27**):

DNA	10 µL
Annealed TruSeq adapters (2 µM each)	1 µL
2× ligation buffer	12 µL
T4 DNA ligase	1 µL

9. Incubate for 20 min at 20ºC in thermocycler.
10. Take 12 µL (half) and save in –80ºC freezer.
11. Mix the other half (12 µL) of the ligation product and mix with 12 µL of 12P XP beads. Incubate for 6 min at room temperature, 5 min in magnetic stand, keep the supernatant (24 µL) and discard the beads.
12. Mix the supernatant with 12 µL of AMPure XP beads and 5 µL 40% of PEG8000. Incubate for 6 min at room temperature, wash twice and elute in 11 µL of DEPC–treated H_2O. Transfer 10 µL to a new tube. Mix with 12 µL of AMPure XP, incubate for 6 min at room temperature, 5 more minutes in the magnetic stand, wash once. Elute in 32 µL of EB (*see* **Note 28**). Transfer 30 µL in a new tube.
13. Save 15 µL of elute in –80ºC freezer.

3.5.4 Library Amplification and Multiplexing

Up to 12 samples can be multiplexed for paired–end sequencing using the adaptors listed in Table 1. The diverse multiplexing options with these specific adaptors are described in Table 2.

1. Prepare the dUTP excision reaction by mixing the following components:

DNA	5 µL
Uracil DNA glycosilase	1 µL

2. Incubate for 30 min at 37ºC.
3. Prepare the amplification reaction by mixing the following components:

Uracil DNA glycosilase–digested DNA	16 µL
5 mM PE primer F	1 µL
5× Phusion HF buffer	6 µL
10 mM dNTPs	1 µL
DEPC–treated H_20	4.5 µL
Phusion Hot Start 2 DNA polymerase	1 µL
5 mM indexed adapter	1 µL

4. Mix, centrifuge briefly at low speed and transfer to thermocycler. Use the following PCR program: 30 s at 94ºC; 14–18 cycles (*see* **Note 29**) of 30 s at 98ºC, 30 s at 65ºC, 30 s at 72ºC; 5 min at 72ºC; and hold at 4ºC.
5. Purify library using 43 µL of AMPure XP beads. Elute with 13 µL of EB. Transfer 12 µL in a new tube.

6. Measure the library concentration by loading 1 μL of purified library DNA in a Qubit fluorometer following the Qubit DNA HS Assay kit protocol (*see* **Note 30**). It is recommended to analyze DNA amplicons in a Bioanalyzer instrument following the DNA HS kit protocol (Fig. 4). If the amount of the band corresponding to primer dimers (~128 bp) is visibly high (Fig. 4), then an additional purification with 1.2 volumes of AMPure XP beads is highly recommended to remove primer dimers.
7. Prepare a 2–10 nM sample in a 20 μL volume for sequencing in a high–throughput sequencer (e.g. Illumina HiSeq 2000).

4 Notes

1. Inflorescence tissue from T4 transgenic *Arabidopsis thaliana* plants (4–6 weeks old, flower stages 1–12) expressing HA–tagged AGO proteins with authentic regulatory sequences, or agroinfiltrated leaves from *Nicotiana benthamiana* plants transiently expressing HA–tagged AGOs with the *Cauliflower mosaic virus* (CaMV) 35S promoter have been analyzed [12]. Target RNA recovery was more efficient when using slicer–deficient AGO forms instead of catalytically active wild–type counterparts [12].
2. The day before the IP do the following: prepare PK buffer, store at room temperature; wash mortars and pestles, store in the cold (4ºC) room together with pipettes and trash containers; prepare a box with 0.2 and 1 mL tips with wide orifice by cutting the top of the tip to allow efficient bead pipetting, store in the cold room; put clean bench paper at the working area in the cold room; prepare IP buffer without leupeptin, aprotonin, PMSF and proteinase inhibitor tablets (these will be added right before starting the IP protocol), store in the cold room; label

15 mL conical and 1.5/2 mL tubes for bead washing, nuclease treatment, IP, and protein and RNA extractions, store in the cold room.

3. Because bead sedimentation usually takes 10–20 min, proceed to the following step while beads sediment.
4. Two grams of *A. thaliana* inflorescences or 5 g of seedlings have been typically processed for AGO RIP–Seq analysis. One gram of *N. benthamiana* agroinfiltrated leaves has been processed for AGO RIP–RT–PCR analysis [12].
5. The addition of an excessive amount of liquid nitrogen may lead to sample freezing. Sample thawing will take several minutes.
6. The nuclease treatment enriches for AGO–protected fragments, as regions of target RNAs not being protected by the AGO will be degraded. Because MNase is active in cell extracts, the treatment is done in filtered homogenates to nuclease–treat both input and IP fractions. The nuclease treatment can be omitted if AGO–bound targets are detected solely by RT–PCR (and not by high–throughput sequencing).
7. Pre–warm an aliquot of PK Buffer to 65ºC, and add 50 U/mL of RNase inhibitor.
8. If reactions are incubated in a Thermomixer (Eppendorf), shake at 750 rpm every 3 min for 15 s. If reactions are incubated in a water bath, mix reactions every 3 min by inverting the tubes several times, and transfer the tubes back to the bath.
9. Each input RNA, nd–input RNA and IP RNA samples will have two tubes and thus two pellets to resuspend. For each class of sample, resuspend one pellet and use this volume to resuspend the second pellet.
10. Nuclease digestion can be verified by polyacrylamide gel electrophoresis, or by loading samples in a RNA 6000 nano chip in a Bioanalyzer instrument (Fig. 2).

11. It is recommended to do sRNA libraries in parallel to analyze AGO–bound sRNAs [13].
12. Ponceau S staining is optional but recommended to check the efficiency of the protein transfer to the membrane. For Ponceau S staining, add enough Ponceau S solution to cover the transferred membrane and shake for 5 min at room temperature. Wash with H_2O to remove the excess of Ponceau S solution. Bands in red corresponding to more abundant proteins become visible. Remove excess H_2O from the membrane by air drying for 5 min maximum. Scan the membrane and keep the image for total protein loading control panel. This staining step does not interfere with antibody probing. The red stain is removed in the following step.
13. As shown in Figure 3, Northern blot detection of miR172 in the IP fraction can be used to confirm sRNA co–IP with *A. thaliana* AGO1.
14. If doing the complete AGO RIP–Seq protocol do not use more than 10–20% of the IP RNA sample for Northern blot checking, as the amount of IP RNA left may be insufficient to obtain enough amount of final DNA amplicon for high–throughput sequencing.
15. Oligonucleotide sequence is antisense to the sRNA that is analyzed. Start by using a DNA probe for sRNA detection as DNA oligonucleotides are cheap and work well most of the times. However, if sRNA detection with a DNA probe fails then use a LNA probe. Order the LNA oligonucleotide with every other three nucleotides locked, including the first one (e.g. an LNA probe to detect *A. thaliana* miR172 is A+TGC+AGC+ATC+ATC+AAG+ATT+CT, where the + indicates the locked nucleotide).

16. If using a LNA probe pre–hybridize and hybridize at ~20ºC below the calculated dissociation temperature (T_d) [T_d (°C) = 4(G+C) + 2(A+T)] for the corresponding ^{32}P–labeled oligonucleotide.
17. This works in the majority of cases. If there is still a background problem, proceed to an additional incubation of the membrane with 0.1× SSC/0.1% SDS for 60 min at 50ºC, or with 0.1× SSC/1% SDS for 60 min at 50ºC.
18. It may be necessary to increase the number of PCR cycles to 35–40 to detect target RNAs in IP fractions.
19. Every time using SPRI beads be sure of mixing very well the bead stock container with a vortex, even if the bead suspension seems uniform. Be careful to not over–dry SPRI beads as this will compromise re–hydration and elution. Mix well with vortex and increase the elution time from 2 to 5 min if beads remain as black chunks in the elution H_2O. Do not try to elute SPRI beads more than once as this will dilute the eluate concentration. One elution is sufficient as the elution efficiency with H_2O or EB is very high. It is usually better to not transfer to a new tube all the eluate, as some beads could be co–pipetted. In this case it is more convenient to leave a small amount of eluate in the tube and transfer a totally clean bead–free eluate.
20. Do not waste IP RNA sample trying to measure its concentration as it is too low.
21. An efficient rRNA depletion is necessary to obtain a high–quality transcript library with low number of rRNA–derived sequences. rRNA depletion can be verified by agarose gel electrophoresis, or by loading samples in a RNA 6000 nano chip in a Bioanalyzer instrument (Fig. 2). Various rRNA depletion kits were tested, with the Ribo–Zero treatment being the most effective.

22. In contrast to other RNA–Seq protocols, no fragmentation step was included because the average size of RNAs after Ribo–Zero treatment according to Bioanalyzer analysis (Fig. 2) was appropriate for high–throughput sequencing (peak around 200 nt).
23. This protocol is suitable for RNA amounts ranging from 0.5–100 ng.
24. RNA XP–based recovery of RNA fragments smaller than 100 bp is increased adding half volume of 100% ethanol. The addition of PEG–8000 also helps.
25. Deoxyuridine triphosphate (dUTP) incorporation in 2nd strand synthesis instead of deoxythymidine triphosphate (dTTP) enforces strand–specificity.
26. Note that when the 12P XP beads are used, the beads are discarded while the supernatant is retained for subsequent applications.
27. To prepare a 20 μM Universal PE adapter mix (20 μM each) mix 20 μL of 50 μM PE adaptor 1, 20 μL of 50 μM PE adaptor 2, 5 μL of 10× hybridization buffer and 5 μLof DEPC–treated H_2O; heat for 5 min at 75ºC; ramp–down to 25ºC by decreasing 1.5ºC/min, hold for 30 min at 25ºC and put on ice. Freeze at –20ºC. To check annealing, load 2.5 μL of annealed oligos (50 μM each, 500 ng each approximately.) on a 4% Metaphor–Agarose gel. Make a 2 μM working solution right before use.
28. Multiple SPRI bead cleanups are done after ligation to remove primer dimers.
29. The number of PCR cycles is variable. For input samples 14 cycles are usually enough. For nuclease–treated IP samples additional cycles (16–18) may be necessary to amplify enough amount of DNA for high–throughput sequencing (2–10 nM). Do not use excessive number of PCR samples as this will lead to low complexity amplicons.

30. Do not quantify with spectrophotometer as it leads to unevenness among samples.

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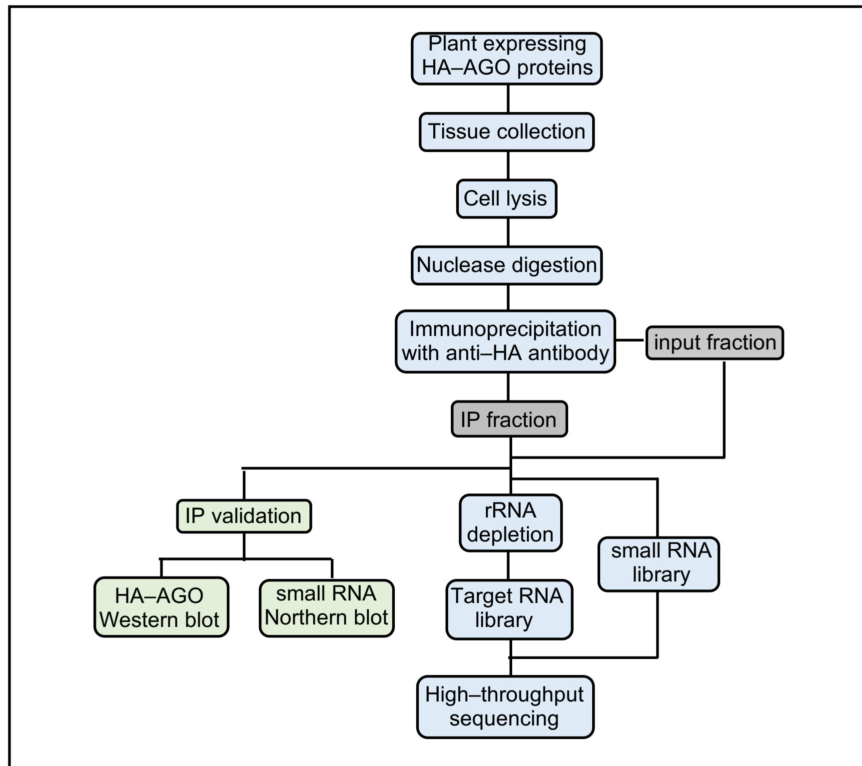

Fig. 1. Flowchart of the analytical steps of the AGO RIP–Seq methodology. Main steps are described in light blue boxes. Steps for the validation of the AGO immunoprecipitation (IP) are described in light green boxes.

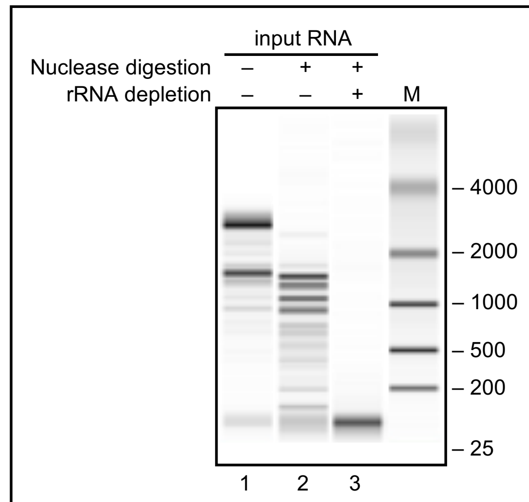

FIGURE 2

Fig. 2. Analysis of nuclease digestion and rRNA depletion. Bioanalyzer analysis of input RNA treated with nuclease and depleted for rRNAs (lane 3). Controls include input RNA untreated (lane 1) or solely digested with nuclease (lane 2). M, molecular weight marker (the size of the bands are indicated in nucleotides).

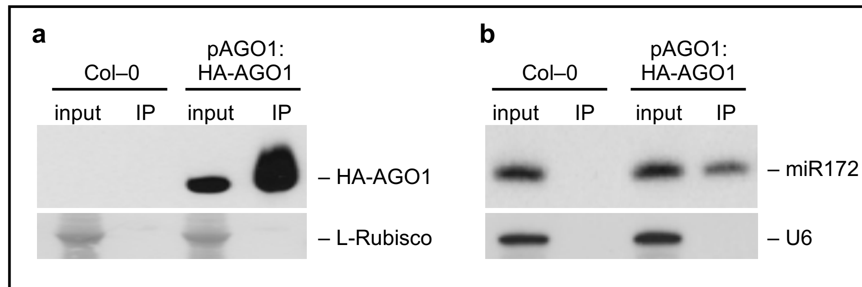

FIGURE 3

Fig. 3. Validation of the AGO immunoprecipitation. Samples from *Arabidopsis thaliana* wild–type Col–0 or transformed with a construct expressing HA–tagged AGO1 forms under endogenous regulatory sequences were analyzed. a) Western blot analysis of HA–AGO1 accumulation in input and IP fractions. L–Rubisco (ribulose–1.5–biphosphate carboxylase/oxygenase) stained membrane is included as input loading and immunoprecipitation control. B) Northern blot analysis of *A. thaliana* miR172 accumulation in input and IP fractions. U6 blot is included as input loading and immunoprecipitation control.

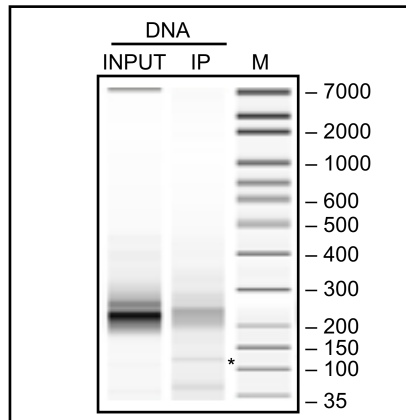

FIGURE 4

Fig. 4. Analysis of the transcript library amplicons. Bioanalyzer analysis of the library amplicons obtained with nuclease–digested input and IP fractions. The asterisk indicates the band corresponding to primer–dimer products. M, molecular weight marker (the size of the bands are indicated in base pairs).

Table 1. Oligonucleotides used in transcript library preparation.

Name	**Nucleotide Sequence**
PE primer-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq adapter 1	A*A*TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT*C*T
TruSeq adapter 2	/5Phos/G*A*TCGGAAGAGCACACGTCTGAACTCCAGTC*A*C
Indexed adapter 1	CAAGCAGAAGACGGCATACGAGATtcgccttaGTGACTGGAGTTCAGACGTGT
Indexed adapter 2	CAAGCAGAAGACGGCATACGAGATctagtacgGTGACTGGAGTTCAGACGTGT
Indexed adapter 3	CAAGCAGAAGACGGCATACGAGATttctgcctGTGACTGGAGTTCAGACGTGT
Indexed adapter 4	CAAGCAGAAGACGGCATACGAGATgctcaggaGTGACTGGAGTTCAGACGTGT
Indexed adapter 5	CAAGCAGAAGACGGCATACGAGATggactcctGTGACTGGAGTTCAGACGTGT
Indexed adapter 6	CAAGCAGAAGACGGCATACGAGATtaggcatgGTGACTGGAGTTCAGACGTGT
Indexed adapter 7	CAAGCAGAAGACGGCATACGAGATctctctacGTGACTGGAGTTCAGACGTGT
Indexed adapter 8	CAAGCAGAAGACGGCATACGAGATcagagaggGTGACTGGAGTTCAGACGTGT
Indexed adapter 9	CAAGCAGAAGACGGCATACGAGATgctacgctGTGACTGGAGTTCAGACGTGT
Indexed adapter 10	CAAGCAGAAGACGGCATACGAGATcgaggctgGTGACTGGAGTTCAGACGTGT
Indexed adapter 11	CAAGCAGAAGACGGCATACGAGATaagaggcaGTGACTGGAGTTCAGACGTGT
Indexed adapter 12	CAAGCAGAAGACGGCATACGAGATgtagaggaGTGACTGGAGTTCAGACGTGT

* - Phosphorothioate Bond
/5Phos/ - 5' primer phosphorylation
Unique index sequences are in low case.

Table 2. Multiplexing of transcript libraries for high–throughput sequencing.

Plex	Indexed adapter selection
1–plex (no pooling)	Any indexed adapter
2–plex	Option A: 1 and 2 Option B: 2 and 4
3-plex	Option A: 1, 2 and 4 Option B: 3, 5 and 6
4– or 5–plex	Option A: 1, 2, 4, and any other indexed adapter Option B: 3, 5, 6, and any other indexed adapter
6–plex	1–6
7–12 plex, single index	1–6 and any other indexed adapter
7–12 plex, dual index	Option A: 1, 2, 4, and any other indexed adapter Option B: 3, 5, 6, and any other indexed adapter