RNA Immunoprecipitation (RIP) Sequencing of Pri-miRNAs Associated with the Dicing Complex in *Arabidopsis*

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[Abstract] RNA immunoprecipitation (RIP) is an antibody-based technique used to map *in vivo* RNA-protein interactions. DBR1, an RNA debranching enzyme, is responsible for the debranching of lariat RNA, for the degradation and turnover of lariat RNAs. It is well known that primary miRNA (Pri-miRNA) is recognized and further processed into mature miRNA by the Dicing complex mainly composed of DCL1 and HYL1. Due to the low abundance of pri-miRNAs, RIP followed qRT-PCR has been widely used to evaluate the binding efficiency of the Dicing complex with pri-miRNAs in previous studies. Therefore, the genome-wide evaluation of the Dicing complex with pri-miRNAs is lacking. With the improvement of high-throughput sequencing technologies, we successfully used RIP-seq to compare the binding efficiency of the Dicing complex with pri-miRNAs between wild-type and the *dbr1-2* mutant in our recent study. In this protocol, we provide a detailed description of RIP-seq using GFP-trap beads in HYL1-YFP and DCL1-YFP transgenic plants between two different genotypes. This method can be used to assess the binding of pri-miRNAs with the Dicing complex in *Arabidopsis*, and it can be applied to other RNA binding proteins in plants.
Keywords: DBR1, RIP, Pri-miRNA, The Dicing complex

Materials and Reagents

1. RNase-free pipette tips
2. RNase-free Eppendorf tubes (Safe-Lock Tubes, Eppendorf, catalog number: 0030120086)
3. 50 ml and 15 ml RNase-free Falcon tubes (Corning Company)
4. Miracloth (Merck, catalog number: 475855-1R)
5. Paper towels
6. Fresh inflorescences including all buds and open flowers together (see Figure 1) of proHYL1::YFP-HYL1/Col-0, proHYL1::YFP-HYL1/*dbr1-2*, proDCL1::DCL1-YFP/Col-0, proDCL1::DCL1-YFP/*dbr1-2* plants grown in 16 h/8 h light/dark condition, 22 °C

Figure 1. A mature plant with the position of the tissue used in this method indicated by a red rectangle. Inflorescences were collected freshly. In general, it is better to use the inflorescences from primary shoot for this experiment.

7. Liquid nitrogen
8. Ice
9. 37% formaldehyde (Sinopharm Chemical Regent, catalog number: 10010018, store in the dark)
10. Diethy pyrocarbonate (DEPC) (Sigma-Aldrich, catalog number: D5758)
11. GFP-Trap agarose beads (Chromotek, catalog number: gta-20)
12. Protein A agarose/Salmon Sperm DNA (Merck, Upstate, catalog number: 16-157)
13. RNase inhibitor (Promega, catalog number: N2611)
14. RQ1 DNase I (Promega, catalog number: M6101)
15. Glycogen (Thermo Fisher Scientific, catalog number: R0551)
16. Proteinase K (Thermo Fisher Scientific, catalog number: EO0491)
17. SuperScript IV (Thermo Fisher Scientific, Invitrogen^{TM}, catalog number: 18090200)
18. Taq DNA polymerase (Takara Bio, catalog number: R500Z)
19. dNTP (Thermo Fisher Scientific, catalog number: R0181)

20. Oligo dT (Thermo Fisher Scientific, catalog number: SO132)
21. DTT (Thermo Fisher Scientific, catalog number: R0861)
22. Acidic Phenol:Chloroform
    a. Chloroform (Acros Organics, catalog number: 423555000)
    b. Acidic Phenol (Sangon Biotech, catalog number: A504195)
23. Ethanol (Acros Organics, catalog number: 397690010)
24. Agarose (Thermo Fisher Scientific, Invitrogen™, catalog number: 16500100)
25. PMSF (Sigma-Aldrich, catalog number: P7626)
26. Protease Inhibitor Cocktail (Roche Diagnostics, catalog number: 11873580001)
27. Illumina TruSeq Stranded Total RNA HT Sample Prep Kit (P/N15031048)
28. $MgCl_2$ (Sangon Biotech, catalog number: MB0331)
29. $CaCl_2$ (Sangon Biotech, catalog number: CT1330)
30. Glycine (Sangon Biotech, catalog number: A610235)
31. EDTA (Sangon Biotech, catalog number: A100105)
32. NaAc (Sangon Biotech, catalog number: ST0827)
33. Sucrose (Sangon Biotech, catalog number: SB0498)
34. HEPES (Sangon Biotech, catalog number: H0511)
35. Tris (Sangon Biotech, catalog number: A600194)
36. SDS (Sigma-Aldrich, catalog number: L5750)
37. NaCl (Sangon Biotech, catalog number: A501218)
38. Ficoll (Sigma-Aldrich, catalog number: F2637)
39. Dextran T40 (Sangon Biotech, catalog number: DB0374)
40. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
41. Primers used in Figure 3:

    Pri-miR156a-F: CAAGAGAAACGCAAAGAAACTGACAG

    Pri-miR156a-R: AAAGAGATCAGCACCGGAATCTGACAG

    Pri-miR158a-F: GTGATGACGCCATTGCTCTTT

    Pri-miR158a-R: TGTGACTTTAGATGCCCTTGTTCA

    Pri-miR159a-F: GGAGCTCTACTTCCATCGTCA

    Pri-miR159a-R: CCACGTTCTCATCAAAACTTTC

    Pri-miR166a-F: GACTCTGGCTCGCTCTATTCA

    Pri-miR166a-R: TGGTCCGAAGACGCTAAAAC

    Pri-miR167a-F: GAAGCTGCCAGCATGATCTA

    Pri-miR167a-R: GGGTTTATAGAAGGGTGCGA

    Pri-miR168aF: GCCTTGCATCAACTGAAT

    Pri-miR168aR: CAAACAAAAGGAGACTAAAGA

    Pri-miR169aF: TGGGTATAGCTAGTGAAACGCG

    Pri-miR169aR: CCTTAGCTTGAGTTCTTGCGA

    Pri-miR171aF: CCGCGCCAATATCTCAGTA

Pri-miR171aR: TGTCTCCATTTCAACACACACA
Pri-miR172aF: ATCTGTTGATGGACGGTGGT
Pri-miR172aR: AATAGTCGTTGATTGCCGATG
Pri-miR319aF: GAGATAGAGAGTTGAACAAATTCTTC
Pri-miR319aR: GTATCCATGATAGTTGAGAAATTTGC

42. Honda Buffer (see Recipes)
43. Nuclei Lysis Buffer(see Recipes)
44. ChIP Dilution Buffer (see Recipes)
45. Binding/Washing Buffer (see Recipes)
46. RIP Elution Buffer (see Recipes)

Equipment

1. Pipettes (Gilson Company)
2. Fume hood
3. Vacuum Pump (Shanghai SENCO Company, model: SHB-3)
4. Microcentrifuge (Thermo)
5. Vortexer (VWR Company)
6. Mortar and Pestle (Fisher)
7. -80 °C Freezer (Thermo)
8. Rotator (Haimen Kylin-Bell Lab Instruments, catalog number: KB-3-D)
9. Mini Gel Electrophoresis Systems (Tanon Company)
10. ChIP-grade Sonifier (Diagenode Bioruptor)
11. Illumina HiSeq 2000 (Shanghai Genegy Company)

Procedure

1. Sample collection

   Collect ~3 g fresh inflorescences of proHYL1::YFP-HYL1 Col-0, proHYL1::YFP-HYL1 *dbr1-2*, and proDCL1::DCL1-YFP Col-0, proDCL1::DCL1-YFP *dbr1-2* plants into a 50 ml RNase-free tub which contains 36 ml RNase-free H_2O.

   Notes:

   a. *Other tissues are also ok to be used for RIP, depending on the expression pattern of the gene of interest. Since there are many chloroplasts in leaf tissue which will interfere with the following steps, inflorescences are more optimal tissues than leaves for a RIP experiment.*
   b. *To avoid protein/RNA/DNA degradation, finish this step as soon as possible, and put the sample tube on ice might help. Also, to make sure each inflorescence immersed in H_2O, we need to invert the tube occasionally.*

2. Sample crosslinking

   Add 1 ml 37% formaldehyde into the tube, and immediately apply vacuum for 30 min at 0.09 Mpa, and then pause the vacuum to mix the tissue by inverting the tube, and repeat vacuum one more time.

   Note: The crosslinking time is dependent on the tissue type. In our case for inflorescences it is 1-1.5 h, and it only needs 10-15 min for young seedlings. During crosslinking process, you will see some tiny bubbles up in the tube, and the tissue should be soaked and translucent after crosslinking.

3. Stop crosslinking
   a. Stop the crosslinking by adding Glycine to a final concentration of 0.125 M (2.5 ml of freshly made 2 M Glycine).
   b. Invert the tube to mix well and apply vacuum for 15 min at 0.09 Mpa. Repeat vacuum one more time.
   c. Take the tube out from the vacuum and rinse the tissue to remove the formaldehyde with RNase-free H_2O for more than 5 times.
   d. To avoid the inconvenience caused by excess water on the tissue during the grinding step, remove the water as much as possible from the inflorescences by pouring the crosslinked tissue onto paper towels.

      Note: You can freeze the crosslinked tissues in liquid nitrogen, and store the sample at -80 °C before taking the next step (It's optimal to take the next step in one month).

4. Cell lysates preparation
   a. Grind the above-crosslinked inflorescences in liquid nitrogen into fine powder in mortar and pestle. Transfer the ground powder to a 50 ml tube containing 30 ml pre-cold Honda Buffer (Recipe 1) supplemented with 8 U/ml RNase inhibitor.

      Note: All the following steps should be manipulated on ice.
   b. Filter the solution through 4 layers of Miracloth (pre-wetted with distilled water) into a fresh pre-cold 50 ml Falcon tube. Repeat once to remove additional debris.

      Note: Squeeze the Miracloth slightly to avoid liquid loss, and finally we have been getting ~30 ml supernatant back.
   c. Centrifuge the filtered solution at 1,750 *x g* for 20 min at 4 °C.
   d. Gently remove the supernatant and thoroughly resuspend the pellet by pipetting up and down in 1 ml of pre-cold Honda Buffer with 8 U/ml RNase inhibitor. Transfer the solution to a 1.5 ml Eppendorf tube on ice.

      Note: To avoid foaming, you have to pipet it very slowly, and keep the tubes on ice when you are doing the resuspension steps.
   e. Centrifuge the solution at 2,500 *x g* for 5 min in a microcentrifuge at 4 °C.
   f. Repeat Steps 4d-4e two more times.

5. Chromatin preparation

a. Gently remove the supernatant and resuspend the pellet thoroughly by pipetting up and down in 300 µl Nuclei Lysis Buffer (Recipe 2) with 160 U/ml RNase inhibitor. Keep 1-2 µl aliquot as the control to detect the sonication efficiency later. Perform sonication on the Diagenode Bioruptor instrument using the following settings: low (L) power output, 30 sec ON/30 sec OFF pulses, 4 °C water bath but no floating ice, position R1 without rotation. Adjust the total sonication time depending on the required chromatin fragmentation. In general, 12-15 min total is required to achieve the extent of 200-1,000 bp fragmentation.

b. Centrifuge the sonicated samples for 10 min at 16,000 *x g* in a microcentrifuge at 4 °C, transfer the supernatant to the fresh tubes. Save 10 µl of each genotype as the Input.

Notes:

i. Together with the aliquot before sonication, take 1-2 µl aliquot to check the sonication efficiency on a 1% agarose gel with ethidium bromide staining. You should make sure the smear bands for each sample are within 200-1,000 bp, with a relative peak around ~500 bp (see Figure 2).

ii. You can freeze the chromatin samples in liquid nitrogen, and store the sample at -80 °C before taking the next step (It is optimal to take the next step in one month).

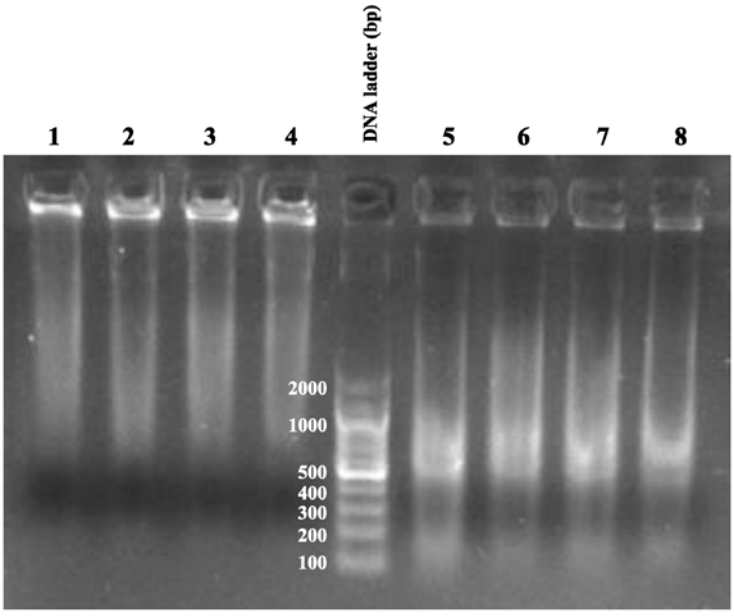

Figure 2. Detection of sonication efficiency by agarose gel analysis. Aliquots (1-2 µl) from Steps 5a (samples prior to sonication, lanes 1-4) and 5b (samples after sonication, lanes 5-8) were analyzed on 1% agarose gel with ethidium bromide staining. The middle lane indicates the DNA ladder to show the size range of DNA before or after sonication.

6. Immunoprecipitation preparation

a. Dilute the chromatin into a total volume of 3 ml ChIP Dilution Buffer with 350 U/ml RNase inhibitor.

Note: No matter how much chromatin will be used in the following steps, please make sure

to dilute the chromatin sample ten times with ChIP Dilution buffer, because 10% SDS in the Nuclei Lysis Buffer should be diluted into 1%. Otherwise, it will disrupt the following reactions.

b. Add 60 U DNase I, 25 mM $MgCl_2$ and 5 mM $CaCl_2$ to the chromatin solution (total 3 ml), and incubate it for 15 min at 37 °C.
c. After incubation, add 20 mM EDTA to stop the reaction.
d. Prewash 40 µl Protein A agarose/Salmon Sperm DNA slurry by adding 1 ml of ChIP Dilution buffer and spinning at 100 *x g* for 1 min at 4 °C, and then remove the supernatants. Repeat this step two more times.
   Note: Please cut the pipette tip when you pipette the Protein A agarose/Salmon Sperm DNA slurry.
e. Pre-clear the sample in a 15 ml falcon tube for 30 min at 4 °C each with 40 µl of prewashed Protein A agarose/Salmon Sperm DNA slurry.
f. Centrifuge the sample at 7,000 *x g* for 10 min at 4 °C.
g. Transfer the supernatant into a new tube, and then aliquot the supernatant into another two Eppendorf tubes, ~1.5 ml for each.
h. Prewash GFP-Trap agarose beads and 25 µl Protein A agarose/Salmon Sperm DNA slurry as described in Step 6d.
i. Add 25 µl of GFP-Trap agarose beads into each sample tube, and 25 µl of Protein A agarose/Salmon Sperm DNA slurry into the tube of the No antibody control.
j. Incubate on a rotator overnight at 4 °C.

7. Wash the immunoprecipitated RNA–wash the beads with the following steps:
   a. Add 1 ml Binding/Washing Buffer + 40 U/ml RNase inhibitor into each tube and shake by hands for 2-3 sec.
   b. Rotate the samples in a rotator at 4 °C for 5 min.
   c. Centrifuge the samples at 100 *x g* for 2 min.
   d. Decant the supernatants.
   e. Repeat Steps 7a-7d one more time.
8. Elute the immunoprecipitated RNA
   a. Add 200 µl RIP Elution Buffer with 40 U/sample RNase inhibitor.
   b. Rotate at room temperature 15 min (gently vortex it several times during rotation process).
   c. Centrifuge at 400 *x g*, 2 min. Save the supernatant.
   d. Repeat the elution at 65 °C for 15 min with gently rotating.
   e. Centrifuge at 400 *x g*, 2 min. Combine the supernatants from twice elutions.
9. Purify the immunoprecipitated RNA
   a. Add 1 µl 20 mg/ml Proteinase K. Incubate for 1 h at 42 °C.
   b. Then add 16 µl 5 M NaCl. Incubate for 1 h at 65 °C.
      Note: Remember to take out the Input samples, and add the RIP Elution Buffer to 400 µl, and perform Proteinase K digestion and reverse crosslinking also.

c. Then add 400 µl acidic Phenol:Chloroform, vortex for 15 sec vigorously, centrifuge at 16,000 *x g* for 5 min at 4 °C.
d. Transfer the aqueous phase (the upper layer) to new tubes.
e. Add 40 µl 3 M NaAc pH 5.2, 1 µl Glycogen and 1 ml ethanol.
f. Leave at -80 °C for overnight.
g. Centrifuge at 16,000 *x g* for 30 min at 4 °C.
h. Wash the pellet with 500 µl 70% ethanol. Centrifuge at 16,000 *x g* for 20 min at 4 °C.
i. Air-dry for 2-5 min, dissolve RNA (you can see the tiny white pellet in the tube bottom) in 100 µl RNase-free-H_2O.

10. Validate the immunoprecipitation efficiency by RT-PCR

Optional: You can use One-step RT-PCR kit for this step.

a. Digest immunoprecipitated RNA samples with RQ1 DNase I as below:

5 µl above immunoprecipitated RNA
10x DNase I buffer 5 µl
RNase inhibitor 0.5 µl
RQ1 DNase I 3 µl
RNase-free H_2O 36.5 µl
Mix by inverting the tubes, spin briefly, incubate at 37 °C for 30 min.

b. Add 250 µl RNase-free H_2O into the tube, 400 µl acidic Phenol:Chloroform, vortex for 15 sec vigorously, centrifuge at 16,000 *x g* for 5 min at 4 °C.
c. Transfer the aqueous phase to new tubes, add 40 µl 3 M NaAc pH 5.2, 1 µl Glycogen and 1 ml ethanol. Leave at -80 °C for overnight, centrifuge at 16,000 *x g* for 30 min at 4 °C.
d. Wash the pellet with 500 µl 70% ethanol, centrifuge at 16,000 *x g* for 20 min at 4 °C.
e. Air-dry for 2-5 min, dissolve the tiny white pellet in 11 µl RNase-free H_2O.
f. cDNA synthesis by using SuperScript IV as below:

11 µl DNase I-digested immunoprecipitated RNA
1 µl Oligo dT
1 µl 10 mM dNTP mix
Mix and briefly centrifuge the components. Heat the RNA-primer mix at 65 °C for 5 min, and then incubate on ice for at least 1 min.
4 µl 5x SSIV Buffer
1 µl 100 mM DTT
1 µl RNase inhibitor
1 µl SuperScript IV
Incubate the above reaction mixture at 50 °C for 10 min, and then inactivate the reaction by incubating it at 80 °C for 10 min. Dilute the cDNA into 50 µl.

g. Perform PCR reactions to detect pri-miRNAs as below:

2 µl cDNA
1.5 µl 10x PCR Buffer

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1.5 µl 2.5 mM dNTP mix

0.5 µl Primer-F (refer to the primer list on the materials)

0.5 µl Primer-R (refer to the primer list on the materials)

0.1 µl Taq

8.9 µl H_2O

Incubate the above reaction mixture as the below procedure:

1) 95 °C for 2 min
2) 95 °C for 30 sec
3) 58 °C for 30 sec
4) 72 °C for 30 sec
5) Go to 2), 35 cycles
6) 72 °C for 10 min
7) 16 °C forever

Detect the PCR products by 2% agarose gel electrophoresis (see Figure 3).

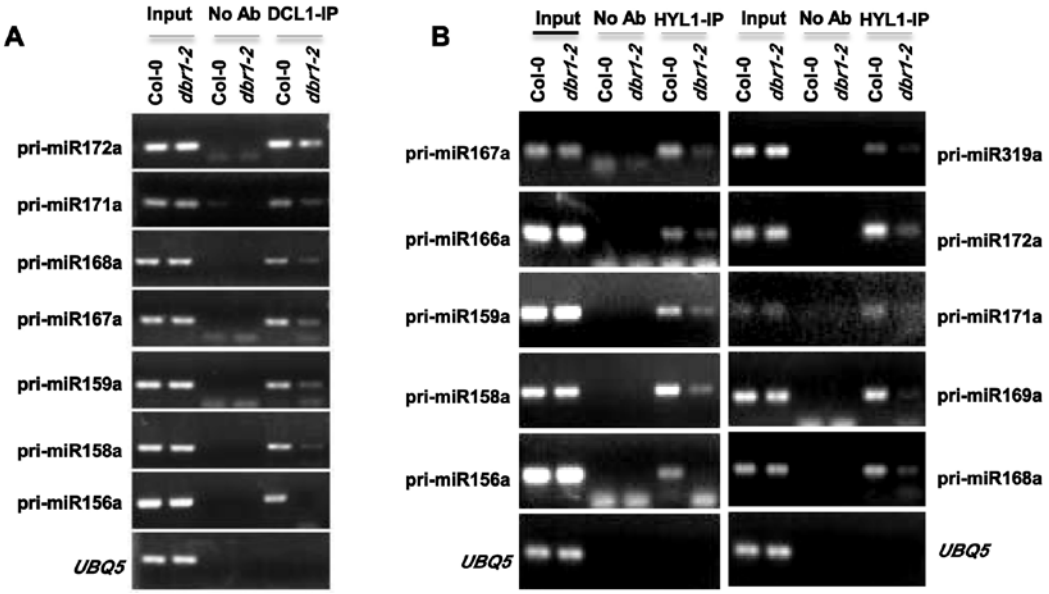

Figure 3. Pri-miRNA binding to DCL1 (A) and HYL1 (B) is reduced in the *dbr1* mutant. DCL1 (A) and HYL1 (B) were immunoprecipitated from inflorescences of proDCL1::DCL1-YFP and proHYL1::YFP-HYL1 in the background of Col-0 and *dbr1-2* using GFP-Trap agarose, respectively. The results shown were reproduced with three biological replicates.

11. Genome-wide detection of pri-miRNA by RNA sequencing (RIP-seq)
    a. Recovered RNAs are used for library preparation with Illumina TruSeq Stranded Total RNA HT Sample Prep Kit (P/N15031048), and then subjected to deep sequencing with Illumina HiSeq 2000.
    b. Align the RIP-seq libraries to the genome with Cufflinks 2 (Trapnell *et al.*, 2010). Use the "bedtools genomecov" command of bedtools (Jagadeeswaran *et al.*, 2012) to calculate

genome coverage of RIP-seq libraries.

c. Then, a custom program is used to calculate the RPKMs (Reads Per Kilo basepairs and per Million sequencing tags) of pre-miRNAs in miRBase, using the genome coverage of RIP-seq libraries. Two examples are shown as below (see Figure 4, Li *et al*., 2016):

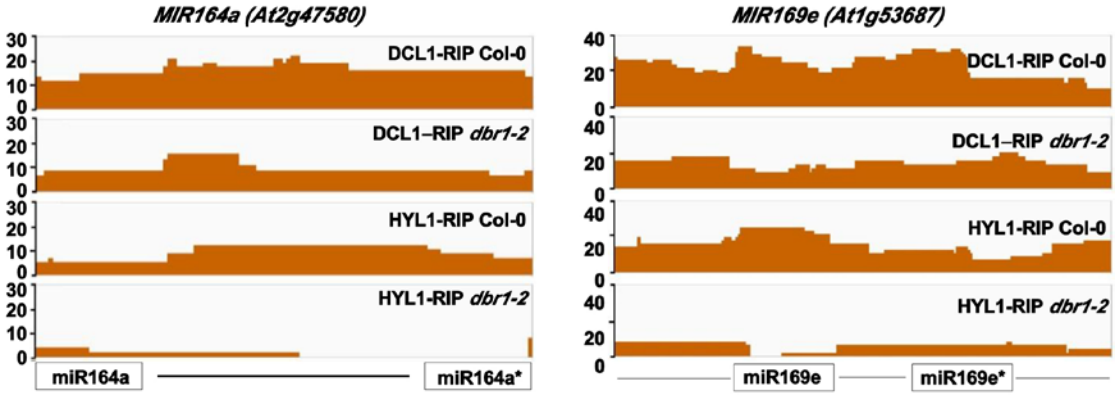

Figure 4. The occupancy of DCL1 and HYL1 at *MIR164a* and *MIR169e*. The coverage regions are shown as normalized peaks. The *x*-axis indicates the relative position of miRNA and miRNA* location. The *y*-axis indicates normalized peaks from the genomic region. Reads counts were normalized to tag per 10 million (TP10M) to adjust for sequencing depth differences of the two RIP-seq libraries.

Recipes

Notes:

a. *All solution should be made with RNase-free water (Water is usually treated with 0.1% DEPC (v/v) for overnight at room temperature and then autoclaved (at least 15 min) to inactivate traces of DEPC).*

b. *DEPC is very toxic; please manipulate it in a hood.*

1. Honda Buffer (freshly made)
   0.44 M Sucrose
   1.25% Ficoll
   2.5% Dextran T40
   20 mM HEPES, pH 7.4 (1 M stock solution)
   10 mM $MgCl_2$ (1 M stock solution)
   0.5% Triton X-100 (20% stock solution)
   5 mM DTT
   1 mM PMSF (added just before use)
   1 tablet Cocktail per 30 ml buffer
   Note: 100 mM PMSF stock is prepared with isopropanol, and store it at -20 °C. Because it

usually loses activity in aqueous solution in less than 30 min, it should be added freshly to the buffer.

2. Nuclei Lysis Buffer (freshly made)
   50 mM Tris-HCl, pH 8 (1 M stock solution)
   10 mM EDTA (0.5 M stock solution)
   1% SDS (10% stock solution)
   1 mM PMSF (the same note as above)
   1x Protease Inhibitor Cocktail (the 50x stock solution is made by dissolving 1 tablet into 1 ml RNase-free H_2O)
3. ChIP Dilution Buffer (freshly made)
   1.1% Triton X-100 (20% stock solution)
   1.2 mM EDTA (0.5 M stock solution)
   16.7 mM Tris-HCl, pH 8 (1 M stock solution)
   167 mM NaCl (5 M stock solution)
   1 mM PMSF (added just before use)
   1x Protease Inhibitor Cocktail (the 50x stock solution is made by dissolving 1 tablet into 1 ml RNase-free H_2O)
4. Binding/Washing Buffer
   150 mM NaCl (5 M stock solution)
   20 mM Tris-HCl, pH 8 (1 M stock solution)
   2 mM EDTA (0.5 M stock solution)
   1% Triton X-100 (20% stock solution)
   0.1% SDS (10% stock solution)
   1 mM PMSF (added just before use)
   1x Protease Inhibitor Cocktail (the 50x stock solution is made by dissolving 1 tablet into 1 ml RNase-free H_2O)
5. RIP Elution Buffer
   100 mM Tris-HCl, pH 8 (1 M stock solution)
   10 mM EDTA (0.5 M stock solution)
   1% SDS (10% stock solution)

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References

1. Li, Z., Wang, S., Cheng, J., Su, C., Zhong, S., Liu, Q., Fang, Y., Yu, Y., Lv, H., Zheng, Y. and

Zheng, B. (2016). Intron lariat RNA inhibits MicroRNA biogenesis by sequestering the dicing complex in *Arabidopsis*. *PLoS Genet* 12(11): e1006422.

2. Jagadeeswaran, G., Nimmakayala, P., Zheng, Y., Gowdu, K., Reddy, U. K. and Sunkar, R. (2012). Characterization of the small RNA component of leaves and fruits from four different cucurbit species. *BMC Genomics* 13: 329.
3. Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J. and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 28(5): 511-515.

