Chapter 17

Identification of Long Noncoding RNA-Protein Interactions Through In Vitro RNA Pull-Down Assay with Plant Nuclear Extracts

Jun Sung Seo and Nam-Hai Chua

Abstract

Recent advances in next-generation sequencing have revealed that majority of the plant genome is transcribed into long noncoding RNA (lncRNA). Many lncRNAs function by interacting with proteins and forming regulatory complexes. RNA-protein interactions are vital in controlling core cellular processes like transcription and translation. Therefore, identifying proteins that interact with lncRNAs is the first step to deciphering lncRNA functions. Here, we describe an RNA-protein pull-down assay, which enables the identification of proteins that interact with an RNA under study. As an example, we describe pull-down of proteins interacting with lncRNA ELENA1, which promotes the enrichment of MED19a on *PR1* promoter to activate *PR1* expression.

Key words Noncoding RNA, RNA-protein interactions, Biotin, In vitro transcription, ELENA1, Pull-down, MED19a

1 Introduction

Majority of the plant genome is transcribed into long noncoding RNA (lncRNA), but only a few lncRNAs have been functionally characterized until now [1, 2]. These few examples support the notion that many lncRNAs function through their interactions with protein partners [2, 3]. Therefore, identification of novel RNA-protein interactions could be highly important in determining unknown functions of lncRNAs. Most of molecular biology tools are protein-centric because of our lack of understanding about functional roles of many ncRNAs. Fewer RNA-centric methods are available for biologists to identify the whole repertoire of RNA-interacting proteins. One of such methods is RNA-protein pull-down assay where one or multiple proteins binding to RNA can be identified. This method (Fig. 1) utilizes in vitro transcription to produce RNA or a part of the RNA of interest which is then

Julia A. Chekanova and Hsiao-Lin V. Wang (eds.), *Plant Long Non-Coding RNAs: Methods and Protocols*, Methods in Molecular Biology, vol. 1933, https://doi.org/10.1007/978-1-4939-9045-0_17,
© Springer Science+Business Media, LLC, part of Springer Nature 2019

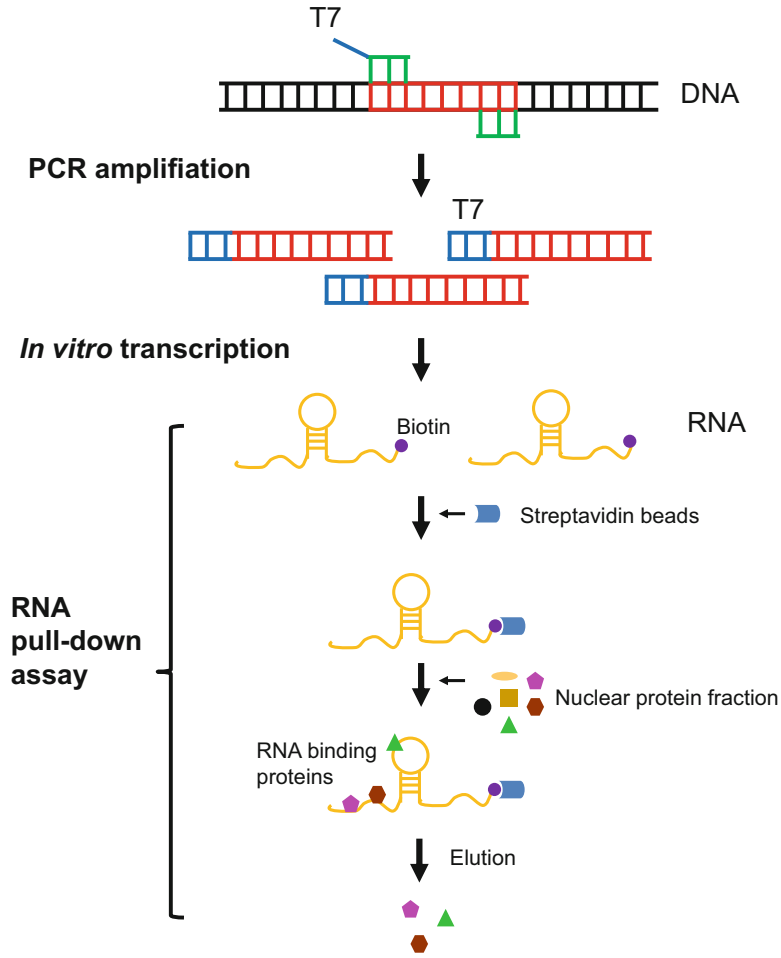

Fig. 1 Work flow of in vitro transcription and RNA-protein pull-down method. The DNA region of interest (shown in red) was amplified and used for in vitro transcription to produce RNA for biotin labeling. Biotin-labeled RNA was captured by streptavidin beads, incubated with nuclear fraction extract to allow for RNA-protein interactions, and the RNA-bound protein was eluted for detection by Western blotting

labeled using biotin. Proteins interacting with the biotin-labeled RNA are retrieved from whole-cell or protein extracts using streptavidin beads. These proteins can be then identified using mass spectrometry or Western blot. In the first step, a DNA template encoding the RNA sequence is produced with an added 5′ T7 RNA polymerase promoter sequence (5′-TAATACGACTCACTATA-3′) [4]. The T7 RNA polymerase promoter sequence is introduced into the DNA template using a polymerase chain reaction (PCR) step and specially designed primers. The T7 promoter is used to transcribe and produce copies of RNA in vitro. The biggest advantage of using in vitro transcription is that it allows synthesis of

different lengths of RNAs with a specified sequence. We used this pull-down assay to identify the interaction between the GFP-tagged target protein, Mediator subunit 19a, and in vitro transcribed ELENA1 lncRNA [5].

2 Materials

Maintain RNase-free environment, and prepare all solutions using ultrapure water, RNase-free disposal materials, and analytical grade reagents.

2.1 DNA Template Preparation Components

1. Genomic or cDNA (100 ng/μL) from *Arabidopsis*.
2. 5 μM stock of primers for PCR: We ordered primers for the amplification of *ELENA1* template (forward, 5′-GAAAT TAATACGACTCACTATAGGG ACAAGTTGACATTTCGAGAG- 3′; reverse, 5′-GCAGTATAAGCCTGTGCTGACTTG-3′) from IDT. But other oligonucleotide-synthesizing companies can also be used (*see* **Note 1**).
3. KOD Hot Start (Novagen) or a suitable alternative DNA polymerase.
4. SYBR Safe DNA gel stain (Invitrogen) or a suitable alternative.
5. TBE buffer: For 1 L of a 10× stock, mix 108 g Tris base, 55 g boric acid, 80 mL 0.5 M EDTA, and 700 mL of dH_2O. Adjust pH to 8.0 and bring to 1 L volume with dH_2O.
6. Agarose gel: For 100 mL 1% gel, 1 g agarose in 100 mL 1× TBE buffer.
7. DNA ladders (1 kb and 100 bp).
8. QIAquick gel extraction kit (Qiagen) or a suitable alternative.
9. Thermocycler.
10. Horizontal gel electrophoresis equipment.
11. UV transilluminator.

2.2 T7 In Vitro Transcription Components

1. T7 RNA polymerase (Roche).
2. 10× Transcription buffer supplied with T7 RNA polymerase (Roche).
3. dNTP mix (dATP, dUTP, dGTP, and dCTP; each 10 mM solution) for unlabeled RNA transcription.
4. RNase inhibitor (RiboLock RNase Inhibitor from Thermo Fisher Scientific).
5. TURBO DNase and 10× TURBO DNase reaction buffer (Ambion).

6. Biotin RNA Labeling Mix, 10× conc. (Roche) for labeled RNA transcription.
7. 5× RNA structure buffer (RSB): 100 mM Tris–HCl (pH 7.0), 500 mM KCl, and 50 mM $MgCl_2$.
8. RNeasy Plant Mini Kit (Qiagen).
9. NanoDrop spectrometer.

2.3 Nuclear Protein Extraction Components

1. Homogenization buffer (HB): 20 mM Tris–HCl (pH 7.4), 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 250 mM sucrose, 1% plant protease inhibitors (Sigma), and add H_2O to 100 mL.
2. Nuclei resuspension buffer (NRB): 20 mM Tris–HCl (pH 7.4), 25% glycerol, 2.5 mM $MgCl_2$, 1% plant protease inhibitors (Sigma), and add H_2O to 100 mL.
3. Nuclei resuspension buffer with 0.2% Triton X-100 (NRBT): 20 mM Tris–HCl (pH 7.4), 25% glycerol, 2.5 mM MgCl2, 0.2% Triton X-100, and add H_2O to 100 mL.
4. Nuclear storage buffer (NSB): 20 mM Tris–HCl (pH 7.4), 25% glycerol, 2.5 mM $MgCl_2$, sucrose 15.1 g, 1% plant protease inhibitors (Sigma), and add H_2O to 100 mL.
5. Quick start Bradford protein assay (bio-rad).
6. Bovine serum albumin (BSA, Sigma-Aldrich).
7. Mortar, pestle, and liquid nitrogen for grinding of plant materials.
8. Bioruptor® Plus sonication device (Diagenode).
9. Benzonase® Nuclease (Sigma).
10. Refrigerated centrifuge (Eppendorf).

2.4 RNA-Protein Pull-Down Components

1. RNA pull-down buffer (RPDB): 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM DTT, 0.2% Nonidet P-40, 8 U/mL Ribolock RNase Inhibitor (Thermo Fisher), and 1% plant protease inhibitors (Sigma) at the day of experiment.
2. Streptavidin Mutein Matrix (Roche).
3. 4× NuPAGE™ LDS Sample Buffer (Thermo Fisher).

2.5 Polyacrylamide Gel Electrophoresis and Western Blot Components

1. Precision Plus Protein™ Dual Color Standards Ladder (Bio-Rad).
2. PVDF membrane (0.45 μm) (Millipore).
3. Filter paper.
4. 10× Tris/glycine/SDS buffer; running buffer (Bio-Rad).
5. 10 × Tris/glycine buffer; transfer buffer (Bio-Rad).
6. TBS: 15 mL 5 M NaCl and 10 mL 1 M Tris (pH 7.5), bring to 500 mL with distilled H_2O.

7. TBST solution: 40 mL 1 M Tris, 60 mL 5 M NaCl, and 1 mL Tween-20, bring to 2 L with distilled H_2O.
8. 7.5% (w/v) blocking solution: Dissolve 7.5 g blotting-grade blocker (Bio-Rad) in 100 mL TBST, and adjust pH 7.6 with NaOH.
9. 5% (w/v) blocking solution: Dissolve 5 g blotting-grade blocker (Bio-Rad) in 100 mL TBST, and adjust pH 7.6 with NaOH.
10. 4% stacking gel: 425 μL 30% polyacrylamide, 625 μL 0.5 M Tris (pH 6.8), 25 μL 10% (w/v) SDS, 1.34 mL H_2O, 25 μL 10% (w/v) APS, and 5 μL TEMED.
11. 10% polyacrylamide gel (1.5 mm) with 4% stacking gel: 3.33 mL 30% polyacrylamide, 3 mL 1.5 M Tris (pH 8.8), 125 μL 10% (w/v) SDS, 4.96 mL H_2O, 125 μL 10% (w/v) APS, and 10 μL TEMED.
12. Primary and secondary antibodies: For example, GFP polyclonal rabbit antibody (Santa Cruz) for primary and ECL Rabbit IgG, HRP-linked whole antibody (Amersham) for secondary antibody.
13. Vertical gel electrophoresis equipment (Bio-Rad).
14. Wet/tank blotting systems (Bio-Rad).
15. Clarity™ ECL Western Blotting Substrate (Bio-Rad) or a suitable alternative.
16. ChemiDoc™ Imaging System (Bio-Rad).

3 Methods

3.1 DNA Template Preparation

1. Order the forward and reverse deoxyribo-oligonucleotide primers for PCR from companies such as IDT. The primer with the same sequence as RNA strand should include T7 RNA polymerase promoter sequence (5′-TAATACGACTCAC TATAGGG- 3′) at its 5′ end enabling its incorporation at the 5′ of the RNA sequence of interest (*see* **Note 1**).
2. Prepare 2× 50 μL PCR reactions for each primer pair as follows: 4 μL each of forward and reverse primers (5 μM stock), 5 μL 10× KOD Hot Start reaction buffer, 1 μL KOD Taq, 100 ng of cDNA or genomic DNA, and RNase-free H_2O (to make a final volume of 50 μL) (*see* **Note 2**).
3. Amplify the DNA through PCR as follows (or in case of using alternative DNA polymerase, according to the vendor's instructions): (a) denaturation, 95 °C, 2 min, and (b) amplification (40 cycles), 95 °C, 20 s; annealing temperature, 20 s; 70 °C, 15 s. (c) Final extension and storage: 70 °C, 5 min; 4 °C ∞.

4. Check the size and purity of the PCR product on a 1% agarose gel containing 0.3 μg/mL SYBR safe DNA gel stain (load 5–10 μL of PCR product). Use a standard UV illuminator to view the gel. If only one band of the desired size is observed, then proceed to **step 6**.
5. If multiple products are observed in **step 4**, you will require purification from a gel (*see* **Note 3**); run the remaining PCR product on an agarose gel, and cut the desired band with a clean scalpel (use suitable UV protection).
6. PCR product cleanup using a Qiagen QIAquick Gel Extraction Kit (*see* **Note 4**).

3.2 T7 RNA Polymerase In Vitro Transcription

1. Set up a 20 μL T7 polymerase reaction by incubating 2 μL of T7 RNA polymerase, 2 μL of 10× reaction buffer, 2 μL of 10× biotin RNA labeling mix, 100–200 ng of purified DNA (PCR product containing T7 promoter sequence), and RNase-free H_2O up to 20 μL (*see* **Note 5**).
2. Incubate the reaction for up to 3 h at 37 °C (*see* **Note 6**).
3. Add 1 μL TURBO DNase to each tube and incubate for 15 min at 37 °C.
4. Purify RNA with RNeasy Plant Mini Kit (Qiagen) (*see* **Note 7**), and elute with 40 μL RNase-free water.
5. Add 10 μL 5× RNA structure buffer to purified RNA.
6. Total 50 μL labeled RNA was heated to 90 °C for 2 min and quenched in the ice for 2 min. Then keep the treated RNA on the bench for 20–30 min to allow proper secondary structure formation.
7. Measure RNA quantity by using NanoDrop spectrometer.

3.3 Nuclear Protein Extraction

1. Grow *Arabidopsis* seedlings (*35S:GFP-MED19a*) on MS media for 2 weeks. Collect approximately 1 g of *Arabidopsis* seedlings, and freeze in liquid nitrogen.
2. Grind the tissue to a fine powder in liquid nitrogen using a cold mortar and pestle. Then collect the powder into a 50 mL conical tube.
3. Add 5 mL cold HB buffer into the powder, and homogenize the mixture by gentle shaking or pipetting (*see* **Note 8**).
4. Filter the homogenate through double layer of Miracloth (*see* **Note 9**).
5. Centrifuge the filtered homogenate at 1500 × *g* at 4 °C for 10 min to pellet the nuclei.
6. Discard the supernatant and add 3 mL NRBT to the pellet. Resuspend the nuclei by pipetting.
7. Centrifuge the sample at 1500 × *g* at 4 °C for 10 min. Repeat **steps 5** and **6** twice more.

8. Discard the supernatant and add 3 mL NRB to the pellet. Resuspend the nuclei by pipetting.
9. Centrifuge at 1500 × *g* at 4 °C for 10 min to pellet the nuclei, and discard the supernatant (*see* **Note 10**).
10. Thaw the pellet on ice. Add 300 μL RPDB to the pellet. Resuspend first with an inoculation loop, and then gently pipet the mix up and down. Avoid foaming at all costs.
11. Sonicate the sample five times, 10 s each, with a sonicator set at low intensity at 4 °C by using Bioruptor® Plus sonication device (*see* **Note 11**).
12. (Optional) Add 2 μL of Benzonase to each sample. Incubate on ice for 1 h (*see* **Note 12**).
13. Centrifuge with 12,000 × *g* for 30 min at 4 °C. Save the supernatant.

3.4 RNA-Protein Pull-Down Assay

1. Mix the streptavidin-agarose beads well before aliquoting 50 μL of beads per reaction.
2. Remove supernatant and wash beads two times with 500 μL H_2O.
3. Remove supernatant, and wash beads three times with 500 μL RNA pull-down buffer.
4. Add nuclear proteins (Subheading 3.3) and RNA pull-down buffer (total reaction volume 200–500 μL).
5. Add 500 ng of labeled RNA and mix gently by pipetting.
6. Incubate at 25 °C for 30 min with rotation or at 4 °C for 2 h (*see* **Note 13**).
7. Add the prepared streptavidin-agarose beads and incubate at 4 °C for 2 h.
8. Remove supernatant and wash twice with 50 μL of 20 mM Tris (pH 7.5).
9. Wash five times with 500 μL of RNA pull-down buffer (dilute 10× in RNase-free H_2O).
10. Remove supernatant completely, and add 25 μL of RNA pull-down buffer, and vortex well (*see* **Note 14**).
11. Add 6.25 μL of 4× NuPAGE™ LDS Sample Buffer, mix well, and boil for 10 min (*see* **Note 15**).
12. Keep cold on ice if proceeding to the next step immediately. For later use, store at −20 °C.

3.5 Western Blot

1. Prepare a 1.5 mm thick 10% polyacrylamide gel with a 4% stacking gel (*see* **Note 16**).
2. Load protein ladder and 30 μL of each protein sample from RNA pull-down assay (positive and negative controls) onto 10% gel.

3. Run the gel at 100 V for approximately 1 h or until the blue front reaches the end of the gel. Remove the gel from the plates and put in a clean plastic container.
4. Soak gel in 1× transfer buffer for 10 min before performing the wet transfer with a PVDF membrane (*see* **Note 17**) by running at 100 V for 1 h (*see* **Note 18**).
5. Block the membrane at room temperature for 1 h with 7.5% blocking solution on an orbital shaker (*see* **Note 19**).
6. Remove 7.5% (w/v) blocking solution, and incubate with the primary antibody, polyclonal anti-GFP, diluted (1:1000–1:5000) in 5% (w/v) blocking solution for 1 h.
7. Wash the membrane three times (each for 10 min) with TBST.
8. Incubate for 1 h at room temperature with secondary antibody diluted (1:5000–1:10,000) in 5% (w/v) blocking solution.
9. Wash the membrane three times (each for 10 min) with TBST.
10. Wash the membrane once with PBS for 5 min.
11. Incubate the membrane with Clarity™ ECL Western Blotting Substrate for 2 min.
12. Scan chemiluminescence of the blot by using ChemiDoc™ Imaging System.

4 Notes

1. When designing primers for the PCR, first design the oligonucleotide sequences (excluding the T7 promoter sequence) to have similar melting temperatures before adding T7 promoter sequence at one end. T7 RNA polymerase has increased efficiency when the T7 promoter sequence is preceded by several nucleotides and immediately followed by GG.
2. cDNA should be used if primers are designed across different exons; however, if the primers are designed to amplify a region of RNA which is not spliced, we have found that genomic DNA works sufficiently well. 2× PCR reactions containing 100 ng genomic DNA should be sufficient to produce 1 μg template DNA.
3. In some cases it might not be possible to produce a single, discrete band after PCR. In such a scenario, the correct size band must be extracted from the gel. For those that do produce a unique band, direct purification of PCR product can be used.
4. Follow the manual provided by Qiagen.

5. To prepare unlabeled lncRNA for competition assay, we used dNTP mix for in vitro transcription of unlabeled lncRNA instead of biotin-labeling kit.
6. For RNA with a strong secondary structure, lower the reaction temperature to 16 °C or 4 °C, and incubate for a longer period (24–72 h) to improve the reaction yield.
7. Follow the manual provided by Qiagen.
8. If the sample is frozen from excess liquid nitrogen, wait until it is thawed enough that it can be homogenized.
9. Once all the homogenate has been filtered, squeeze out the remaining homogenate into the membranes. Always keep the sample on ice.
10. The purpose of this step is to remove the Triton X-100. The nuclei can now be used for any purpose. For example, they can be used to detect nuclear protein using a Western blot. If the nuclei cannot be used immediately, they should be resuspended in 400 μL NSB buffer, quickly frozen in liquid nitrogen, and stored at −80 °C. The nuclear preparation can last for at least half a year.
11. Sonication disrupts the nuclei. The intensity and duration of sonication need to be optimized.
12. Benzonase removes DNA and RNA, thereby releasing DNA-binding proteins. One can infer the relative distribution of proteins between chromatin-bound and chromatin-unbound states based on the Benzonase treatment. Also, the resulting nuclear extracts will not be complicated by the presence of genomic DNA and mRNA. Normally skip this step.
13. To maximize the amount of RNA that binds to the beads, we recommend the use of rotation rather than agitation. And use freshly transcribed RNA for the best result.
14. We found it more efficient to elute in a smaller volume to concentrate the protein samples.
15. To maximize the elution, it is preferable to heat the streptavidin beads with loading buffer.
16. To increase the resolution of the Western band, adjust the polyacrylamide concentration depending on the protein size.
17. Place membranes in methanol for 20 s, and wash with water for 2 min, and then put it in 1× transfer buffer before transferring.
18. We have optimized transfer for the Bio-Rad wet transfer system.
19. Otherwise, you can block the membrane with 5% (w/v) blocking solution overnight.

Acknowledgment

This work was supported by Singapore NRF RSSS Grant (NRF-RSSS-002).

References

1. Liu J, Jung C, Xu J, Wang H, Deng S, Bernad L, Arenas-Huertero C, Chua NH (2012) Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. Plant Cell 24:4333–43345
2. Wang HV, Chekanova JA (2017) Long noncoding RNAs in plants. Adv Exp Med Biol 1008:133–154
3. Chekanova JA (2015) Long non-coding RNAs and their functions in plants. Curr Opin Plant Biol 27:207–216
4. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res 15:8783–8798
5. Seo JS, Sun HX, Park BS, Huang CH, Yeh SD, Jung C, Chua NH (2017) ELF18-INDUCED LONG-NONCODING RNA associates with mediator to enhance expression of innate immune response genes in Arabidopsis. Plant Cell 29:1024–1038