

***In vitro* RNA-dependent RNA Polymerase Assay Using *Arabidopsis* RDR6**

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[Abstract] RNA-dependent RNA polymerases (RdRPs) in eukaryotes convert single-stranded RNAs into double-stranded RNAs, thereby amplifying small interfering RNAs that play crucial roles in the regulation of development, maintenance of genome integrity and antiviral immunity. Here, we describe a method of *in vitro* RdRP assay using recombinant *Arabidopsis* RDR6 prepared by an insect expression system. By using this classical biochemical assay, we revealed that RDR6 has a strong template preference for RNAs lacking a poly(A) tail. This simple method will be applicable to other RdRPs in *Arabidopsis* and different organisms.

Keywords: RNA-dependent RNA polymerase, Double-stranded RNA, RNA silencing, Small RNA, siRNA, RNA-DEPENDENT RNA POLYMERASE6, *Arabidopsis thaliana*

[Background] RNA-dependent RNA polymerase (RdRP) genes have been found in all eukaryotic kingdoms—plants, fungi, protista and animals (Zong *et al.*, 2009). They convert single-stranded RNAs (ssRNAs) into double-stranded RNAs (dsRNAs), thereby amplifying small interfering RNAs (siRNAs) that play crucial roles in various biological processes including regulation of development (Peragine *et al.*, 2004; Li *et al.*, 2005), maintenance of genome integrity (Volpe *et al.*, 2002; Xie *et al.*, 2004) and antiviral immunity (Mourrain *et al.*, 2000; Yu *et al.*, 2003; Garcia-Ruiz *et al.*, 2010; Wang *et al.*, 2010). In addition to this RdRP activity, RdRPs possess another enzymatic activity called terminal nucleotide transferase (TNTase) activity (Curaba and Chen, 2008; Aalto *et al.*, 2010), which adds one or more nucleotides to the 3' end of ssRNAs or dsRNAs in a template-independent manner.

Here, we describe a method for *in vitro* RdRP assay using recombinant *Arabidopsis thaliana* RDR6 prepared by an insect expression system. To accurately discriminate RdRP products from TNTase products, we designed two strategies: 1) elimination of single-stranded TNTase products by treating the reaction mixture with ssRNA-specific RNase I, and 2) electrophoresis of the reaction mixture on a native acrylamide gel, in which double-stranded RdRP products can be distinguished from single-stranded TNTase products based on their different mobility. By using this classical biochemical assay, we revealed that RDR6 has a strong template preference for the RNAs lacking a poly(A) tail (Baeg *et al.*, 2017). This simple method should also be applied to other RdRPs in *Arabidopsis* and different organisms.

Materials and Reagents

A. Preparation of recombinant RDR6

1. 100 mm dish (Corning, catalog number: 430167)
2. 50 ml conical centrifuge tube (Nippon Genetics, catalog number: FG200)
3. Cell counter plate (Neubauer Improved) (FUKAEKASEI and WATSON, WATSON BIOLAB, catalog number: 177-112C)
4. 1.5 ml tube (BM Equipment, BMBio, catalog number: NT-175)
5. *Drosophila melanogaster* Schneider 2 (S2) cells (Schneider, 1972)
6. RDR6 expression plasmid for S2 cells (*pAFW-SUMO-AtRDR6* [Baeg et al., 2017])
7. Anti-FLAG antibody, M2 (Sigma-Aldrich, catalog number: F1804)
8. SUMOstar protease (LifeSensors, catalog number: 4110)
9. Schneider's *Drosophila* medium (Thermo Fisher Scientific, Gibco™, catalog number: 21720001)
10. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 12483020)
11. Antibiotic-Antimycotic (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 15240062)
12. X-tremeGENE HP transfection reagent (Sigma-Aldrich, Roche Diagnostics, catalog number: 06366546001)
13. Dynabeads proteins G (Thermo Fisher Scientific, Invitrogen™, catalog number: 10009D)
14. Triton X-100 (Wako Chemical Pure Industries, catalog number: 169-21105)
15. Dithiothreitol (DTT) (NACALAI TESQUE, catalog number: 14128-04)
16. Glycerol (NACALAI TESQUE, catalog number: 17018-83)
17. Liquid nitrogen
18. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 23227)
19. Sodium chloride (NaCl) (Wako Chemical Pure Industries, catalog number: 190-13921)
20. Potassium chloride (KCl) (NACALAI TESQUE, catalog number: 28514-75)
21. Sodium phosphate dibasic dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: 28-3720)
22. Potassium dihydrogenphosphate (KH_2PO_4) (NACALAI TESQUE, catalog number: 28721-55)
23. HEPES (DOJINDO, catalog number: 340-01376)
24. Magnesium acetate tetrahydrate, $\text{Mg}(\text{OAc})_2$ (Wako Chemical Pure Industries, catalog number: 130-00095)
25. EDTA-free protease inhibitor cocktail (Sigma-Aldrich, catalog number: 11836170001)
26. Potassium acetate (KOAc) (NACALAI TESQUE, catalog number: 28405-05)
27. Phosphate-buffered saline (PBS) (see Recipes)
28. Hypotonic lysis buffer (see Recipes)
29. 1x lysis buffer (see Recipes)

B. Preparation of template RNAs

1. Fluorescent TLC plate
2. 2 ml tube (BM Equipment, BMBio, catalog number: BM-20)
3. Sterilized pipette tips
4. Plastic wrap (AsahiKASEI)
5. T7-Scribe™ Standard RNA IVT Kit (CELLSCRIPT, catalog number: C-AS3107)
6. Distilled water
7. Phenol:chloroform:isoamyl alcohol 25:24:1 mixed, pH 5.2 (NACALAI TESQUE, catalog number: 26058-96)
8. Chloroform (Wako Chemical Pure Industries, catalog number: 035-02616)
9. Isoamyl alcohol (Wako Chemical Pure Industries, catalog number: 135-12015)
10. Ammonium acetate (NACALAI TESQUE, catalog number: 02433-35)
11. 70% ethanol
12. 2-Propanol (NACALAI TESQUE, catalog number: 29113-53)
13. Ethylenediaminetetraacetic acid (EDTA) (NACALAI TESQUE, catalog number: 15105-35)
14. Deionized formamide (NACALAI TESQUE, catalog number: 16229-95)
15. Xylene cyanol (Wako Chemical Pure Industries, catalog number: 240-00463)
16. Tris (Wako Chemical Pure Industries, catalog number: 207-06275)
17. Sodium chloride (NaCl) (Wako Chemical Pure Industries, catalog number: 190-13921)
18. Sodium dodecyl sulfate (SDS) (NACALAI TESQUE, catalog number: 02873-75)
19. Bromophenol blue (Wako Chemical Pure Industries, catalog number: 029-02912)
20. Urea (Wako Chemical Pure Industries, catalog number: 217-00171)
21. SequaGel-UreaGel Concentrate (National Diagnostics, catalog number: EC-830)
22. 2x formamide dye (see Recipes)
23. PK buffer (see Recipes)

C. RNA-dependent RNA polymerase activity assay

1. RNasin® Plus RNase Inhibitor (Promega, catalog number: N2615)
2. α -³²P-UTP (PerkinElmer, catalog number: NEG507H)
3. 100% ethanol (NACALAI TESQUE, catalog number: 14713-53)
4. Ribonucleotide Solution set (New England Biolabs, catalog number: N0450L)
5. Glycogen (NACALAI TESQUE, catalog number: 17110-11)
6. Proteinase K (20 mg/ml) (NACALAI TESQUE, catalog number: 29442-85)
7. Boric acid (H₃BO₃) (NACALAI TESQUE, catalog number: 05215-05)
8. Ethylenediaminetetraacetic acid (EDTA) (NACALAI TESQUE, catalog number: 15105-35)
9. HEPES (DOJINDO, catalog number: 340-01376)
10. Ammonium acetate (NH₄OAc) (NACALAI TESQUE, catalog number: 02433-35)
11. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (Wako Chemical Pure Industries, catalog number: 135-00165)

12. RNase I (Thermo Fisher Scientific, Invitrogen™, catalog number: AM2294)
13. PEG 4000 (Sigma-Aldrich, catalog number: 95904-250G-F)
14. Glycerol (NACALAI TESQUE, catalog number: 17018-83)
15. Tartrazine (NACALAI TESQUE, catalog number: 32706-22)
16. 40(w/v)%-Acrylamide/Bis Mixed Solution(19:1) (NACALAI TESQUE, catalog number: 06140-45)
17. 1 mM NTP mixture (see Recipes)
18. PK buffer (EDTA-) (see Recipes)
19. PK mixture (see Recipes)
20. PK mixture (EDTA-) (see Recipes)
21. 5x TBE (see Recipes)
22. 10x RdRP buffer (see Recipes)
23. 2x RNase I buffer (see Recipes)
24. 2x loading buffer (see Recipes)

Equipment

1. Pipettes (Gilson)
2. Cell incubator (SANYO, catalog number: MIR-153)
3. Centrifuge (TOMY SEKIO, catalog number: MX-301)
4. Vortex mixer (Scientific Industries, model: Vortex-Genie 2, catalog number: SI-0286)
5. Rotator (TAITEC, model: RT-30mini, catalog number: 0057154-000)
6. Magnetic stand (Thermo Fisher Scientific, catalog number: 120.20)
7. Block incubator (TAITEC)
8. UV lamp (UVP, catalog number: 95-0004-11)
9. Gel dryer (Bio-Rad Laboratories, catalog number: 165-1745)
10. Imaging plate cassette (GE Healthcare, catalog number: 63-0035-45)
11. Imaging plate (GE Healthcare, catalog number: 28-9564-74)
12. Typhoon FLA 7000 IP (GE Healthcare, model: Typhoon FLA 7000 IP)

Procedure

A. Preparation of recombinant RDR6

The recombinant RDR6 protein with N-terminal 3xFLAG-tags followed by a SUMOstar protease cleavage site is overexpressed by using *Drosophila* S2 cell expression system. This system produces higher levels of soluble recombinant RDR6 compared to plant cell-free translation systems or *Escherichia coli* expression systems. After lysis of the cells, the recombinant protein is purified by anti-FLAG antibody conjugated magnetic beads, and eluted with SUMOstar protease. Although we use the tag-less recombinant RDR6 protein, the N- or C-terminally tagged RDR6 can

also be used for RdRP activity assay (Devert *et al.*, 2015; Baeg *et al.*, 2017).

1. When S2 cells reach between 1.0×10^7 and 1.5×10^7 cells/ml in a 100-mm dish, collect them into a 50-ml tube(s). Centrifuge at $1,500 \times g$ for 3 min, remove supernatant, and resuspend the cell pellet gently with Schneider's *Drosophila* medium containing 10% FBS. Count the cell numbers using a cell counter plate. Seed 1.5×10^7 cells (1.5×10^6 cells/ml) in a 100-mm dish in 10 ml of Schneider's *Drosophila* medium containing 10% FBS (see Notes 1 and 2).
2. Dilute 10 μ l of 1 μ g/ μ l *pAFW-SUMO-AtRDR6* plasmid with 500 μ l of Schneider's *Drosophila* medium in a 1.5 ml tube. Add 20 μ l of X-tremeGENE HP transfection reagent to the diluent, mix by tapping, and incubate it at room temperature ($\sim 25^\circ\text{C}$) for 10 min.
3. Add the transfection mixture into S2 cells in a dropwise manner, gently agitate the dish in a circular motion to evenly disperse the transfection mixture, and statically incubate at 27°C for 72 h.
4. Collect the cells into a 50-ml conical centrifuge tube(s). Centrifuge in a swinging bucket rotor at $1,500 \times g$ for 3 min at 25°C . Remove the supernatant with a pipette.
5. Resuspend the cell pellet gently in 300 μ l of PBS (see Recipes). Transfer the cell suspension into a 1.5 ml tube. Centrifuge at $1,500 \times g$ for 3 min at 4°C . Remove the supernatant with a pipette completely.
6. Weigh the cells and add an equal amount of pre-chilled hypotonic lysis buffer (see Recipes). Resuspend the cell pellet gently by tapping the bottom of the tube.
7. Incubate the cell suspension on ice for 15 min, then vortex the cell suspension for 30 sec to disrupt the cell membrane.
8. Centrifuge at $17,000 \times g$ for 20 min at 4°C .
9. Transfer the supernatant into the 1.5 ml tube containing Dynabeads protein G coated with anti-FLAG antibody (see Note 3). Mix by tapping, and incubate on a rotator at 4°C for 1 h.
10. Transfer the tube to a magnetic stand. Wash the beads twice with an equal volume of 1x lysis buffer (see Recipes) containing 800 mM NaCl and 1% Triton X-100, and once with an equal volume of 1x lysis buffer.
11. Resuspend the beads with 100 μ l of 1x lysis buffer containing 1 mM DTT, 20% glycerol and 0.05 U per μ l SUMOstar protease. Incubate the slurry on a rotator at 4°C for 20 min.
12. Recover the supernatant in a pre-chilled tube, aliquot it into several tubes on ice, freeze with liquid nitrogen, and store at -80°C . Measure the concentration of purified RDR6 by SDS-PAGE along with the defined dilutions of bovine serum albumin standard (Pierce™ BCA Protein Assay Kit) after Coomassie Brilliant Blue (CBB) staining (Figure 1).

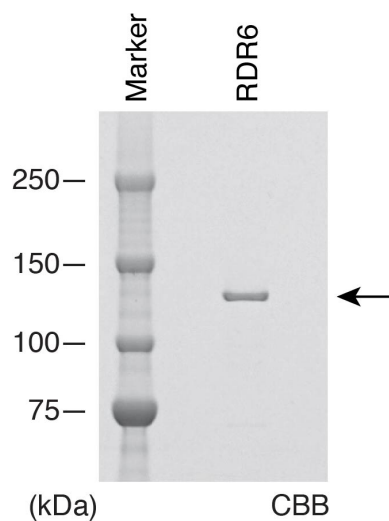


Figure 1. Recombinant RDR6. CBB staining of purified recombinant protein. The arrow indicates purified recombinant RDR6.

B. Preparation of template RNAs

Because *in vitro* transcribed RNAs often contain unwanted shorter or longer byproducts, polyacrylamide gel electrophoresis (PAGE) purification of RNAs is essential for accurate determination of the template specificity of RdRPs. When synthetic oligo RNAs are used as the template for RdRPs, the use of HPLC purification is recommended.

1. Transcribe reporter RNAs from DNA templates at 37 °C for 2 h using T7-Scribe Standard RNA IVT Kit following manufacturer's instructions.
2. Add 1 µl of DNase I, mix thoroughly and incubate at 37 °C for 20 min.
3. Adjust the volume of the reaction mixture to 100 µl with distilled water on ice.
4. Add an equal volume of phenol:chloroform:isoamyl alcohol into the reaction mixture, and mix by vortexing at room temperature (~25 °C). Centrifuge at 17,000 x g for 2 min at 4 °C.
5. Recover the aqueous phase into a new tube with an equal volume of chloroform:isoamyl alcohol, and mix by vortexing at room temperature (~25 °C). Centrifuge at 17,000 x g for 1 min at 4 °C.
6. Recover the aqueous phase into a new chilled tube, add an equal volume of pre-chilled 5 M ammonium acetate, mix the solution by tapping the tube, and incubate on ice for 15 min.
7. Centrifuge at 21,500 x g for 20 min at 4 °C.
8. Remove the supernatant and add 700 µl of 70% ethanol.
9. Centrifuge at 17,000 x g at 4 °C for 3 min and remove the supernatant completely.
10. Place the open tube on ice to air dry.
11. Resuspend the pellet in 40 µl of 1x formamide dye (see Recipes). Incubate the sample at 95 °C for 5 min, mix by vortexing and load onto a urea gel containing 0.5x TBE and appropriate concentration of polyacrylamide (see Notes 4 and 5).
12. Perform electrophoresis (see Note 6).

13. Transfer the gel onto a plastic wrap, cover the gel with another wrap, and place it on a fluorescent TLC plate. Briefly expose the gel to UV light and excise a gel slice containing the RNA of interest (see Note 7). Transfer the gel slice into a 2 ml tube (Figures 2A-2C).

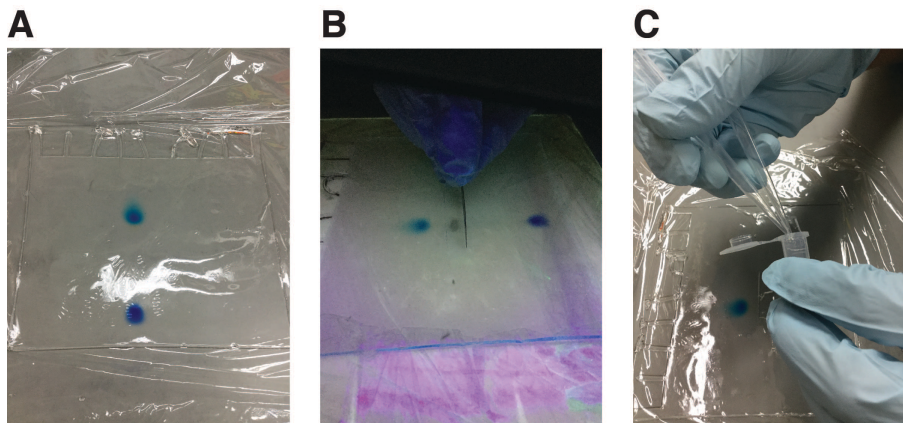


Figure 2. Workflow for RNA purification. A. The gel is transferred onto a plastic wrap after denaturing PAGE. B. Gel excision under UV light; C. A gel slice containing the RNA of interest is transferred into a tube with sterilized pipette tips.

14. Add 700 μ l of PK buffer (see Recipes) and incubate the tube at room temperature ($\sim 25^{\circ}\text{C}$) for 24 h on a rotator.
 15. Transfer the supernatant into a new 1.5 ml tube and add 700 μ l of 2-propanol and mix by vortexing.
 16. Centrifuge at 17,000 $\times g$ at 4°C for 20 min and remove the supernatant carefully.
 17. Add 700 μ l of 70% ethanol.
 18. Centrifuge at 17,000 $\times g$ at 4°C for 3 min and remove the supernatant.
 19. Repeat Steps B17 and B18.
 20. Air dry the pellet for 10 min and dissolve it in distilled water.
 21. Quantify the concentration of the RNA with a spectrophotometer.
- C. RNA-dependent RNA polymerase (RdRP) activity assay
- The flow chart of this assay is shown in Figure 3. After the reaction, the samples are electrophoresed on a urea gel or native gel. In urea PAGE, dsRNAs are denatured into two ssRNAs and migrate based on their molecular weights. Although RNase I treatment is essential for discrimination between RdRP products and TNTase products, this method allows the accurate estimation of the size of newly synthesized complementary strand. In contrast, because dsRNAs retain their double-helical structure in native PAGE, double-stranded RdRP products can be distinguished from single-stranded TNTase products based on their different mobilities without RNase I treatment. A drawback of native PAGE is that the mobility does not reflect the actual size of RNAs. The simultaneous use of both PAGE methods provides more accurate information for the enzymatic properties of RdRPs.

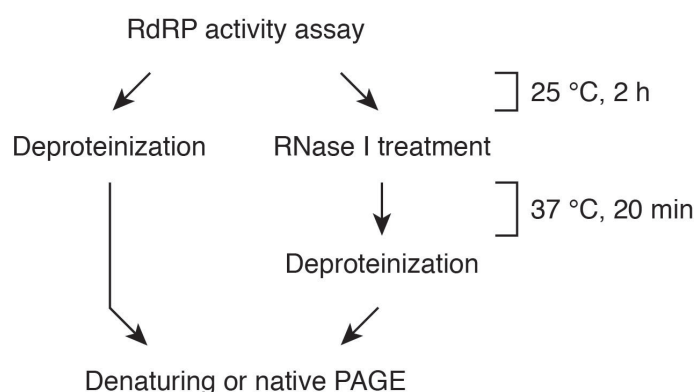


Figure 3. Flow chart of the procedure for *in vitro* RdRP assay

1. Combine the following reagents on ice in the order given: 20 μ l of distilled water, 4 μ l of 10x RdRP buffer (see Recipes), 4 μ l of 5x diluted RNasin, 4 μ l of 1 mM NTP mixture, 2 μ l of 1 μ M template RNA, 2 μ l of α - 32 P-UTP (~3,000 Ci/mmol) and 4 μ l of 100-200 nM RDR6.
2. Incubate the tube at 25 $^{\circ}$ C for 2 h.
3. Split the mixture into 4 tubes (tube A, B, A' and B') containing 10 μ l each. Place them on ice.
4. Add 111 μ l of PK mixture (see Recipes) and 111 μ l of PK mixture (Mg^{2+}) (see Recipes) into tube A and tube A', respectively (see Note 8). Vortex, and incubate at 25 $^{\circ}$ C until Step C6 is completed.
5. Add 10 μ l of RNase I mixture (see Recipes) into tube B and tube B' (see Note 9) and incubate at 37 $^{\circ}$ C for 20 min.
6. Add 111 μ l of PK mixture and 111 μ l of PK mixture (Mg^{2+}) into tube B and tube B', respectively, and vortex.
7. Incubate all tubes at 65 $^{\circ}$ C for 20 min (see Note 10).
8. Add 350 μ l of 100% ethanol to each tube, vortex and centrifuge at 17,000 x g for 10 min at 4 $^{\circ}$ C. Remove the supernatant.
9. Add 700 μ l of 70% ethanol to each tube and centrifuge at 17,000 x g for 3 min at 4 $^{\circ}$ C. Remove the supernatant completely.
10. Air dry the pellet for 10 min.
11. For urea PAGE, dissolve the pellet (tube A and B) in 10 μ l of 2x formamide dye and incubate at 95 $^{\circ}$ C for 5 min. For native PAGE, dissolve the pellet (tube A' and B') in 10 μ l of 1x loading buffer (see Recipes) and keep the samples on ice.
12. Prepare a urea gel containing 0.5x TBE and appropriate concentration of polyacrylamide or a native gel containing 0.5x TBE, 2 mM $MgCl_2$ and appropriate concentration of polyacrylamide (see Note 11).
13. Perform electrophoresis (see Notes 12, 13 and 14).
14. Dry the gel using gel dryer under vacuum at 80 $^{\circ}$ C for 2 h.
15. Expose the dried gel onto an imaging plate for ~12 h. Scan the plate using a laser scanner.

Data analysis

Here, we explain typical results of *in vitro* RdRP assay with the 100-nt RNA without the poly(A) tail. When total RNAs after the RdRP reaction are electrophoresed in a urea gel, multiple smeared bands are generally observed (Figure 4A RNase I-). At this time, these bands cannot be assigned to either TNTase or RdRP products. After ssRNA-specific RNase I treatment, some bands disappear and only a sharp band remains (Figure 4A RNase I+). This RNase I-resistant band is the RdRP product. In contrast, all the other bands erased by the RNase I treatment are single-stranded TNTase products (Figure 4A). When total RNAs after the RdRP reaction are electrophoresed in a native gel, double-stranded RdRP products migrate more slowly than single-stranded TNTase products (Figure 4B RNase I-), which can be validated by digestion of ssRNAs by RNase I treatment (Figure 4B RNase I+). Note that although the optimal concentration of RNase I is determined, RNase I treatment decreases dsRNA signals to some extent (Figure 4B). The signal of dsRNA products in the absence of RNase I treatment in native PAGE reflects the actual RdRP activity of RDR6.

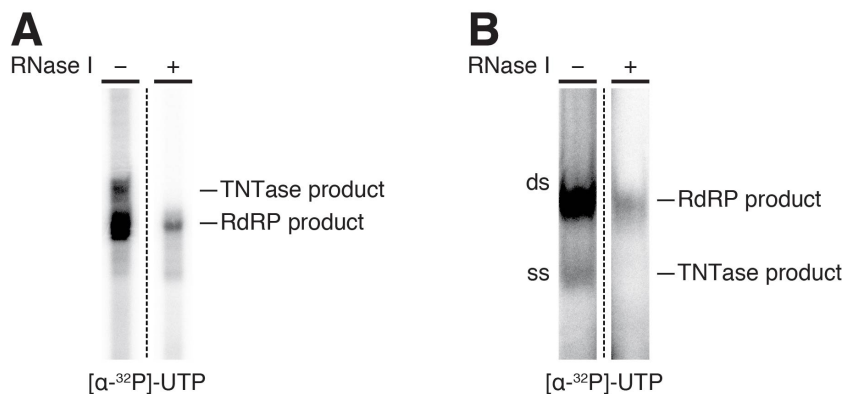


Figure 4. Representative results of *in vitro* RdRP assay. A. A typical banding pattern of RdRP- and TNTase-products from the 100-nt template RNA without the poly(A) tail on an 8% acrylamide-urea gel. B. A typical banding pattern of RdRP- and TNTase-products from the 100-nt template RNA without the poly(A) tail on a 4% acrylamide-native gel.

Notes

1. S2 cells are statically cultured in a 100-mm dish with 10 ml of Schneider's *Drosophila* medium containing 10% FBS and 1x antibiotic-antimycotic in an incubator at 27 °C. For maintenance of cells, passage S2 cells when the cell density reaches between 1.0×10^7 and 1.5×10^7 per ml, and split at a 1:10 dilution into a new 100-mm dish.
2. To obtain high a concentration of recombinant RDR6, we usually prepare 500 ml of S2 cells (1.5×10^6 cells per ml). We aliquot it into 50 dishes (10 ml/dish) for transfection of *pAFW-SUMO-AtRDR6*. After expression of RDR6, S2 cells are collected into twelve 50-ml

- p>
tubes (~40 ml each). After centrifugation, the cell pellet in each tube is resuspended with 300
- μ
- l of PBS, and transferred into a 1.5
- μ
- l tube. At Step A10, the washed magnetic beads in the 12 tubes are collected in a new tube. Then, 100
- μ
- l of 1x lysis buffer containing 1 mM DTT, 20% glycerol and 0.05 U per
- μ
- l SUMOstar protease are added for elution of RDR6.
- To prepare the anti-FLAG antibody conjugated Dynabeads for immunopurification from e.g., 100 μ l of S2 cell extract, 2 μ l of anti-FLAG antibody and 100 μ l slurry of Dynabeads are mixed in a tube by tapping, and incubated on a rotator at 4 $^{\circ}$ C for 1 h. Then, the tube is placed on a magnetic stand, and the supernatant is removed. The beads are washed once with 200 μ l of 1x lysis buffer, and resuspended in 200 μ l of 1x lysis buffer. The supernatant is removed just before incubation with S2 cell extract.
 - We use SequaGel-UreaGel Concentrate to prepare urea gel.
 - We usually use 5%, 8% and 15% acrylamide-urea gels for migration of 400-700 nt, 80-200 nt and < 50 nt RNAs, respectively.
 - We resolve the RNAs at a constant voltage of 500 V in 0.5x TBE as running buffer.
 - If the template RNAs are undetectable by UV shadowing, stain the gel with a nucleic acid staining dye for gel excision.
 - For native PAGE samples (tube A' and B'), we use 'PK buffer (Mg^{2+})' which contains magnesium ions but not EDTA to stabilize double-stranded RNAs (see Recipes).
 - Although RNase I treatment allows discrimination of double-stranded RdRP products from single-stranded TNTase products, the use of a too high concentration of RNase I leads to degradation of dsRNAs. Therefore, the optimal concentration of RNase I should be carefully determined prior to the RdRP activity assay by using ^{32}P body-labeled ss and dsRNA markers as substrates for RNase I.
 - To avoid unwinding of dsRNAs, if the lengths of template RNAs are < 25 nt, it is better to incubate the RNA samples for native PAGE at 25 $^{\circ}$ C for 60 min or at 37 $^{\circ}$ C for 30 min in deproteinization step.
 - We use 40(w/v)%-Acrylamide/Bis Mixed Solution(19:1) to prepare native gel.
 - The running time should be optimized. The RdRP- and TNTase-products from the 100-nt template RNAs are well separated on an 8% acrylamide-urea gel (100 mm (H) x 150 mm (W) x 1 mm (D)) at a constant voltage of 500 V for 40 min or 4% acrylamide-native gel (100 mm (H) x 150 mm (W) x 1 mm (D)) at a constant voltage of 500 V for 40 min.
 - To avoid spontaneous unwinding of double-stranded RNAs, keep the running buffer cold using plastic bars filled with coolant and perform electrophoresis in a cold room (Figure 5).
 - To determine the relative size of the products, 5' radiolabeled template RNAs can be used as the size markers.

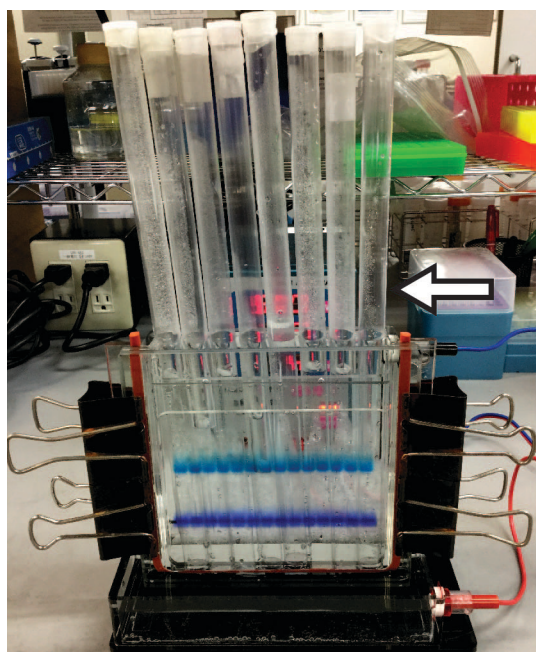


Figure 5. Representative image of native PAGE. The white arrow indicates a plastic bar filled with coolant.

Recipes

1. Phosphate-buffered saline (PBS), pH 7.4
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
2. Hypotonic lysis buffer
10 mM HEPES-KOH, pH 7.4
10 mM KCl
1.5 mM $\text{Mg}(\text{OAc})_2$
5 mM DTT
1x EDTA-free protease inhibitor cocktail
3. 1x lysis buffer
30 mM HEPES-KOH, pH 7.4
100 mM KOAc
2 mM $\text{Mg}(\text{OAc})_2$
4. 2x formamide dye
10 mM EDTA, pH 8.0
98% (w/v) deionized formamide
0.025% (w/v) xylene cyanol
0.025% (w/v) bromophenol blue

5. PK buffer
 - 100 mM Tris-HCl, pH 7.5
 - 200 mM NaCl
 - 2 mM EDTA, pH 8.0
 - 1% (w/v) SDS
6. 1 mM NTP mixture
 - 1 µl of 100 mM ATP in Ribonucleotide Solution set
 - 1 µl of 100 mM UTP in Ribonucleotide Solution set
 - 1 µl of 100 mM GTP in Ribonucleotide Solution set
 - 1 µl of 100 mM CTP in Ribonucleotide Solution set
 - 96 µl of distilled water
7. PK buffer (Mg²⁺)
 - 100 mM Tris-HCl, pH 7.5
 - 200 mM NaCl
 - 1 mM MgCl₂
 - 1% (w/v) SDS
8. PK mixture
 - 2 µl of glycogen
 - 20 µl of Proteinase K
 - 200 µl of PK buffer
9. PK mixture (Mg²⁺)
 - 2 µl of glycogen
 - 20 µl of Proteinase K
 - 200 µl of PK buffer (Mg²⁺)
10. 5x TBE
 - 0.446 M Tris
 - 0.445 M H₃BO₃
 - 0.01 M EDTA, pH 8.0
11. 10x RdRP buffer
 - 500 mM HEPES-KOH, pH 7.6
 - 200 mM NH₄OAc
 - 80 mM MgCl₂
 - 1 mM EDTA, pH 8.0
 - 20% (w/v) PEG 4000
12. 2x RNase I buffer
 - 100 mM Tris-HCl pH 8.0
 - 600 mM NaCl
 - 30 mM MgCl₂

13. RNase mixture
 - 10 µl of 2x RNase I buffer
 - 1 µl of RNase I
 - 9 µl of distilled water
14. 2x loading buffer
 - 4 mM MgCl₂
 - 0.5x TBE
 - 50% (w/v) glycerol
 - 0.04% (w/v) tartrazine
 - 0.02% (w/v) bromophenol blue
 - 0.02% (w/v) xylene cyanol

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