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Purification of Rice Stripe Virus

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[Abstract] Although many spherical and rod-shaped plant virus purification protocols are now available, only a few protocols on filamentous plant virus purification have been published. Here, we report a protocol for large-scale purification of Rice stripe virus (RSV) from RSV-infected rice tissues. RSV virions with high infectivity were first precipitated with polyethylene glycol (PEG) followed by pelleting through primary ultracentrifugation, ultracentrifugation in a glycerol cushion and ultracentrifugation in density gradient. The purified RSV virions can not only be viewed as filamentous particles under an electron microscope, but can also be acquired by insect vector through direct injection into insect body or through membrane feeding prior to transmission to rice plants.

Keywords: Rice stripe virus, Filamentous virion, Density gradient centrifugation, Virion purification, Ultracentrifugation

[Background] Many purification protocols for spherical and rod-shaped viruses have been published (André *et al.*, 2002; Balke *et al.*, 2018). These protocols all rely on chemical precipitations or density gradient centrifugations. However, purification protocols for filamentous viruses are currently limited.

Rice stripe virus (RSV) is a negative-stranded RNA virus and belongs to the genus *Tenuivirus*, the order *Bunyavirales*. RSV often causes severe damages to rice productions in many East Asian countries (Whitfield *et al.*, 2015; Liu *et al.*, 2018). Unlike other members in the order *Bunyavirales* that produce spherical and enveloped virions, RSV virions are filamentous. However, RSV genome encodes a glycoprotein that is not found in purified RSV virions(Toriyama, 1986; Lu *et al.*, 2019). RSV virions are known to carry several copies of RNA-dependent RNA polymerase (RdRp), necessary for virus replication initiation in host plants and insect vectors. Consequently, establishment of a purification method that allows to maintain active RSV RdRp is crucial for future biological assays using purified virions.

Based on a recent report from our laboratory (Lu *et al.*, 2019), we have now developed a purification protocol suitable for producing highly infectious RSV virions. The purified virions can be used for Transmission Electron Microscopy (TEM) and virus transmission assays through insect vectors.

bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

Materials and Reagents

1. Pipette tips
2. Razor blades
3. Pasteur pipette
4. Sterile syringe filters (Millipore, catalog number: SLGV033RB, or equivalent)
5. Sterile 50 ml conical tubes (Corning, catalog number: 430921, or equivalent)
6. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884, or equivalent)
7. Polyethylene glycol (PEG) 6000 (Sigma-Aldrich, catalog number: 81255, or equivalent)
8. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number:S5886, or equivalent)
9. Glycerol (Sigma-Aldrich, catalog number: G5516, or equivalent)
10. $Na_2HPO_4 \cdot 12H_2O$
11. $NaH_2PO4 \cdot 2H_2O$
12. NaOH
13. Coomassie Brilliant Blue G-250 (Sigma-Aldrich, catalog number: 1.15444, or equivalent)
14. Mouse anti-RSV nucleocapsid monoclonal antibody (Provided by Prof.Jianxiang Wu in Zhejiang University)
15. Goat anti-mouse IgG HRP conjugate (Invitrogen, catalog number: 31430, or equivalent)
16. 0.1 M phosphate buffer, pH 7.5 (see Recipes)

Equipment

1. Blender (Philips, catalog number: HR2116, or equivalent)
2. 1 L glass beaker
3. Refrigerated centrifuge (Eppendorf, catalog number: 5810R)
4. 4 °C refrigerator (Haier, catalog number: HYC-391, or equivalent)
5. Pipettes (Eppendorf, catalog number: R42223G, or equivalent)
6. Ice machines (Phcbi, catalog number: SIM-F140AY65-PC, or equivalent)
7. Precision balances (Sartorius, model: Practum313-1CN, or equivalent)
8. Ultracentrifuge (Beckman Coulter, model: Optima XPN-90)
9. Ultra-Clear 26.3 ml tubes for 70Ti rotor (Beckman Coulter, catalog number: 355618)
10. Ultra-Clear 10.4 ml tubes for 90Ti rotor (Beckman Coulter, catalog number: 355603)
11. Ultra-Clear 10 ml tubes for SW32.1 rotor (Beckman Coulter, catalog number: 344061)
12. Formvar and carbon coated 200 mesh Nickel grids (Electron Microscopy Sciences, catalog number: FCF200-Ni)
13. Transmission electron microscope (Hitachi, model: HT-7700)
14. ChemiDoc Touch Imaging System (Bio-Rad, catalog number: 1708370)

bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

Procedure

1. *Laodelphaxstriatellus* carrying RSV was used to transmit the virus to health rice seedling. After inoculation on rice seedling for 48 h, the rice plants were maintained in greenhouse at 30 °C in light (16 h) and 26°C in dark(8 h) for 60 days. The RSV-infected rice leaves showing the typical leaf stripe symptoms were collected (Figure 1A).
2. Keep 0.1 M phosphate buffer (PB), pH 7.5, containing 0.01 M EDTA (PB-EDTA), and a blender at 4 °C.
3. Cut 50 g RSV-infected rice leaves into small pieces (about 2 cm) with razor blades and then ground them in 800 ml chilled PB-EDTA in the chilled blender for 5 min.
4. Filter the homogenate through two layers of gauze to remove large debris.
5. Centrifuge the filtered extract at 8,000 *x g* for 20 min at 4 °C to remove fine cell debris.
6. During centrifugation, weigh PEG 6000 powder (we grind PEG 6000 into fine powders before use) and NaCl according to the supernatant volume.
7. Transfer the supernatant into a sterilized and pre-chilled 1 L glass beaker.
8. Slowly add PEG 6000 and NaCl into the glass beaker till a final concentration of 6% PEG (w/v) and 0.1 M NaCl. Stir the mixture at 4 °C for 4 h and then allow this mixture to stand overnight at 4 °C.
9. Centrifuge this mixture at 8,000 *x g* for 20 min at 4 °C, using an angle rotor in a refrigerated centrifuge, to pellet RSV virions.
10. Resuspend the pellet in 8 ml 0.01 M PB-EDTA and centrifuge again at 8,000 *x g* for 10 min at 4 °C.
11. Transfer the supernatant into a clean pre-chilled centrifuge tube. Repeat Step 9 twice.
12. Combine the three supernatant in a pre-chilled ultra-centrifuge tube for the 70Ti rotor and centrifuge at 150,000 *x g* for 2 h at 4 °C. Discard the supernatant and resuspend the pellet in 6 ml 0.01 M PB.
13. Add 4 ml 20% glycerol solution into a clean ultra-clear centrifuge tube for the 90Ti rotor to form a glycerol cushion.
14. Carefully lay the resuspended pellet solution on top of the glycerol cushion and centrifuge at 150,000 *x g* for 2 h at 4 °C.
15. After centrifugation (Figure 1B), discard the supernatant and resuspend the pellet with 4 ml PB through repeated pipetting.
16. Prepare 10%, 20%, 30% and 40% sucrose solutions. 2 ml 40% sucrose solution was firstly added into the bottom of ultra-centrifuge tube and then slowly add 2 ml each of 30%, 20% and 10% sucrose solutions, sequentially, on the top of 10% sucrose solution with pipettes.
17. Carefully lay the resuspended pellet solution on a 10-40% sucrose density gradient inside a clean ultra-centrifuge tube for the SW32.1 rotor and centrifuge at 70,000 *x g* for 2 h at 4 °C.
18. Place the centrifuge tube under a light source and two separate bands become visible (Figure 1C, M and B). Collect the two bands separately and analyze them by SDS-PAGE (Figure 2A),

bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

Western blot (Figure 2B) and TEM (Figure 3). Add glycerol into the purified virion sample (M band) to reach a final concentration of 30%. Store the sample at -70 °C till further use.

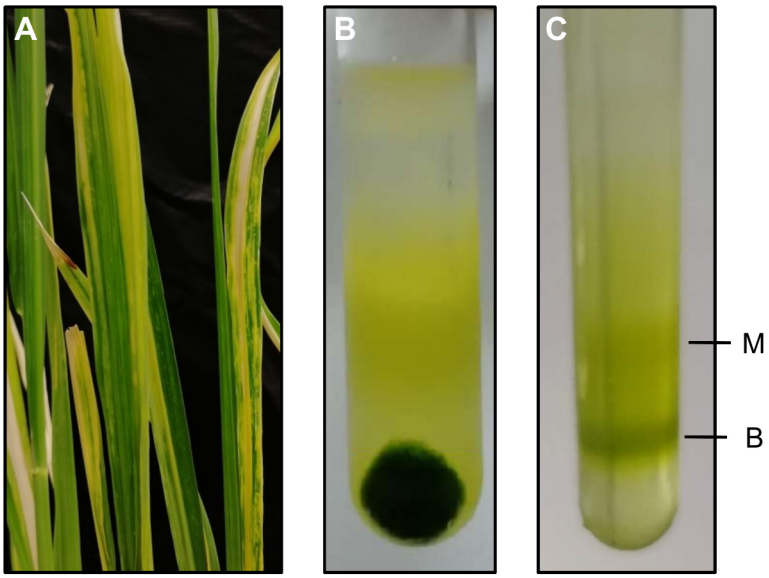

Figure 1. Purification of RSV through ultracentrifugation from RSV-infected rice leaves. A. RSV-infected rice samples showing typical stripe symptoms. B. Pelleting RSV virions through 20% glycerol cushion ultracentrifugation. A dark-green pellet with RSV virions is at the bottom of the centrifuge tube. C. Purification of RSV virions through 10-40% sucrose density gradient ultracentrifugation. The middle and bottom bands with RSV virions are indicated. M, middle band; B, bottom band.

Data analysis

1. SDS-PAGE

   M and B bands are collected separately and analyzed by SDS-PAGE (Figure 2A). Small amounts of samples from the M and B bands are respectively mixed with a loading buffer and boiled at 95 °C for 10 min. These samples are separated in lanes of a 10% SDS-PAGE gel through 50 min electrophoresis at 120 V. The gel is stained with 0.5% Coomassie Brilliant Blue G-250 before imaging.
2. Western blot

   M and B components are further analyzed by Western blot (Figure 2B). After separated by 10% SDS-PAGE through electrophoresis, the proteins are transferred onto nitrocellulose membrane and the blot was probed with a 1:5,000 (v/v) diluted mouse anti-RSV nucleocapsid monoclonal antibody followed by a goat anti-mouse IgG conjugated with HRP. The blot is visualized using the ChemiDoc Touch Imaging System.

bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

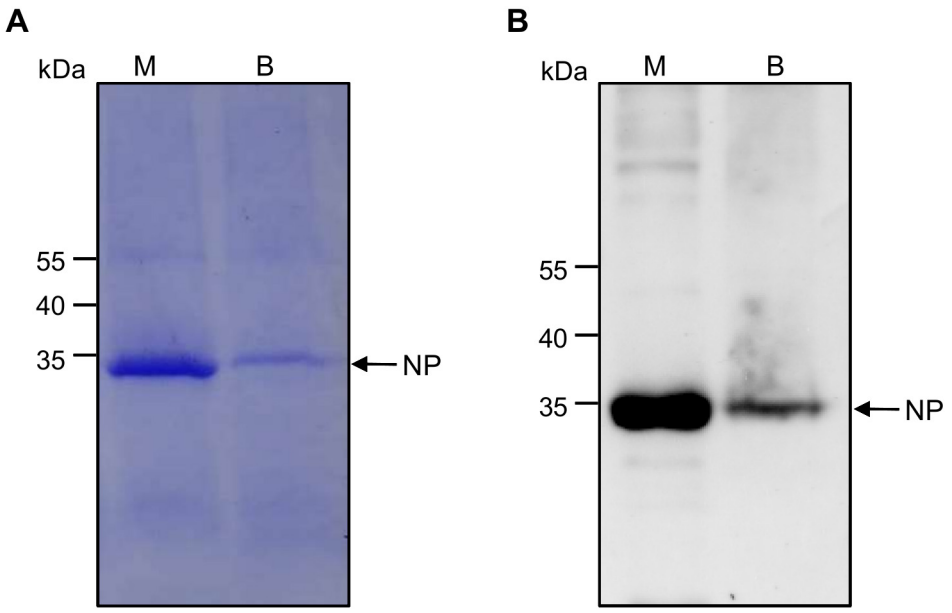

Figure 2. Analysis of M and B band products by SDS-PAGE and Western blot. A. After centrifugation in a 10-40% sucrose density gradient, the M and B bands are collected separately and analyzed in a 10% SDS-PAGE gel through electrophoresis. The gel is stained with Coomassie blue G-250. The band with predicted size for the RSV nucleocapsid protein (NP) is indicated with an arrow.B. Western blot analysis of M and B bands using monoclonal antibody against RSV NP. A band corresponding to the RSV nucleocapsid protein (NP) is indicated with an arrow.

3. Transmission Electron Microscopy

   For TEM, purified RSV sample is diluted 1:50 (v/v) 0.01 M PB and then placed (5 μl per sample) one formvar and carbon coated 200 mesh nickel grid. One minute after, the sample is removed from the grid by touching the edge of the grid with a filter paper. The grid is then stained with a 1% uranyl acetate solution for 2 min. After removal of the uranyl acetate solution, the grid is examined under an electron microscope. RSV virionsare only detected in M band sample (Figure 3).

bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

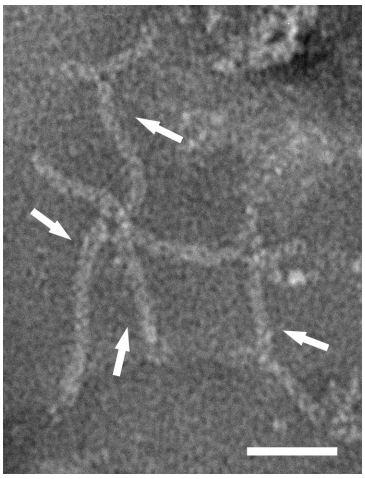

Figure 3. A TEM image showing RSV virions. RSV virions are purified from a RSV-infected rice plant using the above protocol, stained with uranyl acetate solution, and then examined and imaged under an electron microscope. Arrows indicate filamentous RSV virions. Scale bar = 50 nm.

Notes

1. To avoid degradation of virions, each purification step should be done at about 4 °C. The purified virion sample should be immediately stored at -70 °C.
2. Glycerol can affect RSV visualization under an electron microscope. We recommend to use freshly purified RSV for TEM without addition of glycerol.

Recipes

1. 0.1 M phosphate buffer, pH 7.5
   a. Dissloved 21.2g $Na_2HPO_4 \cdot 12H_2O$ and 2.21 g $NaH_2PO_4 \cdot 2H_2O$ in 1 L distilled and sterilized water
   b. Adjust pH value to 7.5 using NaOH
   c. Store the solution at 4 °C refrigerator

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bio-protocol www.bio-protocol.org/e3565 Bio-protocol 10(06):e3565. DOI:10.21769/BioProtoc.3565

Competing interests

The authors declare no conflicts of interest or competing interests.

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