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Infectious Subviral Particle to Membrane Penetration Active Particle (ISVP-to-ISVP*) Conversion Assay for Mammalian Orthoreovirus

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[Abstract] The mammalian orthoreovirus (reovirus) outer capsid undergoes a series of conformational changes prior to or during viral entry. These transitions are necessary for delivering the genome-containing core across host cell membranes. This protocol describes an *in vitro* assay for monitoring the transition into a membrane penetration-active form (*i.e.*, ISVP*).
Keywords: Virology, *Reoviridae*, Mammalian orthoreovirus, Viral entry, Conformational change, Heat inactivation

[Background] Reoviruses are nonenveloped, double-stranded RNA viruses that are composed of two concentric protein shells: the inner capsid (core) and the outer capsid (Dryden *et al.*, 1993; Zhang *et al.*, 2005; Dermody *et al.*, 2013). Following attachment, virions are endocytosed (Borsa *et al.*, 1979; Ehrlich *et al.*, 2004; Maginnis *et al.*, 2006; Maginnis *et al.*, 2008) and host cathepsin proteases degrade the σ3 outer capsid protein (Chang and Zweerink, 1971; Silverstein *et al.*, 1972; Borsa *et al.*, 1981; Sturzenbecker *et al.*, 1987; Dermody *et al.*, 1993; Baer and Dermody, 1997; Ebert *et al.*, 2002). This process generates a metastable intermediate, called infectious subviral particle (ISVP), in which the cell penetration protein, μ1, is exposed (Dryden *et al.*, 1993). ISVPs are produced *in vitro* by treating purified virions with chymotrypsin (Joklik, 1972; Borsa *et al.*, 1973a). The outer capsid undergoes a second conformational change to deposit the genome-containing core into the host cell cytoplasm. As a consequence, the central δ fragment of μ1 adopts a protease sensitive conformation. The altered particle is called ISVP* (Chandran *et al.*, 2002). ISVP-to-ISVP* conversion can be induced *in vitro* using heat (Middleton *et al.*, 2002), large monovalent cations (Borsa *et al.*, 1973b), μ1-derived peptides (Agosto *et al.*, 2008), red blood cells (Chandran *et al.*, 2002; Sarkar and Danthi, 2010), or lipids (Snyder, and Danthi, 2015 and 2016). Thus, questions related to reovirus entry (*e.g.*, the relationship between particle stability and infectivity) are studied using biochemical and cell-based approaches. In this protocol, we describe an *in vitro* assay that recapitulates ISVP-to-ISVP* conversion.

Materials and Reagents

1. Pipette tips
   a. 0.1-10 μl capacity (USA Scientific, catalog number: 1111-3700)
   b. 1-200 μl capacity (VWR, catalog number: 89079-474)
   c. 100-1,250 μl capacity (VWR, catalog number: 53508-924)

Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

2. PCR 8-well tube strips (VWR, catalog number: 20170-004)
3. 50 ml centrifuge tube (VWR, catalog number: 89039-660)
4. 1.7 ml microcentrifuge tubes (MIDSCI, catalog number: AVSS1700)
5. Purified reovirus stocks (see [Berard and Coombs, 2009; Kobayashi *et al.*, 2010] for propagation and purification procedure)
6. Crushed ice
7. Standard SDS-PAGE materials and reagents (*e.g.*, 10% SDS-polyacrylamide mini gels)
8. Coomassie Brilliant Blue stain and destain solutions (Bio-Rad Laboratories, catalog number: 1610435)
9. Bleach (Biz4USA, Janitorial Supplies, catalog number: CLO30966CT)
10. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (MP Biomedicals, catalog number: 02103133)
11. Magnesium chloride hexahydrate ($MgCl_2·6H_2O$) (Sigma-Aldrich, catalog number: M9272)
12. Sodium chloride (NaCl) (Merck, catalog number: SX0420-3)
13. 0.1 N hydrochloric acid (Sigma-Aldrich, catalog number: 2104)
14. 0.1 N sodium hydroxide (Sigma-Aldrich, catalog number: 2105)
15. 4x Laemmli sample buffer (Bio-Rad Laboratories, catalog number: 1610747)
16. *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated chymotrypsin (Worthington Biochemical, catalog number: LS001432)
17. Trypsin (Sigma-Aldrich, catalog number: T6567)
18. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
19. Ultrapure DNase/RNase-free distilled H_2O (Thermo Fisher Scientific, Invitrogen™, catalog number: 10977015)
20. Isopropyl alcohol (Avantor Performance Materials, Macron, catalog number: 3032-02)
21. 50% bleach (see Recipes)
22. Virus storage buffer (VB) (see Recipes)
23. 2 mg/ml *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated chymotrypsin (see Recipes)
24. 100 mM phenylmethylsulfonyl fluoride (PMSF) (see Recipes)
25. 1 mg/ml trypsin (see Recipes)

Equipment

1. Personal protective equipment (PPE)
   a. Laboratory coat
   b. Gloves
   c. Eye protection
2. Biosafety level 2 (BSL-2) laboratory facility
3. BSL-2 certified tissue culture hood
4. Solid and liquid waste containers

Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

5. Autoclave
6. Ice bucket
7. -20 °C freezer
8. Micropipettes
   a. 0.1-2.5 µl capacity (Eppendorf, catalog number: 3123000012)
   b. 2-20 µl capacity (Eppendorf, catalog number: 3123000039)
   c. 20-200 µl capacity (Eppendorf, catalog number: 3123000055)
   d. 100-1,000 µl capacity (Eppendorf, catalog number: 3123000063)
9. Digital pH meter (VWR, model: SB70P)
10. Digital laboratory balance (Mettler Toledo, model: PB1502-S)
11. NanoDrop spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: ND-1000)
12. Hot plate stirrer (VWR, catalog number: 12365-382)
13. Magnetic stir bar (VWR, catalog number: 58948-273)
14. Thermal cycler (Bio-Rad Laboratories, model: S1000™)
15. Analog dry block heater (VWR, catalog number: 12621-110)
16. Thermometer (VWR, catalog number: 89095-566)
17. Gel imaging system (LI-COR, model: Odyssey® Classic)
18. 1,000 ml glass beaker (VWR, catalog number: 89000-212)
19. 1,000 ml graduated cylinder (VWR, catalog number: 65000-012)
20. 1,000 ml storage bottle (VWR, catalog number: 89000-240)
    Note: This product has been discontinued.

Software

1. Image Studio Lite (LI-COR)

Procedure

A. Generation of infectious subviral particles (ISVPs)
   1. Propagate and purify reovirus virions as previously described (Berard and Coombs, 2009; Kobayashi *et al.*, 2010). Using a NanoDrop spectrophotometer, determine particle concentration by measuring the optical density of the purified virus stocks at 260 nm (OD_{260}; 1 unit at OD_{260} = 2.1 x 10^{12} particles/ml) (Smith *et al.*, 1969).
   2. In 1 tube of an 8-well tube strip, dilute 2 x 10^{11} virions into 90 µl of ice cold VB (see Recipes).
   3. Add 10 µl of ice cold 2 mg/ml TLCK-treated chymotrypsin (see Recipes) to the diluted virus. Mix by pipetting up and down 3-4 times.
      Note: For an undigested control, substitute 10 µl of ice cold VB for 10 µl of TLCK-treated chymotrypsin.
   4. Incubate the reaction for 20 min at 32 °C in a thermal cycler.

Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

Note: Under these conditions, σ3 is degraded (Joklik, 1972; Borsa et al., 1973a) and μ1 is cleaved (Nibert and Fields, 1992; Chandran et al., 1999).

5. Following digestion, quench chymotrypsin activity by the addition of 1 μl of 100 mM PMSF (see Recipes). Mix by pipetting up and down 3-4 times.
6. Incubate the reaction for 20 min on ice.
7. To confirm that ISVPs are generated, run 2 x 10^{10} particles per lane on a 10% SDS-polyacrylamide mini gel. Run the gel for 40-45 min at 200 V constant.
8. Visualize the protein bands by Coomassie Brilliant Blue staining (see Data analysis, Figure 1).
9. Store ISVPs on ice, and use within 2-3 h for ISVP-to-ISVP* conversion experiments.

B. ISVP-to-ISVP* conversion assay

1. Add 10 μl of ISVPs to each tube of an 8-well tube strip on ice.
2. Incubate the ISVPs for 1 h on a temperature gradient in a thermal cycler (*i.e.*, each tube is incubated at a different temperature).

   Note: Appropriate temperature ranges will vary based on the incubation time (5 min to 1 h) and the reovirus strain under investigation. Each step in the temperature gradient should be no less than 1 °C. ISVP-to-ISVP conversion typically occurs between 30 °C and 50 °C.*
3. Incubate the heat treated ISVPs for 5 min on ice.
4. Add 0.9 μl of 1 mg/ml trypsin (see Recipes) to each tube of heat treated ISVPs. Mix by pipetting up and down 3-4 times.
5. Incubate the reactions for 30 min on ice.

Note: As a consequence of ISVP-to-ISVP conversion, the central δ fragment of μ1 adopts a protease sensitive conformation (Chandran et al., 2002).*

6. Following digestion, add 3.3 μl of 4x Laemmli sample buffer to each reaction. Mix by pipetting up and down 3-4 times.
7. Boil each reaction for 5 min at 95 °C.
8. To determine the temperature at which ISVP-to-ISVP* conversion occurs, run 2 x 10^{10} particles (*i.e.*, the entire ISVP-to-ISVP* conversion reaction at a given temperature) per lane on a 10% SDS-polyacrylamide mini gel. Run the gel for 40-45 min at 200 V constant.
9. Visualize the protein bands by Coomassie Brilliant Blue staining (see Data analysis, Figure 2).

Data analysis

A. Generation of infectious subviral particles (ISVPs)

1. Record and analyze the results using a gel imaging system and Image Studio Lite software (Figure 1).
   a. Virions contain λ1,2,3, μ1C, σ2, and σ3.
   b. ISVPs contain λ1,2,3, μ1C, δ, and σ2.

Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

Note: The appearance of δ, the loss of μ1C, and the loss of σ3 indicate the transition from virions to ISVPs. λ1,2,3 and σ2 should remain unchanged.

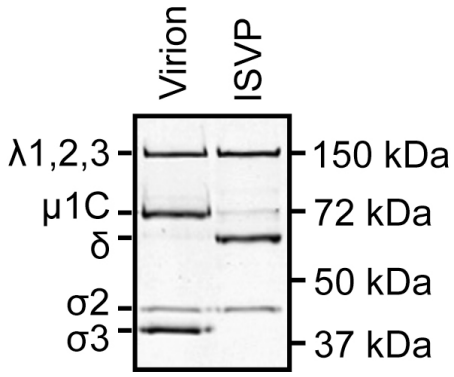

Figure 1. SDS-PAGE gel of reovirus virions and ISVPs

B. ISVP-to-ISVP* conversion assay

1. All ISVP-to-ISVP* experiments should be repeated for at least three independent replicates.
2. Record and analyze the results using a gel imaging system and Image Studio Lite software (Figure 2).
   a. Trypsin treated ISVPs contain λ1,2,3, μ1C, δ, and σ2.
   b. Trypsin treated ISVP*s contain λ1,2,3 and σ2.

*Note: The loss of μ1C and δ indicate the transition from ISVPs to ISVP*s. λ1,2,3 and σ2 should remain unchanged.*

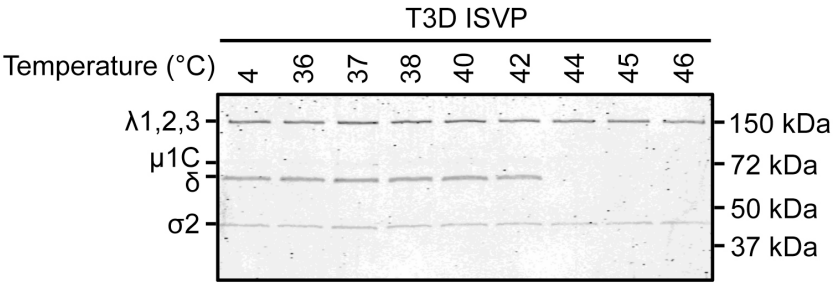

Figure 2. SDS-PAGE gel of heat and trypsin treated reovirus ISVPs. In the experiment shown here, ISVP-to-ISVP* conversion occurred at 44, 45, and 46 °C.

Notes

1. When possible, all procedures are performed in a BSL-2 certified tissue culture hood.
2. Laboratory personnel should use appropriate PPE.
3. All solid waste is autoclaved prior to disposal.
4. All liquid waste is inactivated with 50% bleach prior to disposal.

Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

Recipes

1. 50% bleach
   In a storage bottle, dilute 50 ml of 100% bleach into 50 ml of ultrapure H_2O
2. Virus storage buffer (VB) (10 mM Tris, pH 7.4, 15 mM $MgCl_2$, and 150 mM NaCl)
   a. In a glass beaker, dissolve the following into 900 ml of ultrapure H_2O:
      1.21 g Tris
      3.05 g $MgCl_2 \cdot 6H_2O$
      8.77 g NaCl
   b. Mix at room temperature using a magnetic stir bar on a stir plate
   c. Adjust to pH 7.4 with 0.1 N hydrochloric acid
   d. In a graduated cylinder, bring the final volume up to 1,000 ml with ultrapure water
   e. Transfer the solution to a storage bottle
   f. Sterilize by autoclaving
   g. Store at room temperature
3. 2 mg/ml *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated chymotrypsin
   a. In a centrifuge tube, dissolve 100 mg of TLCK-treated chymotrypsin into 50 ml of ultrapure H_2O
   b. Mix at room temperature by gently inverting the tube until the solution becomes clear
   c. Transfer 1 ml aliquots to microcentrifuge tubes
   d. Store at -20 °C
4. 100 mM phenylmethylsulfonyl fluoride (PMSF)
   a. In a microcentrifuge tube, dissolve 17.4 mg of PMSF into 1 ml of isopropyl alcohol
   b. Mix at room temperature by gently inverting the tube until the solution becomes clear
   c. Store at -20 °C
5. 1 mg/ml trypsin
   a. In a microcentrifuge tube, dissolve 1 mg of trypsin into 1 ml of ultrapure H_2O
   b. Mix at room temperature by gently inverting the tube until the solution becomes clear
   c. Store at -20 °C

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Vol 8, Iss 02, Jan 20, 2018
DOI:10.21769/BioProtoc.2700

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