

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 $\sigma 3$ 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, $\mu 1$, 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 $\mu 1$ 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), $\mu 1$ -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 μ l capacity (USA Scientific, catalog number: 1111-3700)
 - b. 1-200 μ l capacity (VWR, catalog number: 89079-474)
 - c. 100-1,250 μ l capacity (VWR, catalog number: 53508-924)

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 ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (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. α -*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 α -*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

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 (OD₂₆₀; 1 unit at OD₂₆₀ = 2.1 × 10¹² particles/ml) (Smith *et al.*, 1969).
2. In 1 tube of an 8-well tube strip, dilute 2 × 10¹¹ 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.

Note: Under these conditions, $\sigma 3$ is degraded (Joklik, 1972; Borsa et al., 1973a) and $\mu 1$ is cleaved (Nibert and Fields, 1992; Chandran et al., 1999).

5. Following digestion, quench chymotrypsin activity by the addition of 1 μ l 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 2×10^{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 μ l 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 μ l 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 $\mu 1$ adopts a protease sensitive conformation (Chandran et al., 2002).*

6. Following digestion, add 3.3 μ l 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 2×10^{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 $\lambda 1,2,3$, $\mu 1C$, $\sigma 2$, and $\sigma 3$.
 - b. ISVPs contain $\lambda 1,2,3$, $\mu 1C$, δ , and $\sigma 2$.

Note: The appearance of δ , the loss of $\mu 1C$, and the loss of $\sigma 3$ indicate the transition from virions to ISVPs. $\lambda 1,2,3$ and $\sigma 2$ should remain unchanged.

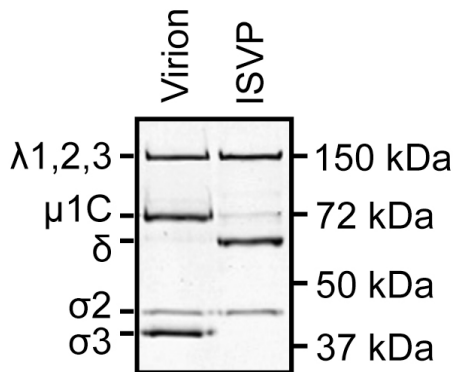


Figure 1. SDS-PAGE gel of reovirus virions and ISVPs

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 $\lambda 1,2,3$, $\mu 1C$, δ , and $\sigma 2$.
 - b. Trypsin treated ISVP*s contain $\lambda 1,2,3$ and $\sigma 2$.

*Note: The loss of $\mu 1C$ and δ indicate the transition from ISVPs to ISVP*s. $\lambda 1,2,3$ and $\sigma 2$ should remain unchanged.*

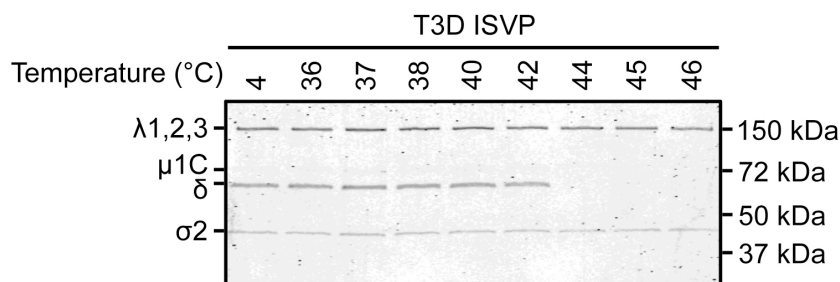


Figure 2. SDS-PAGE gel of heat and trypsin treated reovirus ISVPs. In the experiment shown here, ISVP-to-ISVP* conversion occurred at 44, 45, and 46 °C.

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.

Recipes

1. 50% bleach
In a storage bottle, dilute 50 ml of 100% bleach into 50 ml of ultrapure H₂O
2. Virus storage buffer (VB) (10 mM Tris, pH 7.4, 15 mM MgCl₂, and 150 mM NaCl)
 - a. In a glass beaker, dissolve the following into 900 ml of ultrapure H₂O:
1.21 g Tris
3.05 g MgCl₂·6H₂O
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 Na-*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 H₂O
 - 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 H₂O
 - b. Mix at room temperature by gently inverting the tube until the solution becomes clear
 - c. Store at -20 °C

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