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Infectious Subviral Particle-induced Hemolysis Assay for Mammalian Orthoreovirus

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[Abstract] Mammalian orthoreovirus (reovirus) utilizes pore forming peptides to penetrate host cell membranes. This step is essential for delivering its genome containing core particle during viral entry. This protocol describes an *in vitro* assay for measuring reovirus-induced pore formation.

Keywords: Virology, *Reoviridae*, Mammalian orthoreovirus, Viral entry, Membrane penetration, Hemolysis

[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). Reovirus ISVPs undergo a second conformational change to deposit the genome- containing core into the host cell cytoplasm. The altered particle is called ISVP* (Chandran *et al*., 2002). ISVP-to-ISVP* conversion culminates in the release of μ1-derived pore forming peptides (Nibert *et al*., 1991; Zhang *et al*., 2005; Chandran *et al*., 2002; Odegard *et al*., 2004; Nibert *et al*., 2005; Agosto *et al*., 2006; Ivanovic *et al*., 2008). The released peptides form pores within endosomal membranes, which are thought to mediate core delivery (Agosto *et al*., 2006; Ivanovic *et al*., 2008; Zhang *et al*., 2009).

Many of the conformational changes that define reovirus entry can be recapitulated *in vitro*: (i) ISVPs are produced by digesting purified virions with chymotrypsin (Joklik, 1972; Borsa *et al*., 1973a), and (ii) ISVP* formation can be induced 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 are studied using biochemical and cell-based approaches. In this protocol, we describe an *in vitro* assay that recapitulates ISVP-to-ISVP* conversion and subsequent pore formation.

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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)
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. Vacuum driven and disposable bottle top 0.22 µm filter (Merck, catalog number: SCGPT05RE)
6. Flat bottom, 96-well plate (Greiner Bio One International, catalog number: 655180)
7. Purified reovirus stocks (see Berard and Coombs, 2009; Kobayashi *et al*., 2010 for propagation and purification procedures)
8. Crushed ice
9. Standard SDS-PAGE materials and reagents (*e.g.*, 10% SDS-polyacrylamide mini gels)
10. Coomassie Brilliant Blue stain and destain solutions (Bio-Rad Laboratories, catalog number: 1610435)
11. Citrated bovine calf blood (Colorado Serum Company, catalog number: 31023)
12. Bleach (Biz4USA, Janitorial Supplies, catalog number: CLO30966CT)
13. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (MP Biomedicals, catalog number: 02103133)
14. Sodium chloride (NaCl) (Merck, catalog number: SX0420-3)
15. 0.1 N hydrochloric acid (Sigma-Aldrich, catalog number: 2104)
16. 0.1 N sodium hydroxide (Sigma-Aldrich, catalog number: 2105)
17. *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated chymotrypsin (Worthington Biochemical, catalog number: LS001432)
18. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
19. Isopropyl alcohol (Avantor Performance Materials, Macron, catalog number: 3032-02)
20. Dulbecco's phosphate buffered saline (Thermo Fisher Scientific, Gibco™, catalog number: 21600044)
21. Magnesium chloride hexahydrate ($MgCl_2·6H_2O$) (Sigma-Aldrich, catalog number: M9272)
22. Ultrapure DNase/RNase-free distilled H_2O (Thermo Fisher Scientific, Invitrogen™, catalog number: 10977015)
23. Triton X-100 (TX-100) (Sigma-Aldrich, catalog number: X100)
24. 50% bleach (see Recipes)
25. Virus storage buffer (VB) (see Recipes)
26. 2 mg/ml TLCK-treated chymotrypsin (see Recipes)
27. 100 mM phenylmethylsulfonyl fluoride (PMSF) (see Recipes)
28. Phosphate buffered saline supplemented with 2 mM $MgCl_2$ (PBS^{Mg}) (see Recipes)

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29. 10% Triton X-100 (TX-100) (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. Vacuum pump and aspirator
7. Ice bucket
8. -20 °C freezer
9. 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)
10. Digital pH meter (VWR, model: SB70P)
11. Digital laboratory balance (Mettler Toledo, model: PB1502-S)
12. NanoDrop spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: ND-1000)
13. Hot plate stirrer (VWR, catalog number: 12365-382)
14. Magnetic stir bar (VWR, catalog number: 58948-273)
15. Microcentrifuge (Eppendorf, model: 5424)
16. Thermal cycler (Bio-Rad Laboratories, model: S1000™)
17. Temperature controlled water bath (VWR, catalog number: 89501-466)
18. Gel imaging system (LI-COR, model: Odyssey® Classic)
19. Microplate reader (Molecular Devices, model: FilterMax F5 Multi-Mode)
20. 250 ml glass beaker (VWR, catalog number: 89000-204)
21. 1,000 ml glass beaker (VWR, catalog number: 89000-212)
22. 100 ml graduated cylinder (VWR, catalog number: 65000-006)
23. 1,000 ml graduated cylinder (VWR, catalog number: 65000-012)
24. 100 ml storage bottle (VWR, catalog number: 89000-234)
25. 1,000 ml storage bottle (VWR, catalog number: 89000-240)
    Note: This product has been discontinued.

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Software

1. Image Studio Lite (LI-COR)
2. SoftMax Pro (Molecular Devices)

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.
      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 hemolysis experiments.

B. Preparation of bovine red blood cells (RBCs)
   1. Perform all steps on ice or at 4 °C.
   2. Transfer 1 ml of citrated bovine calf blood to a microcentrifuge tube.
      Note: Citrated bovine calf blood should be used within 2 weeks of the draw date.
   3. Pellet the RBCs by centrifugation at 500 *x g* for 5 min.
      Note: RBCs are the source of membranes for hemolysis experiments.
   4. Aspirate and discard the supernatant.
   5. Resuspend the RBCs in 1 ml of ice cold PBS^{Mg} (see Recipes). Mix by gently pipetting up and down.
   6. Repeat Steps B3-B5 until the supernatant remains clear after pelleting.
   7. Resuspend the washed RBCs in ice cold PBS^{Mg} at a 30% (vol/vol) concentration. Mix by gently

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flicking the side of the tube.

Note: Estimate the RBC pellet volume by using the volume markers on the microcentrifuge tube.

8. Store RBCs on ice, and use immediately for hemolysis experiments

C. ISVP-induced hemolysis assay

1. In separate microcentrifuge tubes, assemble the following reactions on ice:
   a. 33.3 µl VB + 3.7 µl 30% RBCs (0% hemolysis control)
   b. 30.3 µl VB + 3.7 µl 30% RBCs + 3 µl 10% TX-100 (100% hemolysis control, see Recipes)
   c. 3.3 µl VB + 3.7 µl 30% RBCs + 30 µl ISVPs
2. Mix the reactions by gently flicking the side of the tubes.
3. Incubate the reactions for 1 h (T3D reovirus) or for 2 h (T1L reovirus) at 37 °C in a water bath.
   Note: Under these conditions, ISVP-to-ISVP conversion is induced (Chandran et al., 2002; Sarkar and Danthi, 2010) and the µ1-derived pore forming peptides are released (Nibert et al., 1991; Chandran et al., 2002; Odegard et al., 2004; Nibert et al., 2005; Zhang et al., 2005; Agosto et al., 2006; Ivanovic et al., 2008).*
4. Place the reactions on ice for 20 min.
5. Pellet intact RBCs by centrifugation at 500 *x g* for 5 min.
   Note: This step should be performed at 4 °C.
6. Transfer 20 µl of each supernatant to individual wells of a 96-well plate.
7. Dilute each transferred supernatant with 80 µl of VB. Mix by pipetting up and down 3-4 times.
8. To quantify the amount of hemoglobin released (*i.e.*, RBC lysis), measure the absorbance (*A*) of the diluted supernatants at 405 nm using a microplate reader. *A* values are recorded on SoftMax Pro software.
9. Calculate the percent hemolysis (see Data analysis).

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.
   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.

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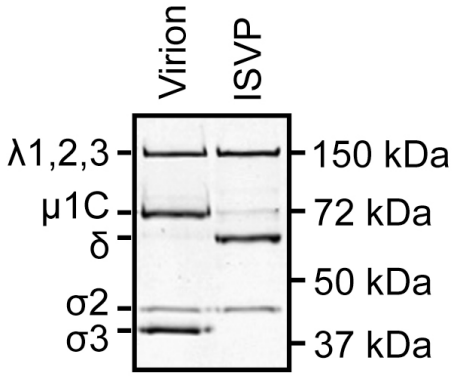

Figure 1. SDS-PAGE gel of reovirus virions and ISVPs

B. ISVP-induced hemolysis assay

1. All hemolysis experiments should be repeated for at least three independent replicates.
2. Calculate the percent hemolysis using the following formula:

   $[(A_{sample} - A_{buffer})/(A_{TX\text{-}100} - A_{buffer})] \times 100$

   a. A_{buffer} represents the supernatant derived from VB and RBCs.
   b. $A_{TX\text{-}100}$ represents the supernatant derived from VB, RBCs, and TX-100.
   c. A_{sample} represents the supernatant derived from VB, RBCs, and ISVPs.
3. When comparing the hemolytic capacity of different reovirus strains, calculate *P* values using Student's *t*-test.
4. Use graphing software to plot percent hemolysis.
   Note: For T3D reovirus, 40-60% hemolysis is typically observed after 1 h incubation at 37 °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 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

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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. Phosphate buffered saline supplemented with 2 mM $MgCl_2$ (PBS^{Mg})
   a. In a glass beaker, dissolve the following into 900 ml of ultrapure H_2O:
      9.55 g Dulbecco's phosphate buffered saline
      0.41 g $MgCl_2 \cdot 6H_2O$
   b. Mix at room temperature using a magnetic stir bar on a stir plate
   c. Adjust to pH 7.4
   d. In a graduated cylinder, bring the final volume up to 1,000 ml with ultrapure water
   e. Sterilize by filtering through a 0.22 µm bottle top filter
   f. Store at room temperature
6. 10% Triton X-100 (TX-100)
   a. In a glass beaker, dissolve the following into 80 ml of ultrapure H_2O:
      0.12 g Tris
      0.31 g $MgCl_2 \cdot 6H_2O$
      0.88 g NaCl
      10 ml of 100% TX-100
   b. Mix at room temperature using a magnetic stir bar on a stir plate
   c. Adjust to pH 7.4
   d. In a graduated cylinder, bring the final volume up to 100 ml with ultrapure water
   e. Transfer the solution to a storage bottle
   f. Sterilize by autoclaving
   g. Store at room temperature

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