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TITLE:

Growth, Purification, and Titration of Oncolytic Herpes Simplex Virus

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Oncolytic virus, cytopathic effect, HSV, virus growth, virus purification, sucrose-gradient method, plaque assay

SUMMARY:

In this manuscript, we describe a simple method of growth, purification, and titration of the oncolytic herpes simplex virus for preclinical use.

ABSTRACT:

Oncolytic viruses (OVs), such as the oncolytic herpes simplex virus (oHSV), are a rapidly growing treatment strategy in the field of cancer immunotherapy. OVs, including oHSV, selectively replicate in and kill cancer cells (sparing healthy/normal cells) while inducing anti-tumor immunity. Because of these unique properties, oHSV-based treatment strategies are being increasingly used for the treatment of cancer, preclinically and clinically, including FDA-approved talimogene laherparevec (T-Vec). Growth, purification, and titration are three essential laboratory techniques for any OVs, including oHSVs, before they can be utilized for experimental studies. This paper describes a simple step-by-step method to amplify oHSV in Vero cells. As oHSVs multiply, they produce a cytopathic effect (CPE) in Vero cells. Once 90–100% of the infected cells show a CPE, they are gently harvested, treated with benzonase and magnesium chloride ($MgCl_2$), filtered, and subjected to purification using the sucrose-gradient method.

Following purification, the number of infectious oHSV (designated as plaque-forming units or PFUs) is determined by a "plaque assay" in Vero cells. The protocol described herein can be used to prepare high-titer oHSV stock for *in vitro* studies in cell culture and *in vivo* animal experiments.

INTRODUCTION:

Oncolytic viruses (OVs) are an emerging and unique form of cancer immunotherapy. OVs selectively replicate in and lyse tumor cells (sparing normal/healthy cells)[1] while inducing anti-tumor immunity[2]. Oncolytic herpes simplex virus (oHSV) is one of the most extensively studied viruses among all OVs. It is furthest along in the clinic, with Talimogene laherparepvec (T-VEC) being the first and only OV to receive FDA approval in the USA for the treatment of advanced melanoma[3]. In addition to T-VEC, many other genetically engineered oHSVs are being tested preclinically and clinically in different cancer types[3-8]. The current advanced recombinant DNA biotechnology has further increased the feasibility of engineering new oHSVs coding for therapeutic transgene(s)[3,5]. An efficient system of oHSV propagation, purification, and titer determination is critical before any (newly developed) oHSV can be tested for *in vitro* and *in vivo* studies. This paper describes a simple step-by-step method of oHSV growth (in Vero cells), purification (by the sucrose-gradient method), and titration (by an oHSV plaque assay in Vero cells) (**Figure 1**). It can be easily adopted in any Biosafety Level 2 (BSL2) laboratory setting to achieve a high-quality viral stock for preclinical studies.

Vero, an African green monkey kidney cell line, is the most commonly used cell line for oHSV propagation[9-13] as Vero cells have a defective antiviral interferon signaling pathway[14]. Other cell lines with inactivated stimulator of interferon genes (STING) signaling can also be used for oHSV growth[12,13]. This protocol utilizes Vero cells for oHSV growth and the plaque assay. Following propagation, oHSV-infected cells are harvested, lysed, and subjected to purification, wherein lysed cells are first treated with benzonase nuclease to degrade host cell DNA, prevent nucleic acid-protein aggregation, and reduce the viscosity of the cell lysate. As proper activation of benzonase often requires Mg^{2+}, 1–2 mM $MgCl_2$ is used in this protocol[15]. The host cell debris from the benzonase-treated cell lysate is further eliminated by serial filtration before high-speed sucrose-gradient centrifugation. A viscous 25% sucrose solution cushion helps to ensure a slower rate of virus migration through the sucrose layer, leaving host cell-related components in the supernatant, thus improving purification and limiting virus loss in the pellet[16]. The purified oHSV is then titrated on Vero cells, and viral plaques are visualized by Giemsa staining[17] or X-gal staining (for LacZ encoding oHSVs)[18].

PROTOCOL:

1) oHSV growth

NOTE: Ensure institutional biosafety committee approval before working with oHSV. This study was conducted under approved IBC Protocol no. 18007. Maintain BSL2 precautions: bleach all pipets, tips, tubes, and other materials that come into contact with the virus. Spray gloves with 70% isopropyl alcohol before hands leave the BSL2 cell culture hood. Always thoroughly wash hands with soap water after working with a virus.

1.1) On day -1, seed low-passage Vero cells in 20 T-150 cm^2 flasks at a density of 7–8 × 10^6 cells/flask in regular Vero cell medium.

NOTE: Vero medium is prepared by supplementing Dulbecco's modified eagle medium (DMEM) with 10% heat-inactivated fetal calf serum (IFCS).

1.2) On Day 0 (cells are 80–90% confluent), add oHSV inoculum on the Vero cells.

1.2.1) Preparation of virus inoculum

1.2.1.1) Use a multiplicity of infection (MOI) of 0.01 (MOI can vary from 0.01 to 0.1 depending on the replication capacity of virus) for virus amplification. Use formula (**1**) to calculate the amount of virus (mL) for 20 flasks.

Amount of virus (mL) for 20 flasks = amount of virus needed (pfu)/titer of virus stock (pfu/mL) (**1**)

1.2.1.2) Use high-glucose Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1% heat-inactivated fetal calf serum (IFCS) to prepare the virus inoculum (7 mL per T-150 cm^2 flask, i.e., ~140 mL for 20 flasks). Add the required amount of oHSV to 140 mL of high-glucose DPBS/1% IFCS solution, vortex for 1 min, and keep the mixture ready for addition to Vero cells.

1.2.2) Wash the T-150 cm^2 flasks 2× with high-glucose DPBS supplemented with 1% IFCS (10 mL/wash). Aspirate the DPBS/1% IFCS and add 7 mL of virus inoculum/T-150 cm^2 flask.

1.2.3) Gently rock the flasks for 5 min using a flask rocker for proper distribution of the inoculum over the Vero monolayer, and then incubate the flasks at 37 °C for 1.5–2 h. Make sure the incubator shelf is level.

1.2.4) Remove the inoculum and add DMEM supplemented with 1% IFCS (25 mL/flask). Incubate the flasks for 2–4 days.

1.3) Check the flasks daily for 90–100% CPE (see **Figure 2**).

1.4) Harvest the oHSV-infected Vero cells.

1.4.1) Collect the culture supernatant (~20 mL) from each flask (leave ~5 mL in each flask) in 50 mL conical centrifuge tubes or media container.

1.4.2) Use a cell scraper to scrape the cells from the bottom of the flasks gently.

NOTE: The cells should quickly come off the flasks.

1.4.3) Add ~15 mL of the culture supernatant (collected in step 1.4.1) to each flask (which brings the volume of ~20 mL in each flask, i.e., 400 mL for 20 flasks) and gently wash the bottom of the flasks a few times using a 10 mL sterile serological pipet.

NOTE: Do **not** pipet vigorously. Aim to keep all cells intact.

1.4.4) Collect the cells (+ medium) into 50 mL conical centrifuge tubes on ice (use 8 tubes to hold 400 mL of harvested cells from 20 flasks).

1.4.5) Spin the cells at 300 × *g* for 10 min at 4 °C and aspirate the supernatant.

1.4.6) Add 1.25 mL (50%) of Virus Buffer (VB) and 1.25 mL (50%) of culture supernatant (collected in step 1.4.1) to each centrifuge tube and re-suspend each pellet thoroughly. Transfer the re-suspended cells from eight 50 mL centrifuge tubes to one 50 mL conical centrifuge tube.

NOTE: Refer to the preparation of VB solution in **Table 1**. Filter-sterilize the VB solution using a media sterilization filter. Use 0.5 mL of VB + 0.5 mL of supernatant per T-150 cm^2 flask for re-suspension, i.e., for 20 flasks (20 mL), use 10 mL of VB and 10 mL of culture supernatant.

1.4.7) Snap-freeze the re-suspended cells using dry ice/100% ethanol and store at -80 °C.

2) oHSV purification

2.1) Snap-freeze (in dry ice and 100% ethanol)/thaw (in a 37 °C warm water bath) the cells followed by water bath-sonication for 1 min for a total of 3 cycles to ensure proper lysis of the cells to release virus in the supernatant.

NOTE: Perform sonication for 1 min using 40 kHz, 120 V power. If a tunable sonicator is not available, use an ultrasonic water bath sonicator. Take a 50 µL aliquot for titration in section 3, which will help to identify which of the following step(s) is responsible for potential virus loss during the purification procedure.

2.2) Treat the cell lysate with Benzonase Nuclease (175 units/mL) + 2 mM $MgCl_2$ (1 M stock = 2 µL/mL), vortex, and incubate for 30 min at 37 °C. Place the tube on ice and perform the following steps at 4 °C.

2.3) Pellet the cell debris by low-speed centrifugation.

2.3.1) Spin the cell lysate at 300 × *g* for 10 min.

2.3.2) Collect the supernatant in a new 50 mL conical centrifuge tube (re-suspend the cell pellet in 0.5 mL of VB, designate it as pellet-1, and store at 4 °C for use in step 2.3.5; see **Figure 1**).

2.3.3) Spin the supernatant (obtained in step 2.3.2) again at 500 × *g* for 10 min.

2.3.4) Collect the supernatant in a new 50 mL conical centrifuge tube (re-suspend the cell pellet in 0.5 mL of VB, designate it as pellet-2, and store at 4 °C for use in step 2.3.5; see **Figure 1**).

2.3.5) Combine the re-suspended pellet-1 (from 2.3.2) and pellet-2 (from 2.3.4) into a new 1.7 mL centrifuge tube, vortex/sonicate (water bath) 2x, and spin at 400 × *g* for 10 min. Collect the supernatant and combine it with the supernatant obtained in step 2.3.4; see **Figure 1**).

NOTE: Perform sonication for 1 min using 40 kHz, 120 V power to prevent viral aggregation before the filtration procedure in step 2.4. Take a 50 µL aliquot of the combined supernatant for titration in section 3.

2.4) Filter the combined supernatant (~21 mL, i.e., 20 mL from 1.4.6, 0.5 mL from 2.3.2, and 0.5 mL from 2.3.4) using the following 3-step filtration method.

2.4.1) Draw 21 mL of the supernatant using a 10 mL syringe (5–7 mL each time for easy passage through the filter) and pass it through a sterile 5 µm polyvinylidene difluoride (PVDF) membrane filter placed on a new 50 mL conical centrifuge tube (labeled as **TUBE 1**).

2.4.1.1) Add 1 mL of VB to a 50 mL conical centrifuge tube emptied in 2.4.1 (to collect the remaining trace amount of virus supernatant), vortex, and pass through same 5 µm PVDF filter placed on **TUBE 1** (bringing the total to 22 mL of filtrate). Proceed to step 2.4.2.

2.4.2) Draw 22 mL of the filtrate from **TUBE 1** (as in 2.4.1) and pass it through a sterile 0.8 µm mixed cellulose ester (MCE) membrane filter placed on a new 50 mL conical centrifuge tube (labeled as **TUBE 2**).

2.4.2.1) Add 1 mL of VB to **TUBE 1** emptied in step 2.4.2, vortex, and pass it through the same 0.8 µm MCE filter placed on **TUBE 2** (bringing the total to 23 mL of filtrate). Proceed to step 2.4.3.

2.4.3) Draw 23 mL of filtrate from **TUBE 2** (as in 2.4.1) and pass it through a sterile 0.45 µm PVDF filter placed on a new 50 mL conical centrifuge tube (labeled as **TUBE 3**).

2.4.3.1) Add 1 mL of VB to **TUBE 2** emptied in step 2.4.3, vortex, and pass it through the same 0.45 µm PVDF filter placed on **TUBE 3** (bringing the total to 24 mL of filtrate).

NOTE: Take a 50 µL aliquot of the filtrate for titration in section 3.

2.5) High-speed centrifugation using the sucrose-gradient method

NOTE: A fixed angle F13-14x50cy rotor was used in this protocol. Both the rotor and the centrifuge must be at 4 °C before proceeding with the following steps.

2.5.1) Add 10 mL of an ice-cold, sterile-filtered 25% sucrose solution (prepared by dissolving 25 g of sucrose powder in 100 mL of Hank's Balanced Salt Solution) in a new 50 mL conical centrifuge tube.

2.5.2) Slowly (3 mL/min) add 24 mL of the virus filtrate (obtained from step 2.4.3.1) on the top of the sucrose layer. Take care to maintain separate layers of the virus filtrate and the sucrose solution.

NOTE: Up to 30 mL of the virus layer can be added over 10 mL of the sucrose solution.

2.5.3) Centrifuge the tube for 90 min at 22,620 × *g* at 4 °C.

2.5.4) Remove the supernatant and the sucrose layer from the 50 mL conical centrifuge tube.

NOTE: The virus pellet should be whitish. Take a 50 µL aliquot of the supernatant and a 50 µL aliquot of the sucrose layer for titration in section 3.

2.6) Re-suspend the pellet in 10% glycerol/PBS solution.

2.6.1) Add 1 mL of sterile 10% glycerol (diluted in PBS) to the 50 mL conical centrifuge tube to cover the pellet.

NOTE: The amount of the re-suspended mixture can vary (such as 0.8–1.2 mL) depending on the pellet size. A smaller volume would give a higher concentration of virus.

2.6.2) Place the 50 mL conical centrifuge tube on ice for 2–4 h. During this period, sonicate/vortex the pellet every 15 min for 30 s to help dislodge/re-suspend the pellet.

2.6.3) Collect the re-suspended pellet (1 mL of 10% glycerol/PBS + the pellet size in a total volume of ~1.3 mL) in a 2 mL microcentrifuge tube. [Optional: Add another 0.3 mL of 10% glycerol/PBS to the same 50 mL conical centrifuge tube to collect the remaining trace amount of the pellet, pipet up and down, and combine with the re-suspended pellet in step 2.6.3 to bring the total volume to ~1.6 mL.]

2.6.3.1) If the suspension in step 2.6.3 is turbid or cloudy (due to cell debris), centrifuge the tube at 500 × *g* for 10 min, transfer the supernatant to a new 2 mL microcentrifuge tube, and proceed to step 2.6.4.

2.6.4) Take a 50 µL aliquot for oHSV titration in section 3. Aliquot the rest of the solution (250 µL/aliquot) into sterile microcentrifuge tubes with screw caps (sealed well for long-term storage), snap-freeze, and store at -80 °C until use (ready for experimental studies once the oHSV titer is determined).

NOTE: All materials that come in contact with the virus must be bleached or treated with ultraviolet radiation before removal from the hood or disposal.

3. oHSV titration and plaque assay

3.1) Seed 1.7–1.8 $\times$ 10^5 Vero cells/well in a 6-well cell culture plate in Vero cell medium (DMEM with 10% IFCS; see step 1.1).

NOTE: Make sure that the cells are homogeneously distributed throughout the well; do not swirl the plate, which can cause accumulation of cells in the middle of the wells. To prevent swirling, slowly rock the plate by hand vertically, then horizontally, and then gently place the plate in the incubator.

3.2) The next day (when the cells reach 70–80% confluency), aspirate the culture medium, and add 1 mL/well of high-glucose PBS supplemented with 1% IFCS. Leave the plate in the cell culture hood until step 3.3 is complete.

3.3) Serially dilute the virus in 5 mL polypropylene tubes using PBS/1% IFCS (**Figure 3**).

NOTE: Use the 50 μL aliquot collected in step 2.6.4 for serial dilution. In the 1st tube (10^{-3} dilution), add 2 μL of the virus in 1998 μL of PBS/1%IFCS, vortex. In the 2nd tube (10^{-5} dilution), take 10 μL from the 1st tube in 990 μL of PBS/1% IFCS, vortex. In the 3rd tube (10^{-6} dilution), take 100 μL from the 2nd tube in 900 μL of PBS/1% IFCS, vortex; continue this 10-fold serial dilution until 10^{-9} dilution. See the details in **Figure 3**.

3.4) Aspirate PBS/1% IFCS, and add 0.7 mL/well of the serially diluted virus (starting from 10^{-5} to 10^{-9}) to Vero cells.

NOTE: Do not let the cells dry.

3.5) Gently rock the plate on a rocker for 5 min at room temperature (to ensure homogeneous distribution of the virus inoculum).

3.6) Incubate the plate for 1.5 h at 37 °C.

NOTE: During this incubation period, prepare 1:1000 dilution of human immunoglobulin G (IgG) in DMEM supplemented with 1% IFCS. For a 6-well plate, prepare 12.5 mL so that 2 mL/well can be used in step 3.7. Adjust the dilution to account for lot variations in the human IgG.

3.7) Remove the virus inoculum from the wells, and add 2 mL/well of 0.1% human IgG (to neutralize the oHSV in the culture medium and prevent the formation of secondary plaques) in DMEM supplemented with 1% IFCS. Incubate the plate at 37 °C for 3–4 days.

NOTE: Clear plaques usually form in 3 days.

3.8) Fixing and staining plates

3.8.1) Remove the supernatant, and fix the cells in pure methanol (1 mL/well) for 5 min. Remove the methanol, and allow the plates to air-dry.

3.8.2) Dilute Giemsa stain (1:5) with deionized water, and add 1 mL of the diluted Giemsa stain per well. Incubate the plate at room temperature for 10–15 min.

3.8.3) Remove the stain, rinse with tap water, and allow the plates to air-dry.

3.8.4) Count the plaques using a dissecting microscope.

3.8.5) Optional for oHSVs with *lacZ* expression:

3.8.5.1) Remove the supernatant, and fix the cells with cold 0.2% glutaraldehyde/2% paraformaldehyde for 5–10 min at room temperature.

3.8.5.2) Remove the fixative solution, and wash the cells 3x with PBS.

3.8.5.3) Add X-gal solution to the cells (1 mL/well), and incubate the plate at 37 °C for 2 h.

NOTE: The X-gal stain should be prepared freshly on the day of staining; long-term storage might lead to fading of color. See the preparation of X-gal solution in **Table 1**.

3.8.5.4) Remove the X-gal stain, and wash the plate with tap water for 1 min.

3.8.5.5) Counter-stain with Neutral Red solution (1 mL/well) for 2 min at room temperature.

NOTE: See preparation of Neutral Red solution in **Table 1**.

3.8.5.6) Wash the plate with tap water for 1 min; allow the plates to air-dry.

3.8.5.7) Count blue plaques using a dissecting microscope (**Figure 4**).

3.9) Calculate the titer by using formula (**2**).

Titer in pfu/mL (plaque-forming units) = Number of plaques/0.7 mL × dilution factor (**2**)

NOTE: For example, if 25 plaques are found in the 10^{-9} dilution well, the titer is $25/0.7 \times 10^9 = 35.7 \times 10^9 = 3.57 \times 10^{10}$ pfu/mL. This is the final oHSV titer of aliquots prepared in step 2.6.4.

REPRESENTATIVE RESULTS:

A brief overview of the entire protocol is depicted in **Figure 1**, which represents the critical steps involved in the growth, purification, and titration of oHSV. CPE in Vero cells can be detected as early as 4 h post-HSV infection[19]. **Figure 2** demonstrates CPE in Vero cells at three different time points following oHSV infection. The level of the CPE is increased over time. In this protocol, 90–100% CPE is usually observed within 48 h of low-MOI oHSV inoculation (which is the best time to harvest cells for purification). However, it can take up to 4 days depending on the oHSV MOI inoculated in step 1.2 and/or the oHSV's replication potential. Beyond this period, cells with CPE can be lysed, leading to the release of the virus in the supernatant. Thus, to obtain a high viral titer, it is critical to harvest CPE-affected cells when they are intact. Another important factor that contributes to the final virus titer is the number or size of the tissue culture flasks used for oHSV amplification. **Figure 3** depicts the process of serial dilution (10^{-3} to 10^{-9}) of a given virus stock (obtained in step 2.6.4) required for titer determination by the plaque assay. For oHSVs with *lacZ* expression, viral plaques can be visualized by X-gal staining (**Figure 4**). In this protocol, 20 T-150 cm^2 tissue culture flasks were used, for which 1.3–1.6 mL of oHSV stock with a titer of 1×10^{10} pfu/mL can be expected.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic presentation of major steps involved in oHSV growth, purification, and the plaque assay. Abbreviations: oHSV = oncolytic herpes simplex virus; CPE = cytopathic effect; VB = Virus Buffer; HBSS = Hank's Balanced Salt Solution; PBS = phosphate-buffered saline.

Figure 2: Cytopathic effect in Vero cells after oHSV infection. Vero cells were inoculated with oHSV coding for mCherry (shown in red fluorescence) at an MOI of 0.01 and imaged (10x magnification) at 36, 48, and 72 h post-virus infection. CPE is identified by rounding of the oHSV-infected cells (indicated by black arrows). Scale bars = 200 μm. Abbreviation: oHSV = oncolytic herpes simplex virus.

Figure 3: A serial dilution of an oHSV stock for plaque assays. See also step 3.3 of the protocol. Abbreviation: oHSV = oncolytic herpes simplex virus.

Figure 4: A representative image of X-gal-stained plaques at 72 h post-oHSV infection. Undiluted (upper well) and diluted (1:10; lower well) oHSV-infected cell culture supernatants added to Vero cells (70–80% confluent), followed by X-gal staining protocol described in section 3.8.5 (excluding counter-staining with Neutral Red). Representative images of an X-gal-stained virus plaque (from left panel) is presented in the middle (4x; scale bars = 1000 μm) and right (10x; scale bars = 200 μm) panels.

Table 1: Solution composition.

DISCUSSION:

The protocol starts with the growth of oHSV in low-passage Vero cells. The confluency of the Vero cell monolayer should be ~80% at the time of virus inoculation as overgrown cells can develop tight fibrous structures that can reduce oHSV entry into Vero cells[20]. Once 90–100% CPE is observed, the culture supernatant is removed, cells are harvested, resuspended in

VB/supernatant (see step 1.4.6), snap-frozen, and stored at -80 °C for later purification. Blaho and colleagues employed a slightly different method of harvesting and storage of infected Vero cells. For instance, the flasks containing the cells and culture media (supplemented with 1% bovine serum albumin and PBS with potassium) are initially stored at -80 °C for at least 15 min, followed by a slow warming up of the flasks at room temperature. The cells are then harvested, mixed with sterile milk, and stored at -80 °C until purification[20]. In this case, sterile milk acts as a stabilizer, and it was demonstrated that the titer of a virus stock is dramatically higher when it is stored in sterile milk/medium than in medium alone[20]. However, in another study, a direct comparison between different stabilizers (including sterile milk) used for the storage of several herpesviruses at -80 °C did not show any significant impact on the final virus titers[21]. Here, the cell pellet was re-suspended in VB constituted with Tris-buffer saline (pH 6.8) and 10% glycerol as a storage stabilizer, which usually gives a high virus titer (as outlined in step 3.10) for experimental studies.

It is critical to remove all cellular- and media-related components from the re-suspended pellet to obtain a high-quality virus stock. The removal of non-viral particles is crucial to avoid potential immune reactions during *in vivo* experiments. Several methods of virus purification have been described including centrifugation[22], different gradient methods[23], filtration[24], and affinity chromatography[25]. Although this protocol is based on high-speed centrifugation using a sucrose-gradient method, a variety of other gradient methods have been used by others, such as iodixanol[11,26], Percoll[27], and Ficoll-Nycodenz[23]. These gradient methods separate the virus by density and require isolation of the band from the gradient, instead of the sucrose cushion where the virus is pelleted. The sucrose-gradient method offers a gentle approach to separate viral particles because it minimizes the risk of disrupting viral envelope proteins while retaining viral infectivity. Despite these advantages, the high osmolarity of the concentrated sucrose solution might dehydrate the viral particles; therefore, the iodixanol gradient method was developed to overcome this drawback. However, the iodixanol gradient method requires ultracentrifuge and collection of the virion band. Other factors that need to be considered during oHSV purification are speed and time of centrifugation and choice of the virus buffer used for long-term storage. This protocol has the limitation that the purity of oHSV is not confirmed; however, a high number of functional virus particles were found in a given purified oHSV stock by titration on Vero cells (see section 3).

oHSV forms plaques on Vero cells (**Figure 4**). The viral plaques can be identified by Giemsa staining, which is an easy and convenient method. Giemsa stains Vero cells, leaving the viral plaques transparent or empty that can be easily visualized (naked eye) and counted using a dissecting microscope. While overlaying the media with agarose or methylcellulose is commonly used during plaque formation (in step 3.7) to prevent the spread of the virus and secondary infections and plaque tails[28], the use of human IgG to neutralize oHSV in the culture supernatant is easier and more convenient. For oHSVs expressing *lacZ*, plaques can be visualized by X-gal staining (**Figure 4**), while fluorescent microscopy is used for fluorescent protein (i.e., green fluorescent protein)-expressing oHSVs[18]. Additional assays to detect oHSV-infected cells include immuno-histochemical or -fluorescence with oHSV-specific antibodies[29] or laser-based scanning of near-infrared fluorophore-conjugated oHSV-specific antibodies[30].

There are critical measures that must be followed to achieve a good virus stock such as maintaining sterility to prevent microbial (bacteria, yeast, or mold) contamination and healthy Vero cells. As the envelope of oHSV is extremely thermosensitive[20], the oHSV stock should be handled in a cryoprotectant such as 10% glycerol. Overall, this protocol can be easily employed and practiced in a laboratory setting, but may not be useful for large-scale virus production.

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DISCLOSURES:

SDR is a co-inventor on patents relating to oncolytic herpes simplex viruses, owned and managed by Georgetown University and Massachusetts General Hospital, which have received royalties from Amgen and ActiVec Inc. and is on the Scientific Advisory Board of EG 427. The other authors have nothing to disclose.

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Figure 1

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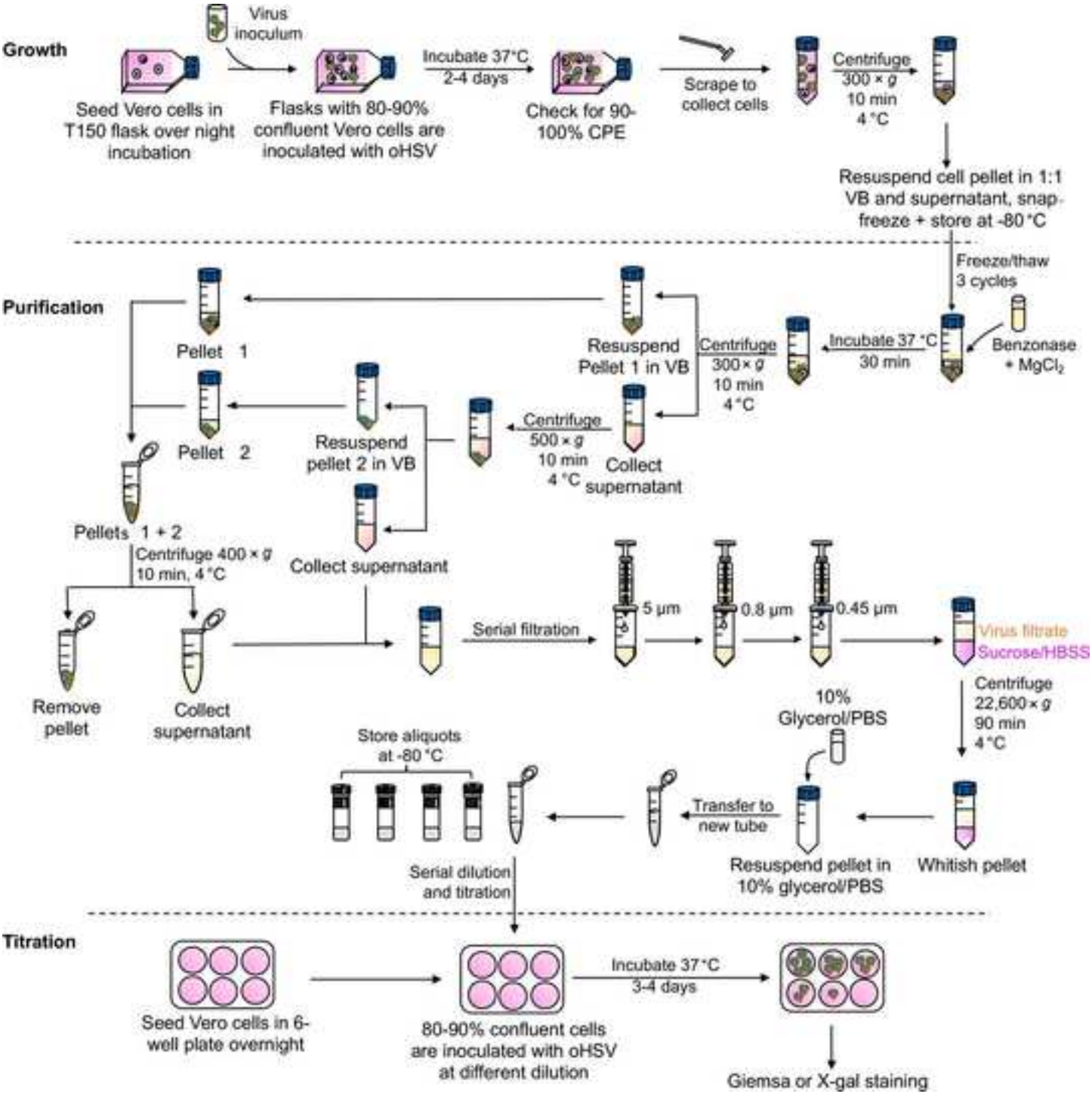

Figure 2

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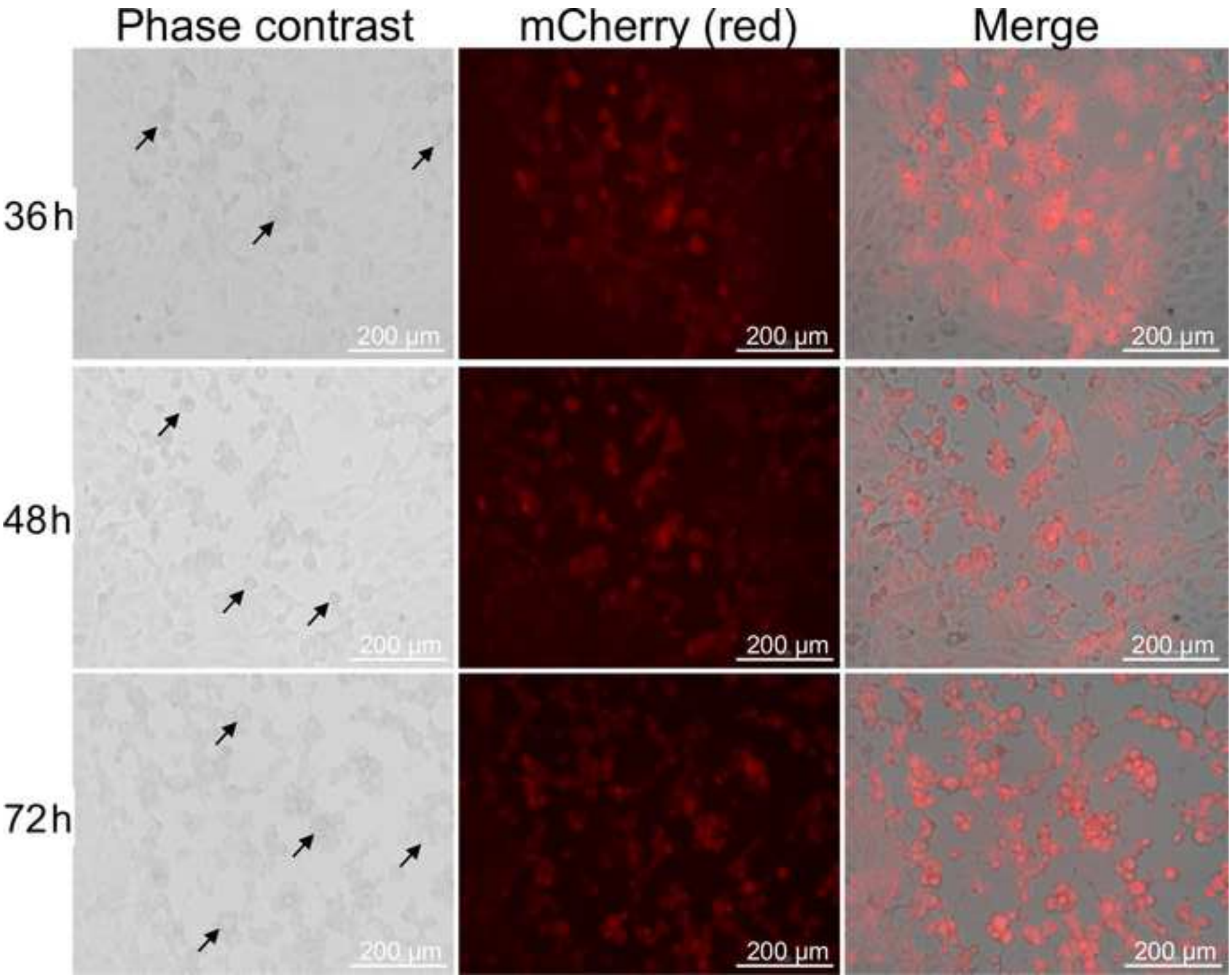


Figure 3

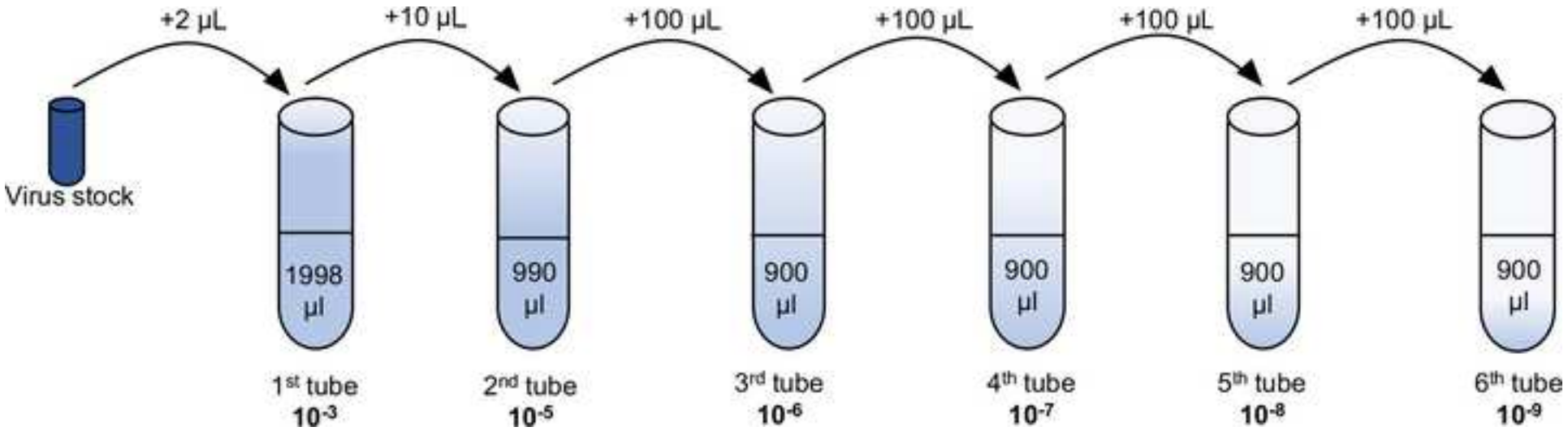
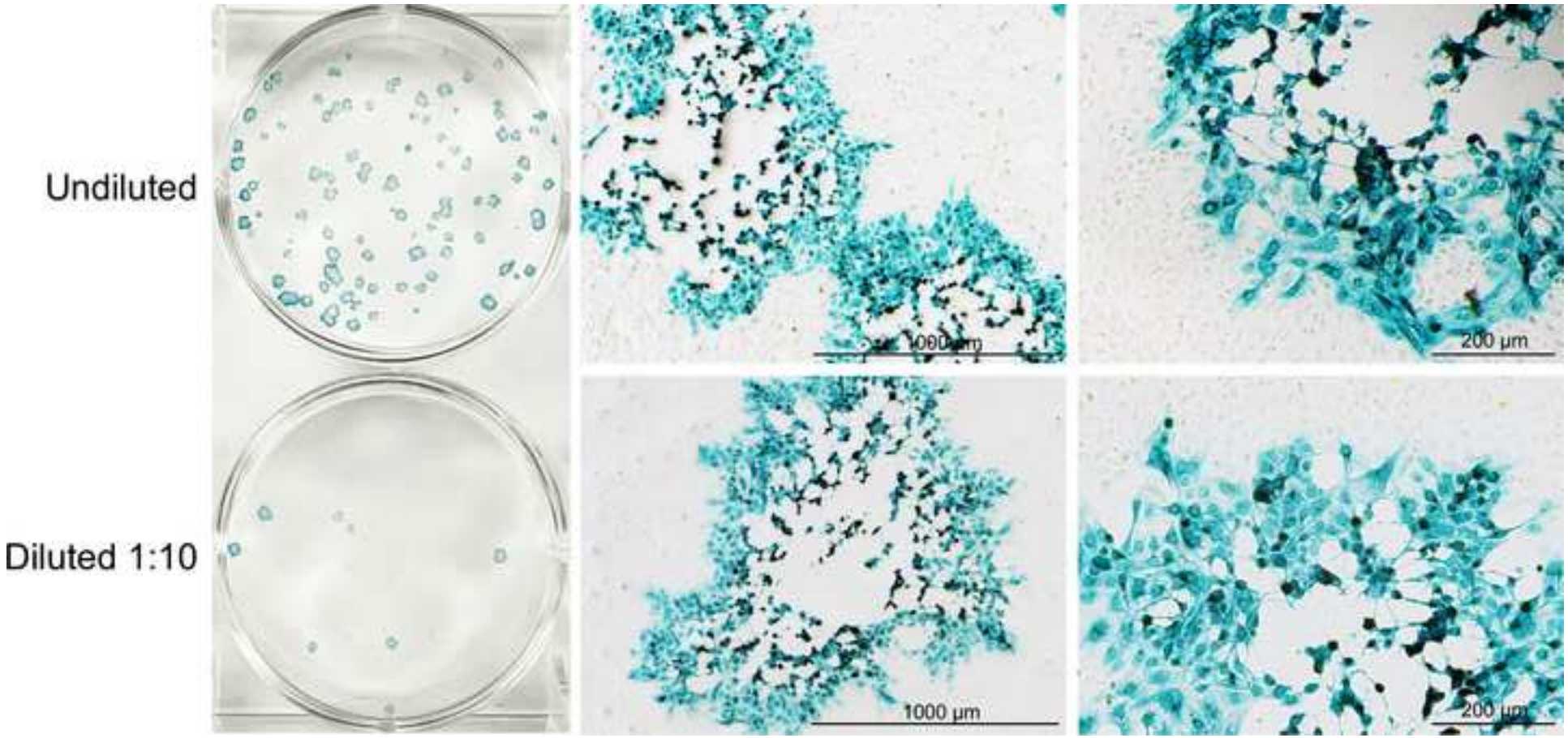


Figure 4



Solution Composition

Preparation of virus buffer	**Quantity**
1 M Sodium chloride	15 mL
1 M Tris-hydrochloride	3 mL
Purified water	132 mL
	adjust pH to 6.8
Preparation of X-gal solution (~6.5 mL for one 6-well plate)	
250 mM potassium ferricyanide	130 µL
250 mM potassium ferrocyanide	130 µL
1 M Magnesium chloride	13 µL
X-gal pre-dissolved (20 mg/mL) in dimethyl sulfoxide (DMSO)	162.5 µL
PBS	6064.5 µL
Preparation of Neutral Red solution for one 6-well plate	
Neutral Red solution	100 µL
Methanol	1 mL
Purified water	7 mL

Table of Materials

Click here to access/download;Table of Materials;Table_Table of Materials.xlsx

Name of Material/Equipment	Company	Catalogue Number
1.7 mL centrifuge tubes	Sigma	CLS3620
15 mL polypropylene centrifuge tubes	Falcon	352097
5 mL polypropylene tubes	Falcon	352063
50 mL polypropylene centrifuge tubes	Falcon	352098
6-well cell culture plates	Falcon	353046
Benzonase Nuclease	Sigma	E8263-25KU
Cell scraper	Fisher Scientific	179693
Dimethyl sulfoxide	Sigma	D2650-100ML
Dulbecco’s Modified Eagle Medium	Corning	MT-10-013-CV
Dulbecco’s Phosphate Buffered Saline	Corning	MT-21-031-CV
Fetal Bovine Serum	Hyclone	SH3007003
Giemsa Stain	Sigma	G3032
Glutaraldehyde	Fisher Scientific	50-262-23
Glycerol	Sigma	G5516
Hank's Balanced Salt Solution (HBSS)	Corning	MT-21-021-CV
High-Glucose Dulbecco’s Phosphate-buffered Saline	Sigma	D4031
Human immune globulin	Gamastan	NDC 13533-335-12
Magnesium chloride	Fisher Chemical	M33-500
Media Sterilization filter, 250 mL	Nalgene, Fisher Scientific	09-740-25E
Media Sterilization filter, 500 mL	Nalgene, Fisher Scientific	09-740-25C
Neutral Red solution	Sigma	N4638
Paraformaldehyde	Fisher scientific	15710S
Plate rocker	Fisher	88861043
Potassium Ferricyanide	Sigma	P8131
Potassium Ferrocyanide	Sigma	P9387
Sodium chloride	Fisher Chemical	S271-3
Sorvall ST 16R Centrifuge	ThermoFisher Scientific	75004381
Sorvall ST 21R Centrifuge	ThermoFisher Scientific	75002446
Sterile Microcentrifuge Tubes with Screw Caps	Fisher Scientific	02-681-371
Sucrose	Fisher Scientific	BP220-1
Syringe Filter, 0.45 PVDF	MilliporeSigma	SLHV033RS
Syringe Filter, 0.8 MCE	MilliporeSigma	SLAA033SS
Syringe filter, 5 µm PVDF	MilliporeSigma	SLSV025LS
T150 culture flask	Falcon	355001
Tris-HCl	MP Biomedicals LLC	816116
Ultrasonic water bath	Branson	CPX-952-116R
X-gal	Corning	46-101-RF

We sincerely thank and appreciate the editor and the reviewers for evaluating this manuscript. Please find below point-by-point responses to each comment (modifications in the manuscript are highlighted by track changes):

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have proofread the manuscript. All abbreviations are defined at first use.

2. Please increase the word count of the abstract ("long abstract") to be between 150 and 300 words.

Response: We have increased the word count of the abstract to 180 words.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Response: References in the text now appear as numbered superscripts, and before punctuation.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Falcon; Durapore; Sorvall F13-14x50cy Rotor etc.

Response: Commercial language has been removed from the manuscript and all commercial products are referenced in Table 1.

5. As this is a virological study, please specify if ethical approval is required and include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the safety guidelines of your institution.

Response: This protocol does not require ethical approval, but typically requires Institutional Biosafety Committee approval and adherence to safety guidelines as indicated in the manuscript in Step 1.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: This protocol was written in the imperative tense during our initial submission. Safety procedures were included in Step 1.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have added few more detailed information in the manuscript (highlighted by track changes).

8. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Response: Texts (up to 3 pages) that need to be filmed are highlighted in yellow.

9. Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Response: We have discussed all figures in the Results section. We have also referenced figures showing the experimental setup in the protocol.
Yes, one paragraph (which refers all of the figures) of text to explain the Representative Results were added during our initial submission.

10. Please consider providing solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.
Response: We have added a new Table 2 for solution composition.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
Response: Scale bars are added in all microscopic images in Fig. 2 and all microscopic images in Figure 4 (two images in the middle panel and two images in the right panel). Two images in the left panel in the Figure 4 are not microscopic images.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ital). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.
Response: We have added JoVE reference style in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript describes in details an user-friendly protocol to produce, purify and titrate herpes viruses. It is well written and in my opinion it is of interest.
The manuscript needs few minor revision for publication.
Response: Thank you.

Major Concerns:
Major revisions are not required
Response: Thank you.

Minor Concerns:
-The authors describe the production of oHSV in Vero cells. Recently a genetic engineering approach has been applied to GMP-grade cell lines to improve the yield of vp. These recent publications could be of interest to scale up viral production and/or to produce low yield viruses. I suggest to include some references [doi: 10.3390/cancers12113407; doi: 10.3390/ijms22020477].
Response: We have added a sentence citing these two references (lines 87-88).

-Step 2.1 and 2.3.5: a note to suggest a set up of sonication protocol depending on available instrument could be useful.
Response: A set-up of sonication protocol has been added as notes in these two steps.

-I suggest to collect aliquots during critical steps of purification to identify those step responsible of potential loss in vp.
Response: We have added this as notes.

-I suggest to insert a note to explain that additional sonication step before and/or filtration (2.4) could help in case of viral aggregation.
Response: An additional sonication step has been incorporated before step 2.4.

Reviewer #2:
Manuscript Summary:
This protocol is well written with sufficient details for others to follow for their own oHSV production, purification, and titration.
Response: Thank you.

Major Concerns:
None.
Response: Thank you.

Minor Concerns:
For the descriptions on the following two sections (1.4.1) Collect culture supernatant (~20 ml) from each flask (leave ~5 ml in each flask) in 50 ml Falcon tubes or media container, then bang flasks to dislodge cells. 1.4.2) Use a cell scraper to gently scrape cells that remain attached to bottom of flasks after banging), banging may not be necessary as cell scraper should be able to efficiently and completely remove cells from flasks. Also banging may not be a safe procedure for handling infectious container.
Response: Thank you. We have removed ‘banging’ from our protocol.