Production and purification of high-titer OrfV for preclinical studies in vaccinology and cancer therapy

Jacob P. van Vloten,[1] Jessica A. Minott,[1] Thomas M. McAusland,[1] Joelle C. Ingrao,[1] Lisa A. Santry,[1] Grant McFadden,[2] James J. Petrik,[3] Byram W. Bridle,[1,4] and Sarah K. Wootton[1,4]

[1]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada; [2]The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA; [3]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada

Poxviruses have been used extensively as vaccine vectors for human and veterinary medicine and have recently entered the clinical realm as immunotherapies for cancer. We present a comprehensive method for producing high-quality lots of the poxvirus *Parapoxvirus ovis* (OrfV) for use in preclinical models of vaccinology and cancer therapy. OrfV is produced using a permissive sheep skin-derived cell line and is released from infected cells by repeated freeze-thaw combined with sonication. We present two methods for isolation and purification of bulk virus. Isolated virus is concentrated to high titer using polyethylene glycol to produce the final *in vivo*-grade product. We also describe methods for quantifying OrfV infectious virions and determining genomic copy number to evaluate virus stocks. The methods herein will provide researchers with the ability to produce high-quality, high-titer OrfV for use in preclinical studies, and support the translation of OrfV-derived technologies into the clinic.

BACKGROUND

The application of poxviruses in human and animal health has expanded dramatically since Edward Jenner first used cowpox as a vaccine against smallpox. The advent of recombinant DNA technology enabled the expansion of poxvirus vaccines to target diverse human and veterinary pathogens, with vaccinia virus strains being the most tested platform.[1] Recently, a new application for poxviruses as oncolytic viruses (OV) has emerged, with *Parapoxvirus ovis* (OrfV) among them.[2,3] OVs are multi-mechanistic immunotherapy tools that fight cancer by selectively targeting and killing tumor cells and by activating a host immune response against the tumor.[4] Since the arrival of T-Vec, the first OV to be Food and Drug Administration approved for treating solid tumors,[5] research has expanded to take advantage of the unique biology of different viral backbones and develop improved immunotherapies.

OrfV is a highly immunogenic poxvirus that targets ungulates as a primary host.[6] Similar to other poxviruses, it has a large double-stranded DNA genome with a central region of conserved genes required for viral genome replication and morphogenesis, which are flanked by regions that encode accessory virulence and immune-modulatory genes.[7] These flanking regions are of considerable interest, as they provide a location for targeted insertion of transgenes for vaccine and cancer therapy and serve as targets for basic knockout studies to modulate the immunogenicity of the viral backbone. OrfV is a known OV capable of infecting multiple types of human and murine cancer cells both *in vitro* and *in vivo*, leading to drastic reductions in tumor burden in preclinical murine models of metastatic melanoma and colon carcinoma.[2]

The OrfV genetic system is amenable to the generation of transgenic viruses, akin to other poxviruses. Recombinant viruses can be generated through homologous recombination between a transfer plasmid and the parental replicating virus in permissive cells.[8] The large genome size and inclusion of non-essential genes provides multiple targets for insertion of therapeutic transgenes and the potential to incorporate multiple transgenes for multivalent vaccine strategies.[9] Recombinant OrfV can and has been used as a vaccine vector targeting a number of pathogens, including rabies, influenza, and herpesvirus,[10–12] demonstrating the value of OrfV as a viral vector system.

Further expanding OrfV-based vaccine and OV technologies will require accessible and reliable methods for producing high-titer virus. OrfV can be grown in cell culture, but the production of high-titer ultrapure virus has been historically challenging and has

Correspondence: Sarah K. Wootton, PhD, Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada.
E-mail: kwootton@uoguelph.ca

restrained *in vivo* preclinical testing. The purity of OrfV propagations is of significant importance because contaminants can alter immunological outcomes, which can make rational design of OVs as immunotherapies challenging. Additionally, both low-titer and low-purity virus prevents systemic administration of OVs, which is critical for targeting metastatic disease.[13] Unfortunately, the literature at the time of this writing is sparse with respect to OrfV production. Therefore, we present a comprehensive method for producing, purifying, quality testing, and titrating OrfV for use in preclinical murine models of vaccination and cancer therapy. This information will endow researchers with the ability to translate OrfV-based technologies from the bench to the bedside.

MATERIALS
Reagents
Cell culture:

- Sheep skin fibroblasts[14]—the authors can provide these cells upon request
- OA3.T (ATCC CRL-6546)
- Complete Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, Cat. #SH30022.01)
  - 10% fetal bovine serum (VWR, PA, USA, Cat. #97068-085)
  - Penicillin-streptomycin cocktail (Fisher Scientific, Cat. #SV30010)
  - 1× non-essential amino acids (Fisher Scientific, Cat. #11140050)
- 0.25% trypsin-ethylene-diamine-tetra-acetic acid (EDTA) (Corning, NY, USA, Cat. #25-052-CI)
- Phosphate-buffered saline (Fisher Scientific, Cat. #SH30256.01)
- MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Basel Switzerland, Cat. # LT07-703)
- Cell culture plates including 150-mm 6-well and 96-well flat-bottomed plates (Corning, NY, USA)

Virus harvest and purification:

- Disposable cell scraper (Fisher Scientific, Cat. #179693PK)
- 50-mL conical tubes (Fisher Scientific, Cat. # 14-432-22)
- 0.3 M NaOH
- 1 M NaOH
- Ultrapure H_2O for buffer preparation
- 36% sucrose-PBS
- 5% sucrose-PBS
- Iodixanol OptiPrep Density Gradient Medium (Sigma-Aldrich, Cat. # D1556)
- 38-mL ultracentrifuge tubes (Beckman Coulter, Cat. #355631 or 344058)
- 250-mL centrifuge bottles, polypropylene (Beckman Coulter, Cat. # 355627), for Type 19 fixed-angle aluminum rotor
- Ultra-clear ultracentrifuge tubes, 13 mL (Beckman Coulter, Cat. #344059)
- Polyethylene glycol (PEG), molecular weight 20,000 g/mol (Sigma-Aldrich, Cat. #81300)
- 3-mL and 5-mL syringes

- 18-gauge sharp Luer-Lok needle (Fisher Scientific, Cat. #14-826-5G)
- 18-gauge blunt-tip Luer-Lok needle (Becton Dickinson, Cat. # 305181)
- Pierce Universal Nuclease for Cell Lysis (Fisher, Cat. #PI88702)

Virus titration

- Multi-channel 10–50-µL and 30–300-µL pipettes
- UltraPure low-melting-point agarose (Fisher Scientific, Cat. #16520050)
- 2× MEM (Temin's modification), no phenol red (Fisher Scientific, Cat. #11935046)
- Proteinase K (Ambion, Cat. #AM2546)
- Phenol/chloroform/isoamyl alcohol (Fisher Scientific, Cat. #BP17521-400)

Equipment
- Biological safety cabinet—all steps which involve virus manipulation must be conducted in the biological safety cabinet
- Clinical and high-speed centrifuges (must accommodate 50-mL conical tubes)
- Ultracentrifuge with swinging bucket SW 41 Ti rotor (methods 1 and 2), SW32 Ti and Type 19 fixed-angle aluminum rotor (method 2 only)
- Probe sonicator (Fisher Scientific, Model FB120)
- Supracap 50 Depth Filter Pall V100P (Pall Laboratory, Cat. #SC050V100)
- Omega Membrane LV Centramate Cassette, 300 kDa (Pall Laboratory, Cat. #OS300T12)
- Slide-A-Lyzer dialysis cassette, 10,000 kDa molecular weight cutoff (Fisher Scientific, Cat. #66380)
- Centramate Cassette Holder (Pall Laboratory, Cat. #CM018V)
- Tubing screw clamp (Pall Laboratory, Cat. #88216)
- Utility pressure gauge (×2) (Cole-Palmer, Cat. #68355-06)
- Male and female Luer-Lok with 1/8 in national pipe thread (NPT; Cole-Palmer, Cat. #41507-44 and -46)
- Female threaded tee fittings, nylon, 1/8 in NPT(F) (Cole-Palmer, Cat. #06349-50)
- Masterflex C-Flex ULTRA tubing, L/S 16, 25 ft (Cole-Palmer, Cat. #06434-16)
- Masterflex L/S Easy-Load Head for Precision Tubing, PARA, SS Rotor (Cole-Palmer, Cat. #UZ-07514-10)
- Masterflex L/S variable-speed drive with remote I/O; 100 rpm (Cole-Palmer, Cat. #UZ-07528-30) or equivalent
- Thermocycler
- Microscope capable of brightfield and fluorescence (optional)

PROCEDURE
Production of high-titer OrfV
- For method overview, refer to Figure 1.

Cell preparation
1. Thaw sheep skin fibroblasts (SSFs) from liquid nitrogen, remove dimethyl sulfoxide, and recover in complete DMEM (cDMEM)
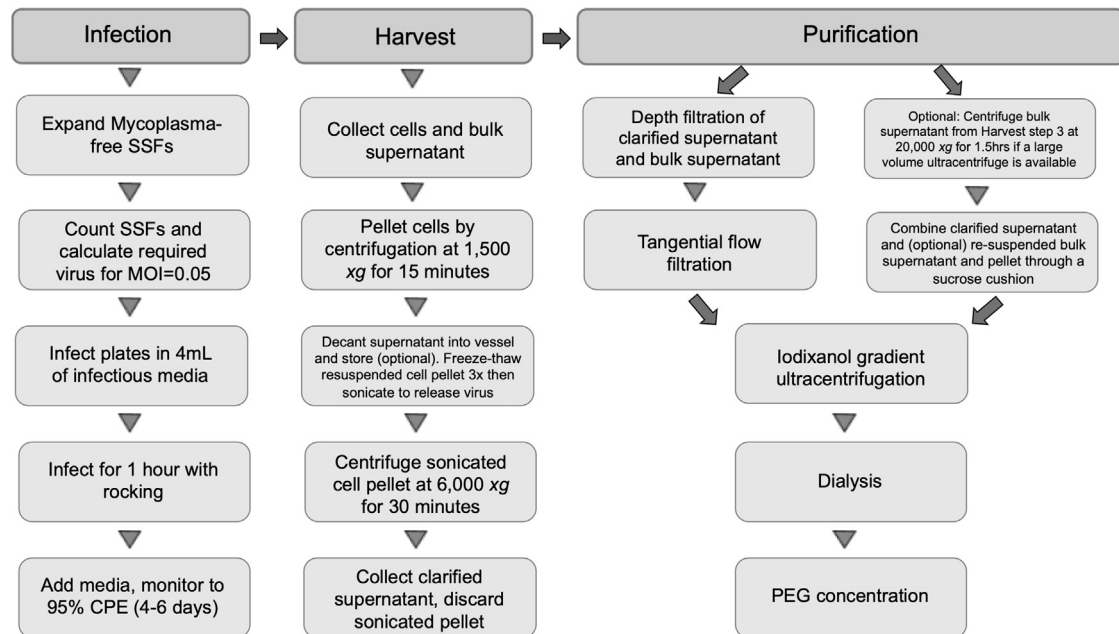
Figure 1. Workflow of the experimental procedure for infection, harvesting, and purifying OrfV

in a plate or flask. Incubate at 37°C, 5% CO_2, and 21% O_2 until cells reach 85%–95% confluency.

- This usually requires 3–5 days.
- We recommend using cells at a low passage number, as cell quality decreases as passage number increases.
- All cells in these experiments were confirmed mycoplasma free using the MycoAlert PLUS Mycoplasma Detection Kit.

2. Seed SSF cells from the recovery plate into larger plates. In this protocol we expand into 3×150-mm plates by seeding 1.5×10^6 cells per plate, but flasks can be used. Incubate until cells reach 85%–95% confluency, which typically takes 3–5 days.

- We recommend seeding SSFs in 20 mL of medium when using 150-mm plates to ensure even coverage of cells and to reduce risk of evaporation.

3. Continue to amplify SSFs to desired propagation size. For a typical preclinical batch, we recommend 50×150-mm plates. To accomplish this, expand the three plates from step 4 to six plates, and then to 15 and finally up to 50 plates, waiting for 85%–95% confluency before moving to the next amplification. SSFs should not be seeded at lower than 1.5×10^6 for 150-mm plates, as lower initial densities reduce growth time.

- Seed an additional three 150-mm plates for each batch produced. One plate is used to count cell number to calculate the amount of infectious virus needed, and the other two are further passaged for virus quantification (see below).

Virus infection

1. Begin virus infection when plates reach 90%–95% confluency.
2. Detach cells from only one of the extra plates by removing medium, washing with PBS, and adding 4 mL of trypsin-EDTA.

a. Neutralize trypsin-EDTA with 6 mL of complete medium for a total of 10 mL.

b. Count cells using a hemocytometer and calculate the total number of cells per 150-mm plate.

c. Multiply the total number of cells to be seeded per plate (1×10^7 cells) by the total number of plates, to obtain the total number of cells for infection.

d. Example: 1×10^7 cells/plate $\times$ 50 plates = 5.0×10^8 cells total.

e. Use a titrated virus stock to calculate the total volume of virus required to infect cells with a multiplicity of infection (MOI) of 0.05. For example:

(Total no. of cells $\times$ MOI)/Stock virus conc.

$= (5.0 \times 10^8 \text{ cells} \times 0.05)/ 1 \times 10^9 \text{ PFU/mL}$

$= 2.5 \times 10^6/1 \times 10^9$

$= 0.0025 \text{ mL or } 2.5 \text{ μL total for propagation}$

3. Add required virus to basal DMEM, for a total volume of 4 mL per plate (e.g., 200 mL for a 50-plate propagation) in 50-mL conical tubes.
4. Remove medium from 150-mm plates of SSF cells and replace with 4 mL of basal DMEM containing virus.
5. Incubate plates at room temperature for 30 min on a rocker to distribute the virus.

- Alternatively, incubate at 37°C, 5% CO_2, and 21% O_2 for 30 min, rocking plates every 10 min by hand.
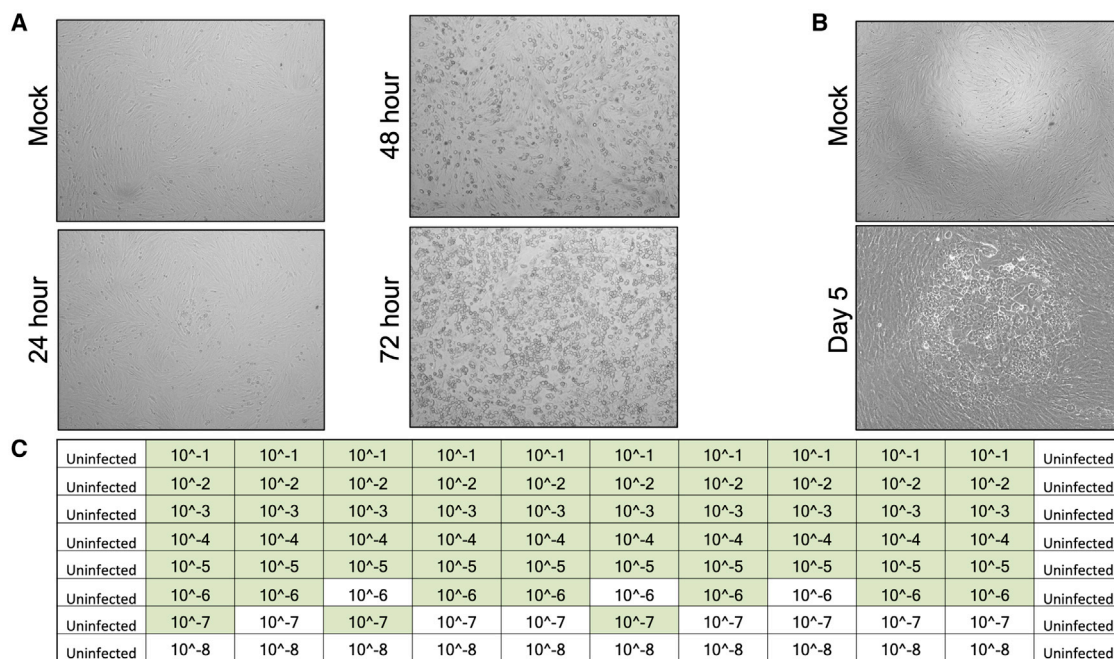
Figure 2. Titering OrfV by TCID$_{50}$

(A) Cytopathic effect generated by OrfV infection of SSFs over a 72-h period. SSFs were infected with an MOI = 0.1 and imaged every 24 h by brightfield microscopy. (B) Typical OrfV plaque phenotype on SSF cells. SSF cells were infected with serial dilutions of a known OrfV stock, overlaid with 1% agarose + complete MEM, and monitored for 5 days before visualization by brightfield microscopy. (C) Typical plate setup for a TCID$_{50}$ titration assay. Green wells depict positive scoring of infected cells, with this example yielding a titer of 1.77×10^8 PFU.

6. Add an additional 12 mL of cDMEM to each plate, for a total of 16 mL per plate.

7. Incubate for 4–6 days, or until 90%–100% of SSF cells are showing signs of cytopathic effect (CPE; Figure 2A).
   - The duration of infection will depend on multiple factors including the health of the SSF cells and the accuracy of the titer of the virus used for the infection. We recommend checking the progress of infection daily.

Virus harvest

1. This process enables recovery of virus from both the supernatant and the cell pellet to maximize propagation yields.

2. Once CPE is observed in 90%–100% of SSF cells, detach cells using a disposable cell scraper.

3. Collect cells and medium in 50-mL conical tubes and incubate on ice.

4. Centrifuge tubes at 1,500 × g for 15 min at 4°C to pellet cell debris.

5. Decant supernatant into a sterile 1-L vessel, seal with parafilm, and store at 4°C.
   - A significant amount of virus will be found secreted in the supernatant, which can be purified using the methods described herein.
   - OrfV can be stored for up to a month at 4°C without significant loss of titer (data not shown).

6. Resuspend cell pellets in 5 mL of clarified virus containing supernatant and pool in 50-mL conical tubes. Fill tubes to a maximum of 45 mL to accommodate expansion during freezing. Store tubes at −80°C.
   a. Tubes can be stored long-term at this step.

7. Freeze-thaw of cell pellet suspension at −80°C should be performed three times to disrupt cell membranes and release intracellular virus.

8. After three freeze-thaw cycles, sonicate the sample for six cycles of 10 s on at an amplitude of 50% followed by 10 s off.
   a. Conical tubes should be placed on ice during sonication to prevent excessive heating of samples.

9. Centrifuge the sonicated cell pellet suspension at 6,000 × g for 30 min at 4°C to separate the virus from cell debris.
   a. This is a critical step, as slower centrifugation speeds fail to remove cell debris and result in clogging of depth filter and tangential flow filtration membranes, resulting in extremely long purification times. Additionally, cell debris not removed by high-speed centrifugation can be carried through to iodixanol gradient separation and can retain virus particles in the wrong bands.

10. Decant supernatant (containing virus) into a sterile vessel. Take care not to dislodge the cell pellet. Discard remaining cell pellets.

11. Add 25 U of nuclease/mL of supernatant (e.g., add 1,250 U of nuclease [Fisher, Cat. #PI88702]) to 50 mL. Incubate at room temperature for 30 min and place on ice.
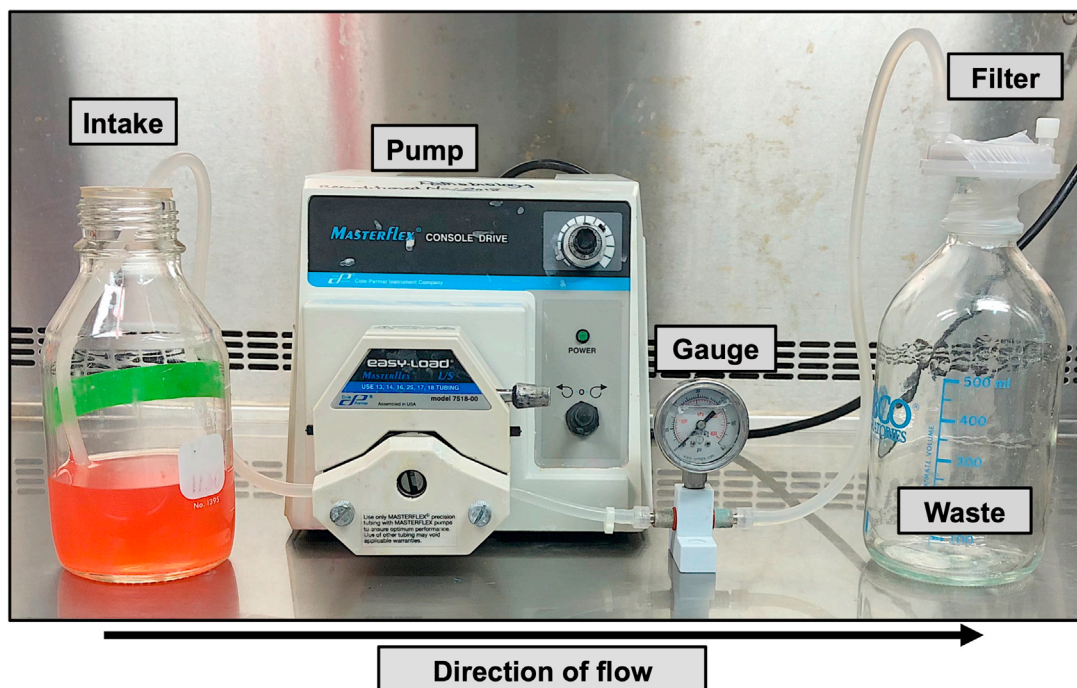
Figure 3. A typical setup for the depth filtration process
Critical components including the intake, pump, pressure gauge, filter, and waste container are labeled.

12. Proceed to method 1 or method 2 for virus concentration.

Method 1: Virus concentration by two-step filtration
Depth filtration
All buffers should be autoclaved or passed through a 0.22-μm filter prior to use. All filtration steps should be performed in a biological safety cabinet.

1. Set up the peristaltic pump and tubing in a biological safety cabinet as per Figure 3. Before attaching the depth filter, sterilize the tubing by running 50 mL of 1 M NaOH-PBS followed by 200 mL of Milli-Q H$_2$O.
2. Attach the depth filter and remove the vent cap. Be sure to include a pressure gauge after the pump outflow but before the depth filter to measure the pressure being applied to the virus going through the depth filter cassette. Slowly pump another 100 mL of PBS through and watch for the release of all air from the depth filter. Once all the air is removed and PBS flows through the top, cap the vent and allow the PBS to continue to flow through.
   - The depth filter can be angled to position the air release at the top to ensure removal of all air from the filter.
   - Stop pump when 5 mL of PBS remains; introducing minor air bubbles is acceptable. Avoid introducing excessive amounts of air or venting will have to be repeated.

3. Before running virus through, place a new sterile bottle at the flow-through to collect the virus as it exits the depth filter. Mix virus supernatants from virus harvest steps 5 and 10 and begin pumping the virus supernatant through the depth filter.
   - Use the pressure gauge to monitor the pressure as virus supernatant moves through the filter. We have not seen loss of virus at pressures up to 15 psi, but the flow rate can dramatically decline as the filter fills with debris. We recommend replacing the filter halfway through to maintain pressures lower than 15 psi and a reasonable flow rate.
4. Chase the final volumes of virus supernatant with 50 mL of PBS without allowing air bubbles to enter the system. This will flush any remaining virus out of the depth filter. Keep virus on ice while setting up the tangential flow filtration equipment.

Pause point: If tangential flow filtration cannot be initiated immediately, store filtered virus at 4°C overnight. Do not freeze filtered virus.

5. Discard the depth filter. Run 50 mL of 1 M NaOH through the tubing to sterilize. Disassemble the tubing and store in 0.5 M NaOH.

Tangential flow filtration (TFF)
Setup and cleaning. As above, all steps should be performed in a biological safety cabinet.

1. Remove the 300 kDa Centramate cassette from the 0.5 M NaOH storage solution and rinse with Milli-Q H$_2$O. Sandwiching the
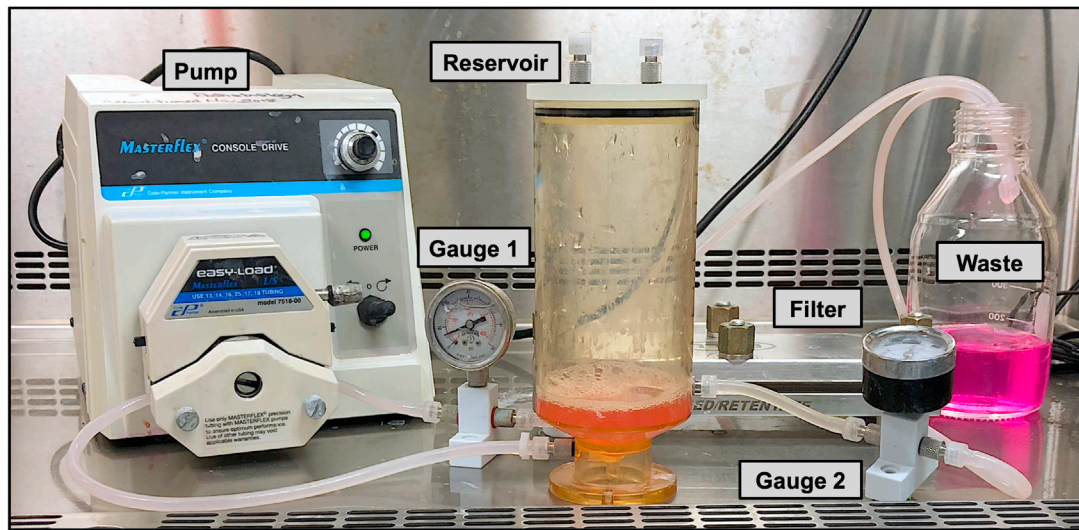
Figure 4. A typical setup for the tangential flow filtration process
Critical components including the pump, reservoirs, pressure gauges, filter, and waste container are labeled.

cassette between two plastic gaskets, set up the Centramate cassette holder following the diagram in Figure 4. Using a torque wrench set to 6 newton-meters, tighten the nut half a rotation and move to the opposite nut—this ensures equal distribution of weight—and then to the nut beside and its opposite. Repeat until all bolts have been tightened fully until the torque wrench clicks, identifying that each bolt is tightened to the torque and that the seal around the cassette is even.

2. Connect the tubing to the peristaltic pump, cassette, and reservoir as shown in Figure 4. Ensure that the cassette is installed so that the filtrate and retentate ports are properly oriented.

3. Install a pressure gauge between the peristaltic pump output and the cassette to monitor the pressure going into the cassette.

4. Sterilize the tubing and the cassette by adding 300 mL of 0.3 M NaOH into the reservoir. Position the tubing running from the filtrate and the retentate so that it goes directly into the waste. Run this for the first 100 mL and then switch tubing so that the filtrate and retentate circulates back into the reservoir for 20 min, then remove the filtrate lines from the reservoir and pump the rest of the 0.3 M NaOH into the waste. This removes debris that may have accumulated in the cassette during storage.

5. Remove any residual 0.3 M NaOH from the system by running 200 mL of ultrapure H_2O from the reservoir, through the cassette, and into the waste. Make sure to properly rinse the entirety of the reservoir, as any remaining NaOH can neutralize virus particles. Repeat this process twice.

6. Stop the pump when 5 mL of the last wash with ultrapure H_2O remains in the reservoir. Add 50 mL of sterile PBS and pump through the system until the reservoir contains only 5 mL of PBS. Remove the waste container and replace with a new sterile waste container.

Virus concentration.

1. Add virus to be purified into the reservoir, making sure retentate lines go back to the reservoir and filtrate lines into a new sterile waste container to avoid inactivation of any virus that might end up in the waste. Begin pump and carefully monitor the pressure gauge, and adjust the flow rate so that pressure is always below 10 psi. At this step, virus will be retained in the system.
   - As virus is concentrated, the pressure of the cassette increases. It is important to regularly check the pressure gauge and adjust the flow rate to keep the pressure below 10 psi.
   - We recommend sampling the waste during virus concentration to ensure that no virus is being lost. Ensure that sampled waste does not contain NaOH-PBS, as this can neutralize the virus.

2. Run virus through the system until only 5–10 mL remains in the reservoir.

Buffer exchange and elution.

3. Exchange the buffer from concentrated virus supernatant to 5% sucrose-PBS for subsequent virus elution. Elution in 5% sucrose-PBS limits the aggregation of virus as it leaves the membrane.

4. Add 50 mL of 5% sucrose-PBS to the reservoir and run until only 5 mL remains in the reservoir.

5. Stop the pump and close the filtrate lines with caps to prevent the flow of liquid into the waste. Detach the retentate line circulating from the cassette back into the reservoir, from the reservoir itself and place in a 50-mL conical tube.

6. Turn on the pump and collect elution 1 in the 50-mL conical tube. Stop the pump when elution 1 is finished.

7. Repeat the elution process another two times by adding 5 mL of 5% sucrose-PBS to the reservoir to produce elution 2 and elution 3. The approximate volume of elutions 1, 2, and 3 are 10 mL, 5 mL, and 5 mL, respectively.

8. Store elutions and waste (for future testing) at 4°C or proceed directly to gradient purification and final concentration.

Cleaning TFF equipment.
1. Move the retentate and filtrate lines to a new waste container. Add 250 mL of 0.3 M NaOH-PBS to the reservoir, and pump 100 mL to remove any debris in the system.
2. Once 100 mL has entered the waste, attach the retentate and filtrate lines to the reservoir to make a closed system and continue to pump for at least 1 h to clean the tubing and cassette.

Pause point: The cleaning process can be extended overnight if the cassette has been used multiple times. Keep in mind that long exposure to high NaOH (>0.5 M) can degrade the filter.

3. Remove the TFF cassette from the Centramate and store the cassette submersed in 0.3 M NaOH at 4°C. Wipe the Centramate down with Milli-Q H_2O to remove excess NaOH and prevent corroding the metal.

Method 2: Virus concentration by two-step density separation
Sucrose cushion purification
1. Distribute the virus containing supernatant that was harvested from the virus-infected cells in step 5 of the virus harvest procedure into 250-mL centrifuge bottles, ensuring each bottle is balanced to 0.01 g.
2. Centrifuge at 20,000 × g for 1.5 h at 4°C to pellet OrfV. We use an ultracentrifuge with a fixed-angle Type 19 rotor, but any high-speed centrifuge capable of achieving 20,000 × g can be used.
   - If a high-speed centrifuge is unavailable, this step can be skipped and the virus containing supernatant that was harvested from the virus-infected cells can instead be used to balance the sucrose cushion in step 5 below, and discarded.
3. Decant the supernatant into a vessel and resuspend the virus pellet in a total of 25 mL of the supernatant. Discard the remaining supernatant.
4. Place 10 mL of 36% sucrose-PBS into autoclaved 38-mL ultracentrifuge tubes.
5. Carefully overlay 25 mL of the cleared lysate from step 11 of the virus harvest on top of the sucrose cushion. Repeat this step until the entire volume of cleared lysate has been loaded on top of a sucrose cushion.
6. Repeat steps 4 and 5 for the resuspended virus pellet obtained in step 3.
7. Centrifuge all sucrose cushion tubes at 55,000 × g for 1.5 h at 4°C in a swinging bucket SW 32 Ti rotor.
8. Decant the supernatant and resuspend and pool viral pellets in a total volume of 7 mL of 5% sucrose-PBS. Store at 4°C.

Purification of preclinical grade OrfV
Iodixanol gradient
Gradient purification removes additional impurities from virus eluted following either two-step filtration or sucrose cushioning and is required to achieve the purity required for *in vivo* experiments.

1. Dilute 60% (w/v) iodixanol (which is the concentration of iodixanol in Opti-Prep) in PBS to generate 15%, 25%, and 35% (v/v) iodixanol-PBS.
2. Create the iodixanol gradient as per Figures 5B and 5C in 13-mL ultra-clear ultracentrifuge tubes. Initially, layer 2 mL of the 60% at the bottom of tube. Then add 1 mL of the 35% gently above the 60% layer. Next, gently layer on 1 mL of the 25% followed by 1 mL of the 15%.
   - Care should be taken to prevent mixing layers of the gradient.
   - For the TFF virus product, we typically only subject elutions 1 and 2 to gradient centrifugation, as they contain the majority of virus.

Pause point: Iodixanol gradients can be stored at 4°C overnight.

3. Gently pipette elutions 1 and 2 from method 1 or the resuspended virus pellet from method 2 on top of the 15% iodixanol gradient. Fill tubes to within 1 cm of the top to avoid collapse during centrifugation; if needed, supplement with 5% sucrose-PBS.
4. Carefully balance each tube inside its ultracentrifuge canister to within 0.01 g.
5. Centrifuge gradients in the SW 41 Ti rotor at 80,000 × g for 3 h at 4°C.
6. OrfV will accumulate as three distinct bands in the 15%, 25%, and 35% iodixanol-PBS bands (Figures 5B and 5C).
7. Extract virus using a P1000 pipette. Remove as much liquid on top of the 15% virus band as possible. Proceed to pipette out the virus band into a new collection tube. Repeat this with the remaining bands. Pool the collected virus.
8. Repeat this step for all gradients poured, and pool the collected virus. Sonicate the pooled virus using a probe sonicator.
   - Conical tubes should be placed on ice during sonication to prevent excessive heating of samples.
   - Sonicator parameters should follow two cycles of 10 s on with 10-s rests in between at an amplitude of 20%.
9. Centrifuge sonicated cell pellet suspension at 1,500 × g for 5 min at 4°C to remove any remaining cell debris from virus, and proceed to dialysis with the decanted supernatant.

Dialysis
1. Pour 1 L of sterile PBS into a 1-L beaker and add a sterile magnetic stir bar.
2. Rehydrate a 3-mL Slide-A-Lyzer dialysis cassette (10,000 kDa cutoff) in PBS for 30 s.
3. Load a 10-mL syringe with virus collected from the gradient after sonication and clarification, and attach an 18-gauge blunt-tip needle.
4. Carefully insert the needle into one of the injection ports of the dialysis cassette, inject the virus, and remove any air before removing the needle. Mark the port used with a sharpie.
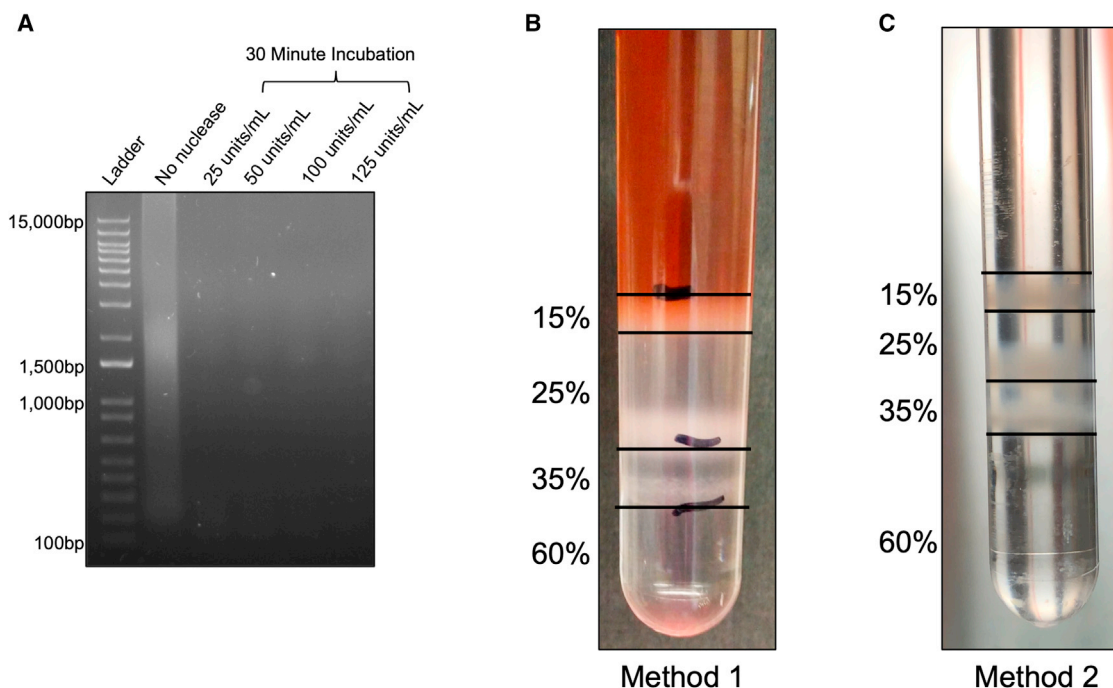
Figure 5. Removal of genomic DNA contamination and OrfV banding patterns

(A) Nuclease treatment of the clarified virus supernatant product collected following sonication followed by high-speed centrifugation to remove contaminant cellular nucleic acid. Nuclease was added at increasing concentrations and incubated on ice for 30 min. Samples were run on a 1% agarose gel with a 1-kb DNA ladder standard. Typical banding pattern of OrfV produced by method 1 (B) and method 2 (C) through a 10%, 25%, and 50% stepwise iodixanol gradient following ultracentrifugation. The first tangential flow filtration elution was overlaid on the 10% layer, and samples were centrifuged at 80,000 × g for 3 h.

- Avoid going back into the same port if needing to access the cassette again for any reason.

5. Place the dialysis cassette in the 1 L of sterile PBS and stir slowly on a magnetic stir plate at room temperature for 2 h.
6. After 2 h, replace the PBS with fresh PBS and transfer the dialysis setup to 4°C, stirring gently for at least 4 h to overnight.

Pause point: After refreshing the PBS, the dialysis can be continued overnight.

PEG concentration

1. Dialysis can result in volumetric expansion of the virus propagation.[15] For preclinical applications, it is desirable to concentrate virus into the smallest possible volume. To accomplish this, proceed with PEG concentration.
2. Load a resealable plastic bag with 25 mL of 40% (w/v) PEG 20,000 in PBS. Place the dialysis cassette in the bag and seal so that all the air is removed and the entirety of the cassette is covered with PEG 20,000.
3. Incubate at room temperature until volume is reduced to a desired level.
   - The duration required to reduce volume is variable, and depends on the amount of starting volume and the desired final volume. We typically concentrate 10 mL into 3 mL in 4.5 h.

We recommend checking the cassette for contraction after the first 2 h and then every hour afterward.

4. Following PEG concentration, gently rinse the cassette in PBS to remove external PEG.
5. Specifically rinse the injection port (different from the one used to inject the virus) to be used with 5 mL of sterile PBS to remove any remaining PEG.
6. Remove the virus by first injecting 5 mL of air into the dialysis cassette using a 10-mL syringe with an 18-gauge blunt-tip needle. Extract the virus, then reload the syringe with 350 μL of sucrose-PBS diluted for a final concentration of 5% sucrose-PBS. Inject sucrose-PBS back into the dialysis cassette. Gently palpate the cassette to remove any residual virus, and once again fill with 2 mL of air and collect all remaining liquid.
7. Aliquot virus (typically 200–500 μL) including one 20-μL aliquot for titration. Store virus at −80°C.

Titration of preclinical grade OrfV

OrfV titration by tissue culture infectious dose 50 (TCID$_{50}$)

1. The TCID$_{50}$ assay for OrfV is optimally performed using SSF cells, which are permissive to OrfV and propagate at a relatively slow rate, allowing time for obvious CPE before monolayers become overly confluent. Additionally, SSF cells have an elongated

phenotype common to fibroblasts, thus making the rounded CPE easy to visualize. See Figure 2B for representative images of SSF cells infected with OrfV or left uninfected.

2. Plate SSF cells at 1×10^4 cells per well in flat-bottomed 96-well cell culture plates in 180 μL of complete DMEM.
   - Ensure that cell viability is at least 95% by trypan blue staining.
3. Prepare serial dilution of virus stock in complete DMEM, ensuring enough volume to infect 12 wells with 20 μL volume, leaving extra volume for two extra wells to account for potential pipetting errors. For a typical virus propagation, we prepare dilutions up to 10^{-9}.
   - It is ideal to titrate a virus stock that has been freeze-thawed only once, as this process can destroy infectious virus particles.
4. Infect ten wells of SSF cells per dilution with 20 μL, following the plate outline in Figure 2C.
5. Incubate at $37°C$, 5% CO_2, and 21% O_2 for 4–7 days.
   - We recommend monitoring the progress of infection every 24 h by brightfield microscopy.
6. Score each well in a dilution that shows CPE as positive and calculate the $TCID_{50}$ using the Spearman-Karber calculation.[16] $TCID_{50}$ values can be converted to plaque-forming units (PFU) by multiplying by a factor of 0.69.[15]

OrfV titration by plaque assay

1. Akin to $TCID_{50}$, titration of OrfV by plaque assay is optimally performed on SSF cells. In addition to the reasons mentioned above, SSF cells form confluent monolayers that resist over-confluency and piling of cells long enough for OrfV plaques to become clearly visible, as depicted in Figure 2B.
2. Plate SSF cells at 5×10^5 cells in 2 mL of cDMEM per well in a 6-well plate and incubate overnight or until cells reach 100% confluency.
3. Prepare serial dilution of virus stock in cDMEM in sufficient volume to infect wells with 500 μL.
   - For a typical virus propagation, we prepare dilutions up to 10^{-9}.
4. Remove medium from SSF cells and infect with 500 μL of diluted virus. Ensure that one well is mock infected with medium only.
5. Incubate virus for 1 h at $37°C$, rocking the plate every 10 min to ensure equal coverage of virus.
6. Prepare 10 mL of 1% low-melting-point agarose in sterile Milli-Q H_2O and melt in a microwave. Keep agarose in a fluidic state by incubating in a $56°C$ water bath until use.
7. Prepare 10 mL of $2\times$ MEM, no phenol red, with 20% fetal bovine serum and $1\times$ penicillin/streptomycin cocktail and equilibrate to room temperature.
8. Following 1 h of incubation, remove infectious media and wash one with 1 mL of PBS per well. Combine 1% agarose and $2\times$ complete MEM at a 1:1 ratio and quickly apply 1.5 mL to each well before solidification occurs. Ensure that no bubbles form, as these will obstruct identification of plaques by microscopy. Allow agarose to solidify before moving plates into incubator.

9. Incubate at $37°C$, 5% CO_2, and 21% O_2 for 4–7 days or until plaques are clearly visible by microscopy.
10. Enumerate plaques at a dilution with 5–50 countable plaques and calculate the PFU by the following formula:

$PFU/mL = (number\ of\ plaques)/dilution \times plating\ volume$
Optional: samples can be stained with nuclear fast red or fixed and then stained with crystal violet to better view plaques.

Quantifying genome copy number by qPCR

1. The ability to detect and quantify OVs in tumors and off-target tissues is extremely valuable for preclinical studies. Quantification of virus genome copy number by qPCR provides increased sensitivity and ease over $TCID_{50}$ and plaque assay for these types of experiments. We describe herein a method to specifically detect and quantify OrfV virus genomes by qPCR using specific primers and synthetic plasmids, using DNA harvested from purified virus stocks as an example.
2. For virus DNA extraction from virus stocks, we recommend starting with at least 1×10^8 virions.
3. Treat virus stock with RQ1 RNase-free DNase to digest contaminant genomic cellular DNA.
   - Add 10 U of DNase I plus 20 μL of Buffer 10X with $MgCl_2$ to 170 μL of virus stock and incubate at $37°C$ for 1 h.
   - Stop the reaction by adding 20 μL of stopping solution and incubating at $65°C$ for 10 min.
4. Extract viral DNA using a commercial kit (e.g., PureLink Viral RNA/DNA Mini Kit, Fisher Scientific, Cat. #12280050), or by the method described below.
   - Add virus stock with 10 μL of Proteinase K (20 mg/mL) and 50 μL of 10% SDS to RNase-free sterile PBS up to a volume of 500 μL.
   - Heat to $65°C$ for 2 h in a heating block.
   - Add equal volume phenol/chloroform/isoamyl alcohol and shake vigorously.
   - Separate phases by centrifugation at $15,000 \times g$ for 10 min at $4°C$.
   - Collect top aqueous layer containing viral DNA without disturbing the interphase, which contains protein and phenol contaminants.
   - Precipitate viral DNA by adding 2.5 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2), incubating at $-80°C$ for 1 h to overnight and centrifuging at $10,000 \times g$ for 30 min. Quantify by Nanodrop or Qubit fluorometric quantification.
5. Purified viral genomic DNA can be quantified by qPCR using the primers provided in Table 1 and the standard plasmid target sequence given in Figures 6A, 6B, and 6E. The standard plasmid contains the target sequences for all three primer pairs.
6. Perform qPCR on isolated virus DNA with primers from Table 1 for OrfV detection, following standard qPCR reaction requirements. We typically perform qPCR on a range of virus DNA dilutions (e.g., 1/100–1/500).

Table 1. OrfV-specific primer pairs for genome copy number quantification by qPCR

Primer name	Genomic target	Sequence 5′-3′	Band size (bp)
ORFV qPCR1 F	viral polymerase	CTTGAGGAAGAGGAACCGG	143
ORFV qPCR1 R		GATGAAGATCACCGCCCAC	
ORFV qPCR2 F	virion core protease	GATCTGCTTGAAGAAGTGGTTC	129
ORFV qPCR2 R		GAAGATGGAGCGCTGGAC	
ORFV qPCR3 F	DNA-binding phosphoprotein	CTGCGCAGTACGAAAGTTAG	131
ORFV qPCR3 R		GATGCTGGGGTCGCTAG	

7. To calculate virus genome copy number, the concentration of the standard plasmid is quantified (e.g., by fluorometric quantitation) and the copy number is calculated using the following formula:

$$\left(\frac{\text{Plasmid concentration } \left(\frac{g}{\mu L} \right)}{\text{Plasmid length } (bp) \times 660} \right) \times 6.022^{23}.$$

8. A standard curve is then generated by diluting the standard plasmid, determining cycle threshold (CT) values at each dilution, and calculating the line of best fit. The CT values of the virus samples are then determined and plotted on the line of best fit to calculate the virus genome copy number (Figure 6D).

Timeline

Approximate time based on 50-plate virus propagation

- Propagating SSF cells to 50 × 15-cm plates: 7–10 days.
- Virus infection: 4–6 days.
- Virus harvest: 2 h.
- Virus concentration using method 1: depth filtration 1–4 h; TFF 2–5 h.
- Virus concentration using method 2: sucrose cushion 1.5–3 h.
- Iodixanol gradient purification: 4 h.
- Dialysis: 12–16 h (overnight).
- PEG concentration: 4 h.
- Titration for infectious units (TCID$_{50}$, plaque assay): 4–7 days.
- Quantifying genome copy number: 6 h.
- Total process: ∼25 days.

Troubleshooting

Extraction of OrfV from cell lysate and removal of cell debris

Cell lysate contaminants carried through the purification and concentration processes can drastically impact titration of virus stocks and the outcome of in vitro and in vivo experiments. Concentrated cellular proteins and DNA can cause death of cell lines used for titration that can resemble CPE. This is less problematic in the pla-

que assay because the visual identification of true virus infection is more easily differentiated. Cell death from contaminants can result in artificially high titers, which in turn impact future experiments and propagations. In vitro experiments performed with contaminated virus stocks are similarly affected. For example, killing assays can be skewed if cell contaminants are lethal to cells or if the actual infectious dose is lower than the calculated value. The use of contaminated stocks in preclinical models for vaccination or cancer can cause acute toxicities and skew immune response data. The instructions detailed below highlight critical points in the OrfV propagation and purification method that prevent accumulation of cellular contaminants in purified virus stocks and ensure consistency for experiments.

The infectious cycle of OrfV results in the majority of virus progeny being produced as intracellular mature virions that do not bud from the infected cell but instead remain associated with internal membranes.[17] Some mature virus acquires a second membrane and buds from infected cells, termed the extracellular mature virus, but this is produced at a much lower ratio than the intracellular mature form.[18] The majority of virus must therefore be released from cells, which occurs optimally by 3× freeze-thaw combined with gentle sonication, which produces a high amount of contaminant cell debris. This includes the release of cellular DNA, which can be removed by nuclease treatment of the clarified virus supernatant (Figure 5A). Vigorous sonication can lead to rapid heating of the sample, so it is strongly advised to avoid high amplitudes, include rest cycles, and always sonicate samples while on ice. Contaminants carried through filtration can cause improper separation of virus from cellular debris during gradient ultracentrifugation. We have observed large bands being pulled to the bottom of the 50% iodixanol gradient, which contain large quantities of virus, if the centrifugation step described above is excluded or reduced in speed or duration. Additionally, we do not recommend using virus directly from TFF elutions for experiments without further purification through an iodixanol gradient. TFF concentrates some contaminants along with virus, which can be detrimental to both in vitro and in vivo experiments.

Controls for virus titration

Uninfected cells. Both the TCID$_{50}$ and plaque assays should contain wells with uninfected cells. For the TCID$_{50}$, an entire row can be committed to this control with ease by using a multi-channel pipette. For the plaque assay, an uninfected control well must be included in each plate as overheated agarose, poor nutrient delivery, and other factors can lead to cell death. Ensure that all wells are given identical volumes of media as infected wells.

Titration standard with known stock. To confirm the quality of virus titration, we recommend titrating a known and trusted standard virus stock in parallel with titration assays. Standard virus stocks should be stored in small-volume aliquots to prevent multiple freeze-thaws. We have observed a loss of up to 30% of infectious particles after a single freeze-thaw of standard virus stocks
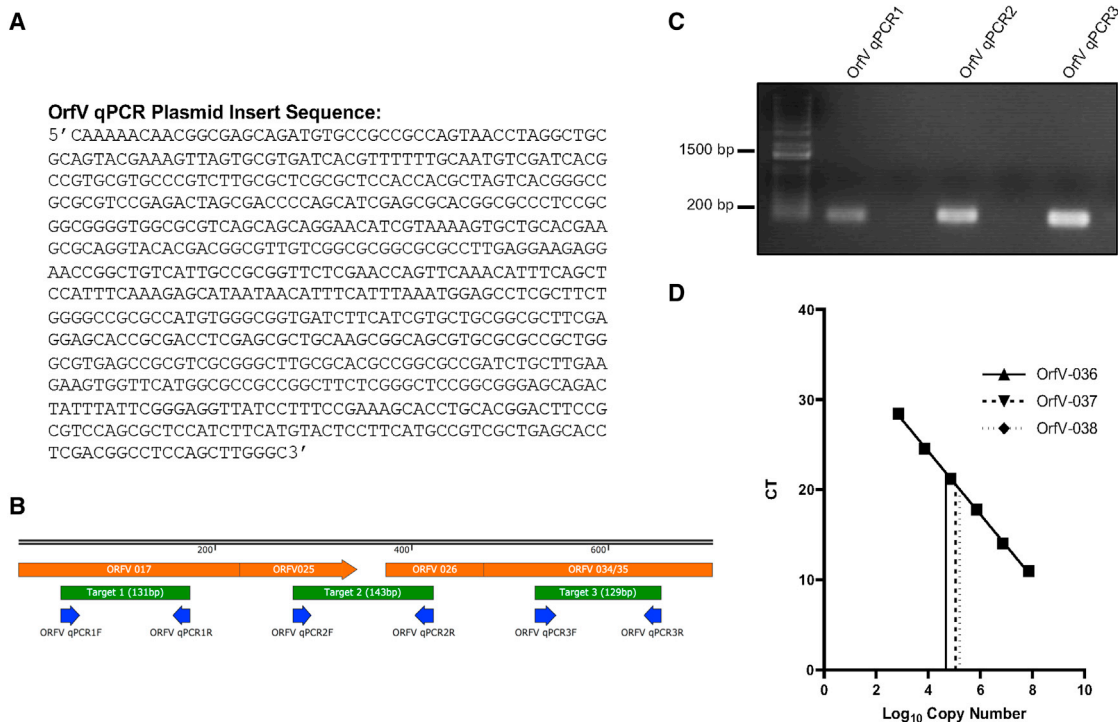
A

OrfV qPCR Plasmid Insert Sequence:
5′CAAAAACAACGGCGAGCAGATGTGCCGCCGCCAGTAACCTAGGCTGC
GCAGTACGAAAGTTAGTGCGTGATCACGTTTTTTGCAATGTCGATCACG
CCGTGCGTGCCCGTCTTGCGCTCGCGCTCCACCACGCTAGTCACGGGCC
GCGCGTCCGAGACTAGCGACCCCAGCATCGAGCGCACGGCGCCCTCCGC
GGCGGGGTGGCGCGTCAGCAGCAGGAACATCGTAAAAGTGCTGCACGAA
GCGCAGGTACACGACGGCGTTGTCGGCGCGGCGCGCCTTGAGGAAGAGG
AACCGGCTGTCATTGCCGCGGTTCTCGAACCAGTTCAAACATTTCAGCT
CCATTTCAAAGAGCATAATAACATTTCATTTAAATGGAGCCTCGCTTCT
GGGGCCGCGCCATGTGGGCGGTGATCTTCATCGTGCTGCGGCGCTTCGA
GGAGCACCGCGACCTCGAGCGCTGCAAGCGGCAGCGTGCGCGCCGCTGG
GCGTGAGCCGCGTCGCGGGCTTGCGCACGCCGGGCGCCGATCTGCTTGAA
GAAGTGGTTCATGGCGCCGCCGGCTTCTCGGGCTCCGGCGGGAGCAGAC
TATTTATTCGGGAGGTTATCCTTTCCGAAAGCACCTGCACGGACTTCCG
CGTCCAGCGCTCCATCTTCATGTACTCCTTCATGCCGTCGCTGAGCACC
TCGACGGCCTCCAGCTTGGGC3′

B

C

D

Figure 6. Titering OrfV genome copy number by qPCR

(A) OrfV target sequence used for generating standard curves for OrfV genome quantification. (B) Expanded view of the OrfV target sequence and primer arrangement showing the target sites with reference to the corresponding virus open reading frame. Primers pairs are shown in blue and amplicons in green including the length in base pairs. (C) Amplification of target regions for qPCR using purified OrfV genomic DNA. (D) Quantification of OrfV genomic DNA copy number from three independent OrfV production runs. The CT values were plotted against the copy number of the pUC57_OrfV_STD standard plasmid (log$_{10}$ transformed). The CT values from each OrfV production run was used to calculate the virus genome copy number. (E) Nucleotide sequence of the OrfV qPCR target region in the pUC57_OrfV_STD standard plasmid.

(Figure 7A), but interestingly have not observed appreciable loss of infectious particles after four subsequent freeze-thaw cycles (Figure 7B).

Testing new virus batches in naive and tumor-bearing animals

Each new batch of OrfV should be tested for toxicities *in vivo*, especially if OrfV is to be delivered intravenously. Contaminants carried through the purification and concentration process can lead to acute toxicity. Mice treated with low-quality batches of OrfV require euthanasia anywhere from several minutes to 48 h following intravenous virus delivery. Mice may have a combination of symptoms including lethargy, hunched posture, ruffled fur, labored breathing, and pinched faces, but adhere to your institution's animal care guidelines. We have never observed neurological signs following systemic administration of OrfV, even at doses as high as 5×10^8 PFU. The maximum dose we have delivered to mice is 1×10^9
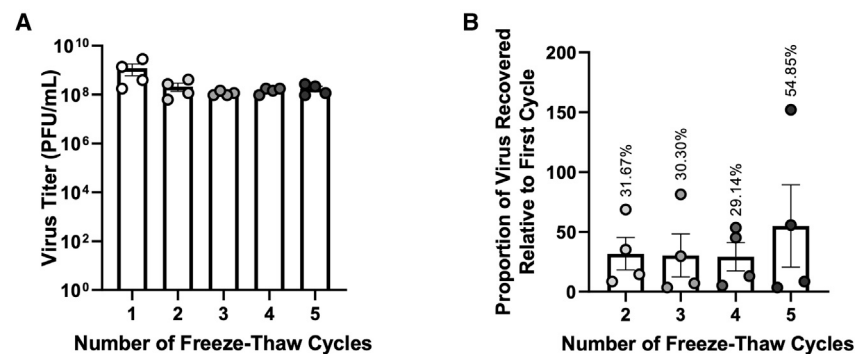
A

B

Figure 7. Impact of multiple freeze-thaws on OrfV titers

(A) Purified OrfV stocks were subjected to multiple freeze-thaw cycles, and virus was titrated by TCID$_{50}$ on SSFs. Data represent log-transformed titers from four independent experimental replicates. (B) Data are presented as the proportion of virus recovered relative to the first freeze-thaw cycle. The mean percent loss of infectious virus is expressed for each consecutive freeze-thaw. Shown are data averages ± SD.
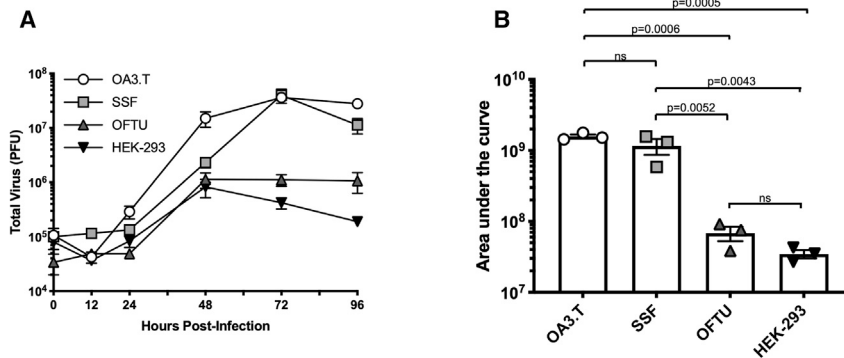
Figure 8. Growth properties of OrfV in various cell lines

(A) Single-step growth curve of OrfV on OA3.T, SSF, OFTU, and HEK-293 cell lines. Cells were infected with OrfV at an MOI = 5. Cell lysates were collected at 12, 24, 48, 72, and 96 h post infection, and virus was isolated by $3\times$ freeze-thaw followed by centrifugation. Data represent experimental triplicate for all time points, and virus was titrated by $TCID_{50}$ on SSF cells. (B) Area under the curve analysis of the single-step growth curves. Shown are data averages ± SD (n = 3/group). Statistical analysis was by one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons.

PFU produced using method 1 (TFF), but a more typical dose for therapy is 5×10^7 PFU. For preclinical cancer models, we strongly recommend conducting this quality control test in animals bearing the experimental tumor model, preferably at the same disease state as experimental treatment is planned. This ensures that virus is safe in mice that may have compromised immune systems due to cancer burden, and factors in dose amplification from tumor-specific replication of the virus.

Anticipated outcomes

Selection of propagation cell lines

To determine an optimal cell line to produce OrfV, we conducted single-step growth curves on SSFs,[14] sheep testes cells (OA3.T, ATCC CRL-6546), primary ovine fetal turbinate cells (OFTu), and human embryonic kidney cells (HEK293, ATCC CRL-1573). Growth curves for each cell line were performed in experimental triplicate at MOI = 5 using the same virus stock for inoculation, and intracellular and extracellular virus was collected at 12, 24, 48, 72, and 96 h post infection (Figure 8A). To calculate the optimal cell line for producing OrfV, we calculated the area under the curve

for all cell lines (Figure 8B). Both the SSFs and OA3.Ts produced the highest titers of OrfV, with no significant difference between the two cell lines. OFTu and HEK293 cells produced significantly less virus than SSF and OA3.T, but did not differ from each other. We recommend production of OrfV on SSF cells, despite OA3.Ts supporting productive virus replication. OA3.Ts are more difficult to culture and have slower replication kinetics than SSF cells (data not shown). Indeed, we have found that the quality of OA3.T cells greatly diminishes after ten passages in cell culture, which is not the case with SSF cells.

Typical batch yields

To determine the typical batch yield of OrfV using the TFF purification method, we produced ten batches and titrated them by $TCID_{50}$. The mean total virus yield was 4.70×10^9 PFU, SD 2.28×10^9 PFU, factoring in a 50×15-cm plate propagation (Figure 9A). Given the mean total virus yield, a single OrfV propagation can generate approximately 80 doses at 5.0×10^7 PFU per dose. Additionally, PEG concentration allows for each dose to be delivered in a small volume. Given the mean total virus yield of 4.06×10^9 PFU, PEG
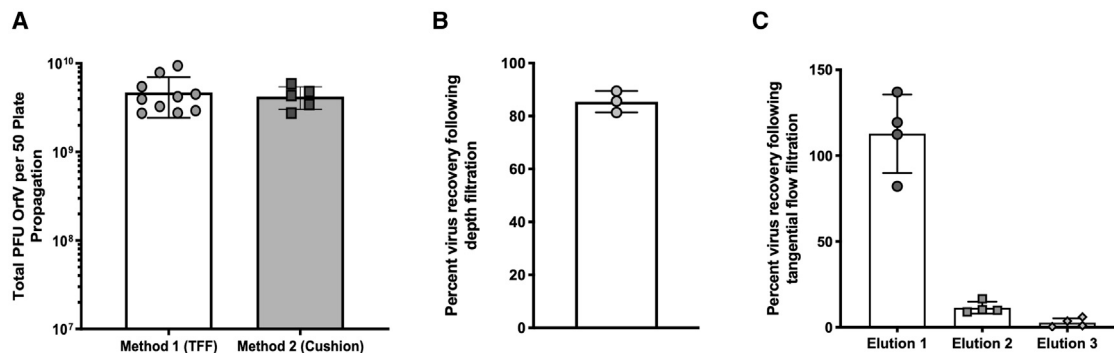

Figure 9. Typical virus yields and recovery efficiencies

(A) Expected total virus yields normalized to a 50-plate propagation. Total virus was determined for each batch following the entire purification and concentration process, representing n = 12 replicates. (B) Percent total recovery of OrfV from depth filtration, relative to input virus. Data were generated from three independent experiments, and input and output samples were titrated in duplicate. (C) Percent total recovery of OrfV from each tangential flow filtration elution, relative to input virus. Data were generated from four independent experiments, and input and elution titers were performed in duplicate. All data shown were titrated by $TCID_{50}$ on SSF cells. Shown are data averages ± SD.

concentration to 2 mL total volume enables delivery of 5.0×10^7 PFU per dose in 25 μL, which is an ideal volume for intratumoral delivery. To determine the typical batch yields using the two-step density separation purification method, we produced five batches and titrated them. The mean total virus yield was 4.23×10^9 PFU, SD 1.21×10^9 PFU, and was not significantly different from that of the TFF method ($p = 0.671$, two-tailed t test). It should be taken into consideration that OrfV batches produced using the TFF purification method have shown greater tolerance *in vivo* at high doses (e.g., 1×10^9 PFU) when compared with batches produced by two-step density separation. However, no differences in batch purity or safety were noted between both methodologies at typical dosing ranges (e.g., 5×10^7 PFU).

Virus recovery from filtration steps

To confirm that depth and TFF are applicable to OrfV, we took samples from multiple propagations and titrated them. The mean percent total virus recovery following depth filtration was 85.45% SD +/−4.03, compared to input virus (Figure 9B). For the TFF, we collected samples from the waste and three consecutive elutions. Through four independently tested propagations, virus was never detectable in the waste (data not shown). The vast majority of virus was collected in the first elution (Figure 9C), with approximately 10% of input virus captured during the second elution and 3% in the final third elution. Using this information, we recommend carrying forward to gradient purification with the first and second elution only.

Confirmation of primers and standard plasmids for pPCR

To quantify OrfV genome copy number in virus stocks and detect OrfV genomes in experimental samples, we designed three independent primer pairs targeting genes for the main virus polymerase subunit, the virion core protease, and a DNA-binding phosphoprotein (Table 1). We selected these genes because they are all part of the "core" set of poxvirus genes and thus are expected to be retained in replication-competent OrfVs, unlike some immune-modulatory or virulence genes. Each primer pair was tested by standard PCR for amplification from purified OrfV genomic DNA (Figure 6C). Next, all three target regions were synthesized (Figures 6A and 6B) into a single plasmid (pUC57_OrfV_STD), which was used to generate a standard curve for detection and quantification of OrfV in samples (see quantifying genome copy number by qPCR). Note that qPCR2 and qPCR3 primers pairs produced a more intense band than that of qPCR1, suggesting better amplification efficiency; thus they are the preferred primer pairs for quantification of genome copy number for this strain of OrfV.

In conclusion, the detailed methods presented here provide researchers with the tools to produce OrfV stocks of high concentration and purity for use in preclinical models of vaccination and cancer therapy. Researchers will also find detailed methods for detecting and quantifying OrfV genomes in virus stocks and experimental samples. We hope that these methods will contribute to the advancement of OrfV as a vaccine and oncolytic vector platform.

AUTHOR CONTRIBUTIONS

Conception and design: J.P.v.V., J.C.I., G.M., J.J.P., S.K.W., and B.W.B.; development of methodology: J.P.v.V., J.A.M., T.M.M., L.A.S., and S.K.W.; acquisition of data: J.P.v.V., T.M.M., and J.C.I.; analysis and interpretation of data: J.P.v.V., J.A.M., T.M.M., J.C.I., G.M., J.J.P., B.W.B., and S.K.W.; writing, review, and/or revision of the manuscript: J.P.v.V., J.A.M., J.C.I., T.M.M., L.A.S., G.M., J.J.P., B.W.B., and S.K.W.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

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