

# Pseudovirus Production

## Production of Papillomaviral Vectors (Pseudoviruses)

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### Citations

#### Original methods for production of BPV1 reporter vectors

•Buck, C.B., Pastrana, D.V., Lowy, D.R., and Schiller, J.T. (2004) Efficient intracellular assembly of papillomaviral vectors. *J Virol*78:751-757

#### Maturation method for production of HPV vectors

•Buck, C.B., Thompson, C.D., Pang, Y.Y., Lowy, D.R., and Schiller, J.T. (2005) Maturation of papillomavirus capsids. *J Virol*79:2839-2846

#### Detailed technical protocol

•Buck, C.B., and Thompson, C.D. (2007) Production of papillomavirus-based gene transfer vectors. *Curr Protoc Cell Biol*Chapter 26:Unit 26.21

#### Improved maturation

•Cardone, G., Moyer, A.L., Cheng, N., Thompson, C.D., Dvoretzky, I., Lowy, D.R., Schiller, J.T., Steven, A.C., Buck, C.B., Trus, B.L. (2014) Maturation of the human papillomavirus 16 capsid *MBio*5:e01104

### Abstract

Papillomavirus-based gene transfer vectors, also known as pseudoviruses (PsV), have become standard tools for studying papillomavirus assembly, cellular entry and neutralization and may have future utility as laboratory gene transfer tools or vaccine vehicles. This protocol outlines methods for PsV production. The production method is based on transfection of a 293 cell line, [293TT](#), engineered to express high levels of SV40 large T antigen. The cells are co-transfected with codon-modified papillomavirus capsid genes (L1 and L2) together with a reporter plasmid (pseudogenome) up to 8 kb in size that contains the SV40 origin of replication. Reporter plasmids entirely lacking papillomavirus sequences can be packaged. However, some reporter plasmids are packaged much more efficiently than others. Rules for what governs plasmid packaging efficiency have not yet been elucidated. Non-infectious virus-like particles (VLPs) can also be produced after transfection of 293TT cells with L1 alone. Efficient purification of the PsV or VLPs is achieved by Optiprep (iodixanol) density gradient ultracentrifugation. Using these methods, it is possible to produce highly purified PsV with yields of at least a billion reporter gene transducing units from a single 75 cm<sup>2</sup> flask of cells.

**Caution:** like other types of viral vectors, papillomavirus PsVs may present potential biohazard risks. Investigators should seek approval from an appropriate regulatory body, such as their institution's biosafety committee or similar advisory committee, prior to engaging in the production of pseudoviruses.

## 1. Introduction

Several methods for in vitro production of papillomavirus virions or pseudovirions (PsV) have been reported. They include production in keratinocyte raft culture, in cultured monolayers of mammalian cells after infection with recombinant vaccinia or Semliki forest virus vectors expressing L1 and L2, or in the test tube after reassembly of capsomers in the presence of plasmid DNA<sup>1234</sup>. However, none of these strategies efficiently produces high infectious titers. Because high titer PsV carrying an easily scored marker gene were unavailable, papillomavirus infection and neutralization assays were laborious, both in terms of production of the infectious capsids and in the conduct of the assays. In this protocol, we provide a flexible procedure for generating purified papillomavirus pseudovirions with titers in excess of  $10^9$  transducing units per ml. We have used PsV produced by this method to develop a simple high throughput [neutralization assay](#) for analysis of HPV-specific antibody responses.

The PsV production strategy outlined in **Subheadings 3.1 and 3.2** is based on maximizing the production of the two PV capsid proteins, L1 and L2, together with a target reporter plasmid (pseudogenome), in mammalian cells. Because expression of native L1 and L2 genes is restricted in cultured mammalian cells, L1 and L2 genes with extensive [codon modification](#) must be used to overcome negative regulatory features of the wild-type ORFs (reviewed in<sup>5</sup>). These codon changes do not alter the primary amino acid sequence of the proteins but do lead to a large increase in capsid protein production. To generate high copy number pseudogenomes for packaging, an SV40 origin of replication (ori) is inserted into the target plasmid and the pseudovirus is produced in cells that express high levels of SV40 large T antigen (LT). 293T is an adenovirus-transformed human embryonic kidney cell line with a stably integrated SV40 genome. The 293T line expresses very low levels of LT due to a splicing bias in favor of small t antigen<sup>6</sup>. We generated a subclone expressing high levels of LT, designated 293TT, by stable transfection of 293T cells with an expression plasmid encoding a cDNA for LT. This line supports high level replication of plasmids containing the SV40 ori. Co-transfection of 293TT cells with plasmids containing strong eukaryotic promoters driving codon modified L1 and L2, together with the pseudogenome plasmid containing the SV40 ori and a marker gene, results in assembly of reporter pseudovirions in the nucleus of the transfected cell. A newer set of methods relies on the infectious [co-propagation](#) of L1/L2 (or L1-only) expression plasmids together with a reporter plasmid of interest. In either system, L2 can be omitted to produce non-infectious virus-like particles (VLPs) composed of L1 alone.

PsV stocks can consist of simple crude extracts of detergent-lysed producer cells. However, for many applications it is desirable to separate PsV capsids from detergent and cellular components. In **Subheading 3.3**, a scheme for papillomavirus PsV and VLP purification is presented. It is based on separation of the capsids from cell debris and detergent by high salt extraction followed by ultracentrifugation through an Optiprep (iodixanol) step gradient. Under the described conditions, Optiprep provides a combined velocity sedimentation and buoyant density gradient. The method produces excellent separation of PV capsids from cell components and also achieves partial separation of DNA-containing PsV from empty capsids. Unlike CsCl, which is often used for gradient purification of virus particles, Optiprep has relatively low osmotic strength, is non-chaotropic and is nontoxic to cells at concentrations of up to 30% wt/vol iodixanol<sup>7</sup>.

Although Optiprep is considered a relatively gentle ultracentrifugation medium, the capsids of most papillomavirus types are too fragile to withstand purification immediately after release from producer cells. It was therefore necessary to devise a method to "mature" the capsids into a more stable conformation. A method for maturing PV capsids by simple overnight incubation of the crude cell lysates at 37°C is presented in **Subheading 3.2.2**. Recently, our group has found that addition of a buffering agent to the cell lysate improves the quality of the mature pseudovirion (see [Improved Maturation protocol](#)).

Using these production, maturation, and purification strategies, it is possible to generate high titer mature PsV stocks with particle to infectivity ratios of less than 10.

## 2. Materials

1. 293TT cells<sup>8</sup> or 293FT cells (Invitrogen# R700-07)(see **Note 1**)
2. DMEM-10: DMEM with 10% 56°C inactivated fetal calf serum, 1% non-essential amino acids, 1% Glutamax-I (Invitrogen)
3. 50 mg/ml Hygromycin B stock (Roche)
4. 0.05% Trypsin / EDTA (Invitrogen)
5. OptiMEM-I (Invitrogen)
6. Lipofectamine 2000 (Invitrogen)
7. Papillomavirus L1 or L1/L2 expression plasmid<sup>910811121314</sup>
8. "Pseudogenome" reporter plasmid (e.g., pYSEAP<sup>11</sup> or pfwB<sup>14</sup>)
9. Siliconized pipette tips (optional) (VWR)
10. Siliconized 1.5 ml screw-cap tubes (Fisher #05-541-63) (preferably – easier to keep sterile) or siliconized 1.5 ml flip-cap tubes (Fisher #0554131)
11. Sterile 2 M MgCl<sub>2</sub>
12. DPBS-Mg: Dulbecco's PBS (Invitrogen #14040-141) supplemented with 9.5 mM MgCl<sub>2</sub> and 1x antibiotic-antimycotic mixture (Invitrogen# 15240-112). Keep sterile. Store at 4°C.
13. 10% Triton X-100 (Pierce #28314). Earlier pseudovirus production protocols used Brij58 (Sigma #P-5884) instead of Triton X-100. However, Triton X-100 is preferable because its stock is more stable than Brij58 stock. Triton X-100 works as well as Brij58 for harvest of HPV reporter vectors and is superior to Brij58 for harvest of polyomavirus-based vectors.
14. Benzonase (Sigma #E1014)
15. Plasmid Safe (Epicentre #E3101K).
16. 1 M ammonium sulfate adjusted to pH 9.0 and filter sterilized.
17. 5 M NaCl.
18. 46% (wt/vol) iodixanol (see "Optiprep Recipes" section at the end of the page). Store at room temperature for up to two months. Protect from light.

\*Although calcium and magnesium have traditionally been added to the Optiprep gradient, we have found that omitting them makes no difference in terms of titer yield or purity.

1. DPBS / 0.8 M NaCl (see "Optiprep Recipes" section at the end of the page). Filter sterilize. Alternatively, add 0.15 volume of sterile 5 M NaCl to DPBS (Invitrogen #14040-141). Store at room temperature. \*Note: Although calcium and magnesium have traditionally been added to the DPBS / 0.8 buffer, we have found that omitting them makes no difference in terms of titer yield.
2. Polyallomer ultracentrifuge tubes (Beckman)
3. Swinging bucket ultracentrifuge rotor rated for >200,000 x g (e.g., SW55ti)
4. SDS-PAGE equipment

### 3. Methods

The methods described below outline (1) culture and transfection of 293TT cells, (2) Harvest of PsV and (3) purification of PsV by ultracentrifugation through an Optiprep gradient.

#### 3.1 Culture and Transfection of 293TT Cells

##### 3.1.1 Thawing 293TT Cells

293TT cells are cultured in DMEM-10. For the initial thaw of the 293TT cells, place the thawed suspension directly into a 225 cm<sup>2</sup> flask with 30 ml of DMEM with a total of 20% FCS. It is not necessary (or desirable) to spin the cells out of the freezing medium. Like other types of 293 cell lines, 293TT do not adhere tightly. It may take as many as three days for the cells to attach after thawing. If cells do not attach after two days, it may help to spin them out of the medium, wash once with calcium-free PBS, then pellet and resuspend for five minutes at 37°C in 1 ml of trypsin/EDTA. Resuspend the trypsin-treated cells in 30 ml of DMEM-10 and plate in a 225 cm<sup>2</sup> flask.

##### 3.1.2 Passaging 293TT Cells

Split 293TT cells 1:5 to 1:20 when they reach 95% confluence. Detach cells by first gently rinsing the flask once with several ml of trypsin. It is important to add the trypsin along the top edge of the culture surface, allowing it to run toward the bottom of the flask in a sheet of fluid. Remove the first rinse of trypsin and sheet ~2 ml of fresh trypsin down the flask. Incubate the cells with trypsin in a humidified 37°C incubator for 5-10 minutes. 293TT cells adhere more tightly to one another than to standard culture plastic. It is therefore important to trypsinize the cells thoroughly. Insufficient trypsinization leads to lytic shredding of cell clumps during trituration (resuspension). Inactivate trypsin by adding 10 ml of DMEM-10. Resuspend the cells and transfer a portion of the cell suspension directly into a fresh flask. It is not necessary (or desirable) to spin the cells out of the residual trypsin, since the trypsin is functionally inactivated by the fetal calf serum in DMEM-10.

After the cells have fully recovered from thawing, DMEM-10 can be supplemented with 250 µg/ml hygromycin B (Roche) to promote maintenance of T antigen expression. Although 293TT cells can typically be passaged for several months without alteration of PsV production or titration characteristics, early passages should be frozen in aliquots for long-term storage.

293TT cells are sensitive to DMSO and freezing them improperly will result in failure to attach after thawing. It is important to chill all components needed to freeze down the cells prior to starting. This will minimize the length of time that the 293TT cells are exposed to DMSO. Begin with a T-225 flask of 293TT cells grown to ~80% confluence. Chill a rack with 8-10 labeled cryotubes to -20 °C. Chill a "Mr. Frosty" isopropanol bath to 4°C. Make 10 ml of 2x freeze medium (fetal calf serum + 18% DMSO) and chill on ice. Remove 5 ml of supernatant (conditioned medium) from the flask and save. Trypsinize the cells as described above (using 1-2 ml of trypsin), then resuspend in the reserved conditioned medium. Chill the suspension on ice. Mix the chilled cell suspension 1:1 with the chilled 2x freeze medium. Distribute 1.5 ml of suspension into each chilled cryotube. Place aliquots in Mr. Frosty and place at -80 °C overnight, then transfer aliquots into liquid nitrogen for long-term storage.

##### 3.1.3 Transfection

Use Invitrogen's Lipofectamine2000 essentially according to the package insert.

1. Preplate 7 million 293TT cells in 20 ml of DMEM-10 (no hygromycin or antibiotics) in a 75 cm<sup>2</sup> flask 16 hours prior to transfection. Ideally, the culture should be at high density (complete or nearly complete confluence) at the time of re-plating. The pre-plated cells should be ~40% confluent at the time of transfection. Higher cell density will result in reduced transfection efficiency. In general it appears that cells with a free edge attached to bare culture plastic are most efficiently transfected. Cells growing in the middle of islands (surrounded by other cells) seem not to be transfected well.
2. Mix 38 µg of DNA (for example, 19 µg each of p16sheLL and pYSEAP (see **Notes 2 and 3**)) with 2 ml of OptiMEM-I.
3. In a separate tube mix 85 µl of Lipofectamine 2000 with 2ml of OptiMEM-I.
4. Incubate the two mixtures separately at room temperature for 10 to 20 minutes, then combine and incubate for at least an additional 20 minutes.
5. Add the resulting lipid/DNA complexes directly to the preplated cell culture. It is not necessary to change medium prior to transfection (in fact, doing so appears to decrease transfection efficiency). From this point forward treat cells and all their products like an infectious agent (**Note 4**).
6. Incubate the cells with the lipid/DNA complexes overnight. The next morning, remove the complexes and add fresh DMEM-10 pre-warmed to 37°C. Add the fresh DMEM-10 to the upper surface of the flask to avoid dislodging cells. Previous versions of this protocol specified incubating the cells with the lipid/DNA complexes for 4-6 hours. Although the overnight incubation causes noticeable cytotoxicity, we have found that the overall titer yields are nevertheless somewhat better from overnight-transfected cultures.

7. After the media change, return flask to the incubator for ~30 hours (i.e., a total of 48 hours after initial addition of the lipid/DNA complexes). It is not necessary to split the culture.

### 3.2 Harvest and Maturation

In this section, PsV are released from 293TT cells by detergent lysis. Although the PsV are infectious immediately after release, pseudovirions must be given time to mature prior to purification. Maturation is accomplished by simple overnight incubation of the cell lysate at 37°C. The matured PsV is solubilized by addition of sodium chloride to the lysate, which allows clarification of the lysate by low-speed centrifugation.

Note that the protocol below, which uses the broad spectrum nuclease Benzonase, is most appropriate if overall yield of recombinant capsids is the primary goal. If the primary goal is harvest of only the infectious pseudovirions, it may be preferable to use the [Revised Production protocol](#).

#### 3.2.1 Collect Cells

Collect producer cells by trypsinization about 48 hours after transfection (**Note 5**). If there are floating cells in the culture, collect them by centrifugation. Resuspend the cells in 10ml of DMEM-10 and transfer to a conical tube (with collected floaters, if applicable). Rinse the flask with another few ml of fresh DMEM-10. Expect about 25 million cells from a confluent 75 cm<sup>2</sup> flask.

Spin down the cells and discard the supernatant. Partially resuspend the cell pellet in residual fluid by gently agitating the tube. Using a 2 ml pipet, transfer the suspension into a siliconized (see **Note 6**) 1.5 ml screw-cap tube. Rinse the original tube with 0.5 ml of DPBS and combine with suspended cells. Repeat the DPBS rinse, if necessary. Spin down the cells in the siliconized tube and discard supernatant.

#### 3.2.2 Cell Lysis and Capsid Maturation

This step of the protocol, in which cells are suspended in PBS and lysed with detergent, is surprisingly important. The cells must be suspended at very high concentration (>100 million cells per ml). If lysis is performed at too low a density, proteins in the lysate aggregate non-specifically. This results in entrapment of virions in large aggregates that can be lost during the initial low-speed clarification. The aggregation also seems to interfere with pseudovirus infectivity (presumably due to sequestration of the pseudovirions inside large particles). The large aggregates also drag free DNA and free reporter proteins (e.g., NanoLuc protein produced by the transfected cells) down the Optiprep gradient. Surprisingly, the line between an appropriately high-density lysate and an inappropriately low-density lysate is very sharp. When performing this step of the protocol, it is always better to err on the side of a higher density cell lysate than risk the negative consequences (low infectious yield, severely "dirty" pseudovirions contaminated with a wealth of host cell proteins) of lysing cells at even slightly lower than ideal density.

The diameter of the transfected 293TT "producer" cells at the time of harvest can vary wildly. It appears that cells that have replicated the transfected plasmids to very high copy number swell up like beach balls. Because of this problem, cell counts are less reliable than simply performing a gross estimate of the volume of the cell pellet. To estimate the volume of the cell pellet, load 1 ml of DPBS (or other fluid) into a 1ml pipet (i.e., long clear plastic pipet with volume markings on it). Dispense the liquid into a dummy tube until the volume in the dummy tube matches the height of cell pellet. It may be necessary to shake the bubble out of the bottom of the dummy tube. Record the volume that has been dispensed out of the 1ml pipet. This represents the volume of the cell pellet. Add 1.4 pellet volumes of DPBS+ 9.5mM MgCl<sub>2</sub> and PSF (or Primocin) to the siliconized tube. For polyomaviruses, add 1.2 pellet volumes of DPBS (to allow for addition of neuraminidase). Suspend the cells by briefly vortexing the tube.

As an example, a 225 cm<sup>2</sup> flask might give a 220µl pellet of cells. The cell pellet would then be suspended by adding 300 µl of DPBS+Mg for HPV pseudoviruses (or 250 µl of DPBS+Mg for polyomavirus pseudoviruses). For calculation purposes (below), the total volume of the suspension could be considered about 500µl.

To lyse the cell suspension, add 1/20<sup>th</sup> of a volume of 10% Triton X-100 (Pierce #28314)(i.e., a final Triton concentration 0.5%). Add 0.1% Benzonase. Add 0.1% Plasmid Safe. Note: in some situations, it may be preferable to use the ["Revised Production"](#) harvest method. In that method, an RNase cocktail is added instead of Benzonase and Plasmid Safe.

Add 1/40<sup>th</sup> volume of 1 M ammonium sulfate pH 9. See [Improved Maturation protocol](#) for details. If making dye-conjugated capsids, do NOT add ammonium sulfate, since it will quench the amine-reactive group on the fluorochrome. The lysate can instead be buffered by adding 40 mM sodium phosphate starting from a 1 M stock pH'd to 7.5.

Incubate cell lysate at 37°C overnight (see **Note 7**). It may be helpful to mix the tube by inversion once or twice during the course of the incubation. Since it is possible for contaminating microbes to grow during the maturation period, it is essential to use aseptic techniques while preparing the cell lysate. If the cell suspension is dense enough, there should be very little clear fluid above the settled cell suspension the next morning. A lot of clear fluid visible above the cell suspension may be a sign that the cell density was too low and nonspecific protein aggregation may have occurred.

After maturation, the lysate can be stored overnight at 4 °C or frozen long-term at -80 °C. Although freezing the matured lysate does not result in significant loss of infectious titer, freeze-thawed lysates sometimes show greater amounts of contaminating cellular proteins after Optiprep purification. Thus, it's ideal to proceed to Optiprep purification immediately after maturation.

#### 3.2.3 Salt Extraction

1. Chill the matured lysate on ice for 5 minutes. This step is likely optional.
2. Bring salt concentration up to 850 mM by adding 0.17 volume of 5M NaCl. Do NOT add salt if using the [Revised Production](#) method. Incubate salted pellet on ice for 10 minutes. If desired, a sample of salt-treated lysate can be withdrawn for titring (see **Note 10**). Crude stock must be diluted at least 1:1000 to avoid detergent toxicity to the target cells.
3. Clarify the salt lysate by spinning for 5 minutes at 5,000 x g in a refrigerated microcentrifuge.
4. Transfer the clarified supernatant into a fresh siliconized screw-cap tube.
5. Re-extract the pellet material by re-suspending it in two pellet volumes of DPBS / 0.8 M NaCl. Centrifuge at 5,000 x g for 5 minutes and transfer this second clarified supe into the same tube with the first supe.
6. Re-clarify the pooled supes at 5,000 x g for 5 minutes.
7. Layer the double-clarified supernatant(s) onto an Optiprep gradient (**Subheading 3.3**) or see **Note 8** for alternative purification methods.

### 3.3 Optiprep Purification

In this section, PsV is purified by ultracentrifugation through an Optiprep step gradient. The section also outlines methods for biochemical analysis of the purified PsV.

#### 3.3.1 Preparation of Optiprep Gradients

Optiprep is a trade name for 60% (wt/vol) iodixanol solution. Use DPBS / 0.8M NaCl to dilute 46% (wt/vol) iodixanol (see **Materials**) to 27%, 33%, and 39%. Use 50 ml conical centrifuge tubes to allow easier syringe draws (see below). Pour Optiprep gradients in thin wall polyallomer 5 ml tubes (e.g., Beckman 326819, if using an SW55 series rotor) by underlaying (27% then 33% then 39%) ~1.4 ml steps using a 3ml syringe fitted with a long needle. Note: it is possible to process up to 3 ml of clarified cell lysate by reducing the total volume of the gradient to ~2 ml (i.e., 0.7 ml per step). If necessary, pour a balance gradient. The gradients can be allowed to diffuse at room temperature for 1 to 4 hours.

#### 3.3.2 Ultracentrifugation

Gently layer clarified cell lysate (**Subheading 3.2.3**) onto the Optiprep gradient. The tubes should be full or (nearly full) and the tubes/buckets should be balanced to within  $\pm 5$  mg. Spin for 3.5 hours at 16°C at 50,000 rpm (234,000 x g) in an SW55ti rotor. Set the acceleration and deceleration to "slow." Too rapid of an acceleration/deceleration may stir the gradients. Other types of rotors can be used successfully, for example SW40.1Ti at 40,000 rpm for 4.75 hours or SW32 at 32,000 rpm for 5.75 hours.

#### 3.3.2 Fraction Collection

The L1 band may be faintly visible as a light gray layer a little over a third of the way up the gradient. Collect gradient fractions by puncturing the bottom of the tube slightly off center with a syringe needle. If a 25 gauge syringe needle is used, perform a simple in-and-out puncture without any rocking or rotating of the needle. A 26-gauge syringe creates a very small hole with frustratingly slow flow rates. It is therefore necessary to gently rotate the 26-gauge syringe after puncturing.

Drip fractions into siliconized microcentrifuge tubes. Collect the first ~750  $\mu$ l as one fraction, then collect 6 to 8 drop (~250  $\mu$ l) fractions up to fraction 10. Discard the top ~2 ml of the gradient.

#### 3.3.3 Screening Fractions

The simplest method for screening fractions is to look for the presence of encapsidated DNA in core fractions of the gradient. The capsid is freely permeable to small solutes, including high-sensitivity fluorescent DNA stains, such as Quant-iT PicoGreen dsDNA Assay reagent (Invitrogen# P7589). Note that for the [Revised Production](#) method, there is sometimes too little encapsidated DNA to be accurately detected using PicoGreen.

To screen fractions using PicoGreen, transfer 5  $\mu$ l of fraction material into separate wells in a 96-well plate suitable for fluorescence measurements (e.g., Nunc# 237105). Add 150  $\mu$ l per well of PicoGreen diluted 1:200 in 1x TE supplied with the PicoGreen kit. Optional: supplement the PicoGreen working mixture with 0.1% proteinase K stock (e.g., Qiagen# 19131). Mix the PicoGreen with the fraction samples either by trituration or by patting and swirling the plate. Optional: incubate the plate in a 65 °C oven for 20 minutes. Read the plate on a fluorescent plate reader at OD 500/520 (or thereabout – see PicoGreen kit insert). There should be a small histogram of DNA signal in the core fractions of the gradient, with a larger ramp of DNA signal (unencapsidated DNA) toward the top of the gradient. The proteinase K and heating are not strictly necessary, but together tend to increase the signal from encapsidated DNA – perhaps by releasing it from chromatin packing.

Since L1 is the major protein present in core fractions of the gradient, fractions containing PsV can be identified by SDS-PAGE minigel analysis of a few  $\mu$ l of each fraction. SimplyBlue (Invitrogen) offers a rapid and sensitive method for staining SDS-PAGE gels. Fractions containing significant amounts of L1 (55 kD) should be pooled, aliquoted and frozen at -80°C. Peak L1 content is generally found between fractions 4 and 8. SDS-PAGE

analysis of bovine serum albumin standards (BioRad) ranging from 2 µg to 50 ng or BCA protein assay (Pierce) can be used to examine L1 yield. Overall L1 yield should be about 200 µg of L1 from a 75 cm<sup>2</sup> flask transfected with p16L1-GFP. **Notes 9 and 10** discuss additional methods for screening fractions.

#### 4 Notes

1. Invitrogen recently began selling a 293 cell line, [293FT](#), that is conceptually similar to 293TT. 293FT work reasonably well for pseudovirus production, but don't perform as well in the neutralization assay. We suspect that 293FT express SV40 T antigen at lower average levels than 293TT. Alternatively, the presence of SV40 small t antigen in 293TT (but not in 293FT) may be a factor in the better performance of 293TT cells. It is also conceivable that the 293T parent line lacks innate antiviral defenses that remain active in the 293 parent line used to make 293FT cells<sup>15</sup>. 293TT cells are [available](#) through the NCI's Developmental Therapeutics Program.
2. Maps of plasmids useful for PsV production are available at [our website](#). Care should be taken when re-transforming the plasmids since the collection uses a wide variety of different drug resistance markers. It is currently possible to generate PsV for BPV1, CRPV, and HPVs 5, 6, 11, 16, 18, 31 and 45. Extensive efforts to generate high titer PsV stocks for HPVs 1 and 2 have so far been unsuccessful.
3. Although essentially any plasmid under 8 kb in size can be packaged by L1 and L2, PsV production efficiency varies with different reporter plasmids for reasons that are not fully understood. The presence of an SV40 ori on the target plasmid is not strictly required, but does typically augment titer yield by 5- to 10-fold. PsV carrying GFP reporter plasmids (e.g., pfwB<sup>14</sup>) are convenient since they can be easily titered by fluorescence activated cell sorting (FACS). [Neutralization assays](#) typically use a reporter plasmid encoding secreted alkaline phosphatase (SEAP)<sup>11,12</sup>. Reporter plasmids based on pCDNA3 (Invitrogen) and pCIneo (Promega) are also packaged with relatively high efficiency.
4. Because PsV are capable of transferring foreign DNA. It is important to note that the promiscuity of packaging by L1 and L2 can lead to generation of PsV with encapsidated fragments of cellular DNA, possibly including SV40 large Tumor antigen, adenovirus oncogenes, or unknown oncogenes present in 293TT cells. In a small set of experiments, we found that mature PsV can be inactivated by 70% ethanol. At a recent HPV meeting, Meyers and colleagues had a poster suggesting that isopropanol is more effective than ethanol for inactivating the infectivity of pseudoviruses and native virions. Like many other types of non-enveloped virus particles, they are resistant to a wide spectrum of physical insults, including various detergents and proteases, prolonged heating at 56°C, 50mM EDTA, and sodium chloride up to at least 2 M<sup>16</sup> (and unpublished results). PsV are probably also resistant to desiccation<sup>16</sup>. High-titer PsV should never be harvested using tip sonication, which can create potentially dangerous aerosols.
5. In most cases, substantial amounts of PsV are generated within 24 hours post-transfection. However, maximum titer yield is typically ~44 hours post-transfection. Although the SV40 ori<sup>+</sup> plasmid DNA content within the cell increases about 5-fold between 44 and 52 hours, very little additional PsV titer appears during this period, particularly if the L1+L2 plasmid is not also replicating (e.g., pSheLL). Titer yield at 72 hours is generally poor, presumably due to cell death triggered by over-replication of SV40 ori<sup>+</sup> plasmid DNA.
6. It is important to use siliconized tubes since PsV adsorb nonspecifically to polypropylene<sup>17,18</sup>. Long-term storage of purified PsV or VLPs at 4°C can result in loss due to nonspecific sticking, even in siliconized and polystyrene tubes (see [also Note 9](#)). Since the capsids are only in contact with pipet tips very briefly, the use of siliconized tips is preferred, but not essential.
7. During the overnight incubation of the cell lysate, pseudovirions "mature" into a more stable configuration. During maturation, disulfide bonds gradually form between L1 molecules and the capsid becomes condensed and shows improved regularity in electron micrographs<sup>14</sup>. Although the immature pseudovirions are infectious immediately after cell lysis, they are too fragile to withstand purification. It is sometimes practical to use crude cell lysates as PsV stock. If purification is not necessary, the maturation step can be omitted and the stock can be frozen in aliquots after addition of salt ([Subheading 3.2.3](#)).
8. Several alternative protocols for handling PsV are available at our lab [website](#). For preparation of large amounts of PsV it may be desirable to concentrate the clarified lysate using [equilibrium ultracentrifugation](#). If ultracentrifugation equipment is unavailable, VLPs or PsV can be partially purified by [gel filtration using agarose bead columns](#). Gel filtration can also be used to remove Optiprep from purified PsV stocks. Some of our earlier protocols suggested concentrating PsV using Amicon centrifugal filter units. However, EM analysis of PsV concentrated this way showed dramatic distortion of capsid structure, leading us to abandon the method.
9. For some papillomavirus types (particularly BPV1) a significant percentage of L1 particles may lack encapsidated DNA. Empty capsids migrate to the higher-density fractions (i.e. toward the bottom of the tube) relative to DNA-filled infectious PsV. For some applications, it may be desirable to achieve a lower particle to infectivity ratio by discarding fractions containing empty capsids. If gradient SDS-PAGE gels are available (e.g., NuPAGE precast 4-12% Bis-Tris / MOPS gels (Invitrogen)), it is often possible discriminate between the two types of particles by visualization of histone-sized proteins (~15 kD) in fractions with significant PsV titer. Another rapid method for screening fractions is to [extract encapsidated DNA](#) from 20 µl of each fraction using microcentrifuge silica columns (e.g., PCR Purification Kit (Qiagen)). In most instances the extracted DNA can be easily visualized by agarose gel electrophoresis followed by [staining](#) with ethidium bromide or SYBR Green I (Sigma). Finally, the fractions can be titered for [SEAP](#) or GFP transducing activity individually (see [Note 10](#)). A drawback to titrating the fractions is that they must be stored during the two to three days it takes to perform the titration. In order to avoid loss by non-specific adsorption to the tube walls (see [Note 6](#)), the fractions (or aliquots of pooled fractions) should be stored at -80°C. Alternatively, it is possible to saturate nonspecific binding by adding 10% FCS to PsV stocks, allowing storage for up to a month at 4°C. Although mature PsV stocks typically suffer less than a 25% loss of titer during freezing, repeated freeze-thaw cycles should be avoided. PsV stocks are stable at -80°C for at least a year.
10. If a GFP-expressing plasmid (e.g., p16L1-GFP or pfwB) was included in the transfection, it is possible to titer the stock using a FACS machine. For a pSheLL + pfwB stock, titer yield should be at least a billion GFP-transducing units per 75 cm<sup>2</sup> flask. GFP-based titration should be performed as follows:
  - Pre-plate 293TT cells in DMEM-10 in a 24-well plate at 1 x 10<sup>5</sup> cells in 0.5 ml per well. Incubate overnight. Alternatively, preplate 2 x 10<sup>5</sup> cells in 0.5 ml of DMEM-10 an hour or two in advance. Cells should be <50% confluent.
  - Use a 2.5 µl pipettor with short siliconized tips (BioPlas) to add 1 µl of PsV stock directly to a well of preplated cells. It may be necessary to dilute the stock 1:10 or 1:100 into DPBS/0.8M NaCl (use siliconized tubes and tips). Incubate cells 44 to 52 hours.
  - Trypsinize cells and resuspend in DPBS with 1% FCS. Analyze cells by FACS. Adjust an FL1 marker region to exclude at least 99.8% of untransduced control cells. Choose a PsV dilution that gives between 1 and 25% of cells falling in the FL1+ marker region. For titer (in transducing units per ml), use the formula [fraction of cells FL1+] x [200,000 cells inoculated] x [1000 µl/ml] x [stock dilution (if any)].

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## Optiprep Recipes

### Make 200 ml of DPBS / 0.8M salt:

153.5 ml of water  
 20 ml of 10x PBS  
 25 ml of 5M NaCl  
 90 µl of 2M CaCl<sub>2</sub>  
 50 µl of 2M MgCl<sub>2</sub>  
 420 µl of 1M KCl

### Make 50ml of 46% Optiprep in PBS / 0.8M salt:

38.3 ml of 60% Optiprep  
 5 ml 10x PBS

6.5 ml of 5M NaCl

23  $\mu$ l of 2M  $\text{CaCl}_2$

13 $\mu$ l of 2M  $\text{MgCl}_2$

100 $\mu$ l of 1M KCl

ml DPBS/0.8 / 46% Optiprep

27%:            9.3     /   13.2

33%:            6.4     /   16.1

39%:            3.4     /   19.0

## Numbers

- 1 pg of a 5.7kb plasmid has 171k copies.

- 1 L1 VLP weighs 72 pentamers x 5 L1/pentamer x 56 kD/L1 = 20 megaDaltons

- 1 ng of VLPs has 30 million particles

- L1 monomer at 1 mg/ml is 18  $\mu$ M

- virion density in Optiprep: empties = 1.25 g/ml; fulls = 1.20

- virion density in cesium = 1.32 g/ml

- 1 diploid human cell has 6.4 pg of DNA

- ATCC says 293 have modal chromosome # of 64 (occurs in 30% of cells). So they probably have a lot more than the normal 6.4pg of DNA per cell.