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Production of Furin-cleaved Papillomavirus Pseudovirions and their use for *in vitro* neutralization assays of L1 or L2-specific antibodies

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Abstract

Immunization with Human Papillomavirus (HPV) L1 virus-like particles or L2 capsid protein elicits neutralizing antibodies that mediate protection. A high throughput and sensitive *in vitro* neutralization assay is therefore valuable for prophylactic HPV vaccine studies. Over several hours during infection of the genital tract, virions take on a distinct intermediate conformation, including a required furin cleavage of L2 at its N-terminus. This intermediate is an important target for neutralization by L2-specific antibody, but it is very transiently exposed during *in vitro* infection of most cell lines resulting in insensitive measurement for L2, but not L1-specific neutralizing antibodies. To model this intermediate, we describe a protocol to generate furin-cleaved HPV pseudovirions (fc-PsV) which deliver an encapsidated reporter plasmid to facilitate infectivity measurements. We also describe a protocol for use of fc-PsV in a high throughput *in vitro* neutralization assay for the sensitive measurement of both L1 and L2-specific neutralizing antibodies.

Keywords

furin-cleavage; papillomavirus; antibody; human papillomavirus; HPV neutralization assay; HPV L2

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INTRODUCTION

The development of papillomavirus (PV) virions containing reporter constructs (generally called pseudovirions (PsV) (Buck et al., 2005) and their application for *in vitro* neutralization assays (Pastrana et al., 2004), has greatly facilitated the development of prophylactic HPV vaccines and measurement of their immunogenicity. The licensed preventive HPV vaccines are based on the seminal discovery that the L1 major capsid protein alone or co-expression with the minor capsid L2 protein will co-assemble into VLP (Kirnbauer et al., 1992; Zhou et al., 1993). Under both circumstances, these L1 or L1/L2 VLPs resemble empty HPV virions upon examination by an electron microscope, and when used for vaccination, elicit high titer type-specific neutralizing antibody responses (Harro et al., 2001).

Early research on the biology and immunology of HPV virions was hampered by the inability to readily generate quantities of virions in standard tissue culture, the absence of a simple and quantitative readout for infectivity and their inability to produce disease in an animal model. HPV PsVs carrying reporter plasmids have proved as a valuable surrogate of authentic papillomavirus for many such studies (Buck and Thompson, 2007) because they can be readily manufactured, have simple and flexible readouts of infectivity both in tissue culture and animals, including mice (Roberts et al., 2007).

Although highly successful and efficacious, the current licensed HPV vaccines provide mostly type-restricted and there remain logistical challenges for their global implementation including cost, and need for a cold chain, and needle-based delivery. Consequently there are ongoing efforts to develop second-generation HPV vaccines based upon L1, L2 or both capsid proteins. L2 is a promising vaccine antigen because of its potential to protect against multiple oncogenic HPV types (Alphs et al., 2008; Gambhira et al., 2007a; Gambhira et al., 2007b; Pastrana et al., 2005; Roden et al., 1994). However, because L2 does not form a VLP, it is weakly immunogenic relative to L1. Nonetheless, preclinical vaccination studies with L2 are sufficient to provide full prophylaxis against a large inoculum of HPV genotypes.

The standard *in vitro* neutralization assay developed by Buck and colleagues (Pastrana et al., 2004) often shows serum titers from L2 vaccinated mice to either be very low, or sometimes undetectable. Yet, passive transfer of the same anti-L2-serum antibodies can confer complete protection against experimental viral challenge in naive mice (Wang et al., 2014a). The discrepant findings for L2-specific antibodies between the *in vitro* neutralization assay (Pastrana et al., 2004) and animal protection studies (Roberts et al., 2007) suggest that the currently utilized *in vitro* PsV neutralization assay is not sensitive for anti-L2 neutralizing antibodies, although it detects L1-specific neutralizing antibodies with great sensitivity. Studies by Day *et al* utilizing HPV PsV identified spatio-temporal differences in the HPV L2-epitope exposure between the *in vitro* infection of 293TT cells used for neutralization studies (Day et al., 2008a; Day et al., 2007), and infection of basal keratinocytes in the mouse challenge model (Day et al., 2008a). This may reflect differences in the primary receptor in 293TT monolayers versus the basement membrane *in vivo*, the local

concentration of extracellular furin and the kinetics of infection, which together may account for the differences in the efficacy of L2-specific neutralizing antibodies *in vitro* versus *in vivo*.

Micro-trauma of the cervical epithelium, which results in the exposure of the basement membrane and access to basal keratinocytes, is crucial for HPV infection. Infection of epithelial cells is initiated first through the primary binding of the viruses to heparin-sulfate proteoglycans on the basement membrane. Binding to the basement membrane triggers a conformational change in the capsid and the subsequent exposure of previously buried L2 epitopes (Buck et al., 2005; Day et al., 2008a). The L2 protein contains a conserved furin-cleavage site (amino acid [AA] ~9–11) which, when exposed on the capsid surface, is cleaved by extracellular furin, further exposing a broadly neutralizing epitope (AA 17–36 defined by the RG1 monoclonal antibody). Once it achieves this infectious intermediate state, it enters host keratinocytes via an unknown secondary receptor (reviewed in (Buck and Trus, 2012; Wang and Roden, 2013)). This process, including the exposure of L2 neutralizing epitopes appears to occur differently or with distinct kinetics in the L1-based *in vitro* neutralization assay, limiting the detection sensitivity of anti-L2 neutralizing antibodies (Day et al., 2012). To improve the detection of anti-L2 antibodies, we first developed *Basic Protocol 1*, to generate the furin-cleaved HPV pseudovirions (HPV fcPsV) (Wang et al., 2014a) as a surrogate of the putative infectious intermediate, and in *Basic Protocol 2*, we describe their application in a furin-cleaved HPV PsV neutralization assay (FC-PBNA).

BASIC PROTOCOL 1 PRODUCTION OF FURIN-CLEAVED PSEUDOVIRIONS (FC-PSV)

The method of producing furin-cleaved PsV (fcPsV) is a modification of the original HPV PsV production protocol developed by Buck and colleagues (Buck and Thompson, 2007) (Figure 1A). Our method also relies on transfection of the codon-optimized HPV L1 and L2 capsid genes along with a reporter plasmid (see *Critical Parameters*). These constructs are introduced into 293TTF cell line, a human embryonic kidney cell line, 293T, which stably expresses an additional SV40 Large T antigen (Pastrana et al., 2004) as well as an additional human furin gene (NM_002569.2). The 293TTF cells co-transfected with the L1/L2 expression vector and reporter plasmid (which is encapsidated) are optimally maintained for 48 h prior to harvest, lysis and an *in vitro* maturation step. During the maturation step, we have found that raising the calcium concentration increases the activity of furin in the 293TTF cells (Anderson et al., 2002; Anderson et al., 1997), and cleavage of L2 in the PsV (Wang et al., 2014a) (Figure 1B). In this section, we describe 1) transfection of 293TTF cells with HPV codon-optimized viral capsids and reporter plasmid; 2) harvesting/lysing of transfected 293TTF cells; and 3) maturation and purification of fcPsV particles (*Basic Protocol 1*). A support protocol to use qPCR to determine full virion particle concentration (i.e. encapsidated reporter plasmid) is included. Western blot-based determination of furin cleavage and infection assays for further analysis of fc-PsV preparations are discussed in *Critical Parameters*.

Materials

**See Reagents and Solutions for solutions required for the production of fcPsV, and the culture media used for the maintenance of 293TTF cells.*

293TTF cells [See Background Information]

293TTF culture medium (see recipe)

OptiMEM[®] I reduced serum medium (Invitrogen[™]/Life Technologies, cat# 11058-021)

Mirus TransIT[®]-2020 transfection reagent (Mirus Bio LLC, cat# MIR 5404)

HPV types specific L1 and L2 vector plasmid and a reporter plasmid

Note: Plasmids can be obtained from (Addgene: http://www.addgene.org/Richard_Roden or http://www.addgene.org/John_Schiller/).

Siliconized pipette tips (VWR, cat#60828-914) *Optional*

Siliconized 1.5 ml screw-cap tubes (Fisher Scientific, cat# 05-541-63)

OptiPrep[™] density gradient medium (60% w/v, Sigma, cat# D1556)

PBS (Invitrogen[™]/Life Technologies, cat# 10010-023)

0.05% trypsin-EDTA

0.25% trypsin/EDTA

DPBS (Invitrogen[™]/Life Technologies, cat# 14040)

DPBS with Mg⁺⁺ and Ca⁺⁺ (Invitrogen[™]/Life Technologies, cat# 14287-080),

10X DPBS (Invitrogen[™]/Life Technologies, cat# 14200-075)

DPBS-10mM MgCl₂ with antibiotics (see recipe)

DPBS-0.8M NaCl (see recipe)

5M NaCl (KD Medical, cat# RGF-3270)

1M CaCl₂ (KD Medical, cat# PMS-0614)

1M KCl (KD Medical, cat# PMB-0730)

1M MgCl₂ (KD Medical, cat# PMS-0630)

Distilled water (Invitrogen[™]/Life Technologies, cat# 15230-147)

15 mL Polyallomer ultracentrifuge tubes (Beckman Coulter, cat# 331374) or 5 mL Polyallomer ultracentrifuge tubes (Beckman Coulter, cat# 326819)

Swinging bucket ultracentrifuge rotor rated for >200,000g (e.g., SW55ti for 5 mL tube or SW40ti for 15 mL tube).

Microfuge (eg, Eppendorf Centrifuge 5417R), *Need to use it at 4°C*

Centrifuge (e.g., Thermo Scientific Sorval Legend RT+)

Ultracentrifuge (e.g., Beckman Coulter Optima L-80 XP Ultracentrifuge)

T-225 flask (BD, cat# 353138)

T-150 flask (Corning, cat# 430825)

BD 1 mL TB syringe with 25G needle (BD, cat# 309626)

Pipetting needles with blunt end and standard hub (Popper and sons, cat# 7936)

Brij58 (Sigma-Aldrich, cat# P-5884)

Lysis buffer (see recipe in Reagents and Solutions)

Additional reagents and equipment for SDS PAGE (see Appendix 3M), Western blotting (see UNIT 14A.2), cell counting with hemocytometer (see Appendix A)

Preparation of 293TTF cells for transfection (Day 1)

1. Prepare flasks of 293TTF cells in 293TTF media that are growing in the exponential phase.
Note: Avoid using overgrown cells. We recommend using cells when the flask is generally 60–80% confluent. Size of the flask for the maintenance of 293TTF cells should be determined according to the laboratory's needs. Incubator should be set to 37°C with 5% CO₂. 293TTF media should also contain 2 µg/mL of puromycin for optimal furin expression.
2. Aspirate the media in the flasks.
3. Add 5–10 mL of sterile PBS gently to the side of the flasks to avoid detaching cells, and gently spread it around the flasks.
4. Aspirate the PBS.
5. Add 3 mL of 0.05% trypsin-EDTA, and gently spread the solution to distribute the solution evenly.
6. Place the flasks into a 37°C, 5% CO₂ tissue culture incubator for 3–5 minutes.
7. Take the flasks out and tap on the side to detach the cells: Check under the microscope to ensure that all cells have detached.
8. Add 10–15 mL of 293TTF culture medium, and gently rinse the flask with the media before transferring the cells to sterile 50 mL conical tubes.
9. Adjust volume of the tubes to 40–45 mL with culture media and centrifuge at $300 \times g$ at room temperature for 5 minutes.
10. Aspirate the media, loosen the cell pellet, and repeat Step 9 one more time.
11. Aspirate the media, and resuspend in 25 mL of the culture media.
12. Count cells by hemocytometer using trypan blue.
13. Prepare four to six T150cm² flasks per HPV type, and plate 7×10^6 293TTF cells per flask in 20mL of culture media with 2 µg/mL of puromycin.

Note: If T225 flasks are used, prepare four to six flasks per HPV type and plate 21×10^6 293TTF cells per flask in 30 mL

14. Incubate the flasks in a 37°C, 5% CO₂ tissue culture incubator overnight.

Transfection of 293TTF cells (Day 2)

15. The next day, prepare transfection mixture as per MirusIT-2020 (MirusBio LLC, cat# MIR 5404) manufacturer's protocol. In this protocol, T150cm² flask is utilized as an example, and the following culture media volume and DNA quantities are required per flask:

Note: We also recommend measuring DNA concentration of each construct on the day of the transfection. Transfection conditions can be scaled up or down depending on quantity desired, and is dependent on the size of the cell culture flask. Different transfection reagents (e.g. Lipofectamine 2000, Invitrogen™/Life Technologies, cat# 11668-019) can be used, but transfection conditions need to be optimized for each respective transfection reagent. 56.25 µg of HPV and reporter DNAs (112.5 µg total) is added into the transfection complexes for a T225 flask.

16. Add the entire volume of the transfection solution shown in Table 1 into one flask of 293TTF cells. Scale the reagents up or down depending on number of flasks used.

Note: Adjust the concentration of puromycin to 2 µg/mL for optimal furin expression in 293TTF cells, after addition of transfection complex solution.

17. Incubate for 48 hours in a 37°C, 5% CO₂ tissue culture incubator.

*Note: While most manufacturer's transfection protocols suggest that transfection can occur up to 72 hours, **it is important not to exceed 48 hours incubation**, (Buck and Thompson, 2007). As major cell death and low yield occurs beyond 48 hours, potentially due to over-replication of SV40 ori⁺ reporter plasmid DNA, thereby reducing the yield of cells for subsequent HPV fcPsV extraction.*

Harvesting of transfected-293TTF cells (Day 4)

18. Label both 50 mL conical tubes and 1.5 mL siliconized tubes; one for each transfection flask.
19. Collect the media from each flask into the labeled 50 mL conical tubes.
20. Gently wash the transfected 293TTF cells with 2–5 mL of sterile PBS by swirling around in the flask, and then aspirate the PBS.
21. Add 3 mL of 0.25% trypsin/EDTA per flask, and incubate at 37°C for 5 minutes.

Note: It has been observed that transfected 293TTF cells are more adherent compared to regular 293TTF cells, hence the higher concentration of trypsin (0.25%) is utilized to detach all cells.

22. After the incubation, ensure that all the cells are detached. If cells remain adhered, tap the flask gently to detach the cells.
23. Add 25 mL of media collected in Step 19 into each flask, and collect the trypsin-treated cells per flask into the same 50 mL conical tubes.
24. Centrifuge the harvested 293TTF cells at $300 \times g$ for 10 minutes at room temperature.
25. Carefully aspirate the media.
26. Add 1mL of DPBS-10mM $MgCl_2$ with antibiotics to each conical tube to re-suspend the 293TTF cells.
27. Transfer all the resuspended cells into the 1.5 mL siliconized tube (one for each conical tube).
28. Centrifuge the siliconized tubes at $300 \times g$ for 10 minutes at $4^\circ C$ using a microfuge (e.g., Eppendorf Centrifuge 5417R).

Note: During this centrifugation or during centrifugation in Step 24, prepare Lysis Buffer (must be prepared fresh for each experiment. See Reagents and Solutions for the recipe for the Lysis Buffer).

29. After centrifugation, manually remove the supernatant using a 1000 μL pipette. Keep the cell pellet for cell lysis and virus maturation.

Cell lysis and virus maturation (Day 4)

30. Estimate the volume of the cell pellet, and add an equal volume of the Lysis Buffer to the pellet.
31. Gently re-suspend the cells via flicking or tapping the tube (Do not vortex).
32. Based on the total volume (volume of the cell pellet + equal volume of the Lysis Buffer), calculate the amount of 1M $CaCl_2$ solution to be added to the lysate to make a final concentration of 5mM of $CaCl_2$.

Note: For example, for 1 mL of the total volume, add 5 μL of 1M stock of $CaCl_2$ (1/200 dilution) to the sample tubes. At this point, based on published data (Buck et al., 2005), the pseudovirion particles produced in 293TTF cells are still in the immature state.

33. Incubate the lysates in a $37^\circ C$ water bath for 48 hours for maturation. It may be helpful to mix the lysates by gentle inversion once or twice during the first couple hours (seal around the edge of the cap with a piece of parafilm to prevent the influx of water).

*Note: **This is the most important step in the production of fcPsV**, as it is during the maturation phase where exposed L2 in the immature PsV particles*

are being cleaved (Figure 1B). Addition of 5mM CaCl₂ helps to increase the overall calcium content, and improves furin activity in cell lysates over-expressing furin (Anderson et al., 2002). We have also shown that 48 hours is the optimal time period for the maturation and for the cleavage of L2 by furin (Wang et al., 2014a).

Preparation of OptiPrep density gradients and ultracentrifugation of matured fcPsV particles (Day 6)

Prior to starting this phase, make sure that the ultracentrifuge is cooled to 16°C and ready for use.

34. Prepare the OptiPrep density gradient solution (based on the stock concentration of 60%) by first diluting it to 46% by mixing 46.05mL of 60% OptiPrep solution with 13.95mL of DPBS-0.8M NaCl.
35. Using DPBS-0.8M NaCl, further dilute the 46% OptiPrep to prepare 39%, 33% and 27% solutions.

Note: Excess gradient solutions can be stored at 4°C for future use (up to 2 weeks). These layering volumes in Table 2 are based on the utilization of 15mL Beckman Ultra-clear polyallomer tubes in which a SW-40Ti ultra-centrifuge rotor is used to purify the HPV fcPsV. The layering volumes can be adjusted based on Ultra-centrifuge equipment utilized. (See Choice of rotors in Critical Parameters).

Prepare the OptiPrep gradient solution by layering these three solutions (3 mL each) with different densities on top of each other (starting with 39%, 33%, and then 27%) in thin wall ULTRA-CLEAR polyallomer 15 mL tubes (e.g., Beckman, cat# 331374). After layering each gradient layer, mark the interface between the layers (Figure 2).

36. Prepare siliconized tubes by labeling them with appropriate information, and place them on ice for 5–10 minutes
37. Take the tubes containing the matured lysates from the 37°C water bath, and put them on ice for 10 minutes.
38. Centrifuge the matured lysates at 10,000 × g for 10 minutes, at 4°C.
39. Transfer the clarified supernatant into a fresh siliconized tubes prepared in Step 36.
40. Add 1mL of DPBS to the pellet in the siliconized tube, resuspend the pellet by flicking the tube gently, and repeat Steps 38 and 39.
41. Load the pooled clarified supernatant onto the Opti-Prep gradient by using a pipette with 200 µL siliconized tips by gently dispensing down on the side of the polyallomer ultracentrifuge tube.

Note: In some previous HPV PsV protocols, DPBS-0.8M NaCl is utilized. In this protocol, the NaCl is omitted because the Lysis Buffer does not contain

any DNase. Hence, the addition of any excess salt will result in release of DNA from chromatin, making the lysate too viscous for subsequent purification (see *Critical Parameters*).

42. Centrifuge at $28,000 \times g$ for 16 hours in a SW40.1Ti (or SW55Ti $36,600 \times g$) rotor at 16°C in an ultracentrifuge. Ensure that acceleration and deceleration is set at “slow”.

Collection of fcPsV particles (Day 7)

43. Following 16 hours of ultracentrifugation, remove tubes and securely fasten on a clamp ring or retort stand.
44. Hold a dark object behind the clamped tube to check whether an opaque layer of fcPsV particles can be visualized between the interface of the 33% and 39% OptiPrep layer (Fig 2, arrow B).

Note: It may be difficult to visualize the band. However, the marking of the interface between the OptiPrep layers prior to cell lysate loading and purification should help in providing an idea on where the optimal HPV fcPsV fraction resides.

45. Label 9 siliconized tubes.
46. Ensure that the sample tube is firmly secured on the clamp, and collect 9 fractions from the bottom of the tube by piercing a hole on the bottom with a 25 gauge needle.

Note: Precaution should be taken when using needles. Ensure that protective gear is used. We use a hexarmor glove on the hand stabilizing the polyallomer ultracentrifuge tube.

47. Collect 250 μL for each fraction.

Note: With the 15 mL polyallomer tube, the peak fractions that contain the functionally active fcPsV particles will generally be in Fractions 5–6. If using 5 mL tubes, one could collect eight 250 μL fractions, the peak fractions will generally be in Fractions 3–4.

48. Save 50 μL from each fraction, and determine the fraction with the highest yield of L1/L2-capsids (expected MW 50kDa and 75kDa respectively) by loading 10–20 μL of each fraction onto a 4–20%SDS-PAGE gradient gel followed by coomassie blue staining (see *Critical Parameters*). This step also assesses the purity of the fractions.
49. Fractions with the highest presence of HPV L1/L2- capsids following SDS-PAGE and coomassie blue staining suggest that they contain the highest amount of purified infectious virions. Pool as appropriate and make 20–50 μL aliquots in siliconized tubes and store at -80°C . Also, keep the other fractions at -80°C until the below procedures have been completed.

Our experience has been that the fraction that contains the highest amount of L1 protein will generally contain the highest amount of reporter plasmid.

50. Using the aliquots, perform the following downstream experiments to determine the following properties:

- a.** Biological activity of fractions, i.e. optimal viral titer (see *Critical Parameters, Assessment of optimal viral titer via Infectivity Assays*). Furin-cleaved properties of the fcPsVs are measured via infectivity assays using furin-deficient cells or usage of furin inhibitor can be performed also on this aliquot (see *Critical Parameters, Assessment of Furin-cleavage*). When setting up the assay for the very first time, it is recommended that one determines an optimal cell number to be used.

Note: To be used in the FC-PBNA assay described in Basic Protocol 2, the fcPsV to be used can be diluted to 1:10 in the Assay Media (see recipe in Reagents and Solutions), aliquotted, and stored at -80° . As a precaution, we recommend that you only use these vials once (do not re-freeze). Therefore, the volume to aliquot should be based on your general experimental needs. Typically, this 1:10 solution will further be diluted to 1:1000 as a starting dilution factor to be used in a final volume of 200 μ L in a 96-well plate.

- b.** Viral reporter genomic equivalents via qPCR (see *Support protocol 1*)
- c.** Degree of L2 protein cleavage via Western blotting (see *Critical Parameters, Percentage of L2 cleavage*).

SUPPORT PROTOCOL 1

Assessment of encapsidated reporter plasmid or 'Viral Genomic Equivalents (VGE)'

This protocol describes the detection of encapsidated reporter plasmid as viral genomic equivalents (VGE) as an approach to standardize the amount of fc-PsV regardless of HPV type for subsequent *in vitro* or *in vivo* studies. The method involves extraction of reporter plasmid from a given volume of HPV fcPsV virions and performing qPCR analysis to detect encapsidated reporter plasmid molecules versus a known standard. Below, we outline the steps to perform quantitative polymerase chain reaction (qPCR) analysis to detect HPV fcPsV viral reporter plasmid genomic equivalents with a known standard.

Additional Materials

Machine able to perform qPCR

Known quantity (i.e 5–50 nanograms) of reporter DNA to be utilized for the standard curve during qPCR analysis. (e.g. SEAP: pYSEAP construct, or LUCIFERASE: pcDNA-luciferase plasmid from: <http://home.ccr.cancer.gov/LCO/plasmids.asp>)

PureLink Viral RNA/DNA extraction kit (Invitrogen, cat# 12280-050)

Forward and Reverse Primers for SEAP/Luciferase plasmid (500nM)

Luciferase Firefly Forward: TTG ACC GCC TGA AGT CTC TGA

Luciferase Firefly Reverse: ACA CCT GCG TCG AAG ATG TTG

SEAP Forward: GTA CCC AGA TGA CTA CAG CCA AG

SEAP Reverse: GGT GGA TCT CGT ATT TCA TGT CT

SsoFast EvaGreen Supermix (Bio-Rad, cat# 172–5200)

Optical seals for qPCR plates (Microseal 'B' Adhesive Seals, Optical, BioRad #MSB-1001)

96-well plate for qPCR

qPCR reaction buffer (e.g. EVAgreen Supermix, Bio-Rad, cat#172–5200)

Viral Extraction and setting up of qPCR—

1. Extract the reporter genome plasmid from a known volume of HPV fcPsV sample.

Note: The kit utilized for HPV fcPsV reporter genome extraction is PureLink Viral RNA/DNA extraction kit (Invitrogen, cat#12280-050). However, other extraction kits can also be utilized. In general, 10–15µL of purified fraction from the aliquot is used for viral extraction.

2. For qPCR, a reaction volume of 20µL is utilized. All standards and samples are analysed using either 2 or 3 reactions, respectively
3. For simplicity, a 2.2X or 3.3X reaction volume of master mix is made for each standard or sample to be tested, respectively (See Table 3).
4. After making the reactions, aliquot 20 µL/well of total reaction of the respective standard/sample onto the qPCR plate. Discard the excess reaction.
5. Seal the plate with the plate sealer (BioRad, #MSB-1001) and centrifuge the plate for approximately 5 minutes to ensure all contents are at the bottom of the plate well.
6. Load the plate into a qPCR machine.
7. Set qPCR cycle conditions according to conditions previously optimized using dilution series of reporter DNA to assess assay sensitivity and linearity (conditions will vary based upon buffer, machine and dye system used), and run the reaction.
8. Following the qPCR, the VGE (copy number) can be calculated using the known amounts of reporter plasmids from the Standard C_T values as well as the C_T values from the samples tested.

Particle: infectivity ratio can subsequently be extrapolated based on calculations previously whereby it was estimated that there are 30,000 capsids per picogram of L1 protein (Handisurya et al., 2012).

FURIN-CLEAVED HPV PSEUDOVIRUS NEUTRALIZATION ASSAY (FC-PBNA)

The basic concept of this assay is the same as that of the well-established L1-PBNA originally developed by Buck and colleagues at NCI (Pastrana et al., 2004). This L1-PBNA uses 293TT cells as target cells, and uses PsV particles (not furin pre-cleaved) to infect 293TT cells (Figure 3A). However, this assay was found to be suboptimal for the detection of anti-L2 antibodies and it has been hypothesized to be due to insufficient L2 epitope exposure (Day et al., 2012); therefore, we have developed the current assay using fcPsV particles and LoVoT cells. The fcPsV particles have a far greater amount of L2 epitopes exposed, compared to regular uncleaved HPV PsVs (Day et al., 2008a; Day et al., 2008b; Wang et al., 2014a). Briefly, FC-PBNA uses fcPsV particles of HPV types of your choice to infect a furin-deficient cell line (LoVo) that overexpresses the SV40 Large-T antigen (LoVoT) (Figure 3B). The LoVoT cell line was created specifically for this assay (Wang et al., 2014a; Wang et al., 2014b). As LoVoT cells are furin-deficient (and infection requires furin cleavage of L2), only those PsV particles whose L2 proteins have already been cleaved during the production (*Basic Protocol 1*) can infect, but will still be hindered by the presence of HPV capsid neutralizing antibodies (Figure 3). Also, the cell line has been transfected with SV40 Large-T antigen (Wang et al., 2014a) which allows the reporter plasmid to be amplified in the target cells, as it contains the SV40 origin of replication. The FC-PBNA can be performed using HPV fcPsV that deliver SEAP (*Basic Protocol 2*), luciferase (*Alternate Protocol 1*), or GFP reporter plasmids (Figure 3). For choices of reporter genes, and their advantages and disadvantages, see *Critical Parameters* under “*Choices of reporter genes*”.

BASIC PROTOCOL 2: PERFORMING FC-PBNA WITH SECRETED ALKALINE PHOSPHATASE

This protocol describes the use of a secreted alkaline phosphatase (SEAP) reporter plasmid encapsidated within fcPsV particles for the FC-PBNA, followed by the description of an assay to detect SEAP as a measure of infectivity. Prior to conducting the FC-PBNA assay, fcPsV particles should be diluted to 1:10 in Assay Media, aliquotted into siliconized flip-cap 1.5 mL tubes, and stored at -80°C (as described in *Basic Protocol 1*). In addition, the dilution factor of the fcPsV to be used in the neutralization assay must have been predetermined for each preparation, and especially for different HPV types (see *Critical Parameters* under “*Assessment of optimal viral titer via Infectivity Assays*”). A dilution factor of the virion preparation that would lead to approximately 100-fold increase in signal over background (i.e. signal-to-noise ratio) is recommended.

Materials

LoVoT cells (see Background Information)

Furin-cleaved pseudovirions (fcPsV) particles (see *Basic Protocol 1*)

Serum samples or Antibody samples (See *Critical Parameters*)

Assay Media (see recipe in Reagents and Solutions),

*Note: DMEM must be **phenol red-free** for SEAP-based assays.*

1× PBS without Ca⁺⁺ or Mg⁺⁺ (Life Technologies, cat# 14190)

0.05% Trypsin/EDTA (Life Technologies, cat# 25300)

0.4% trypan blue stain (Life Technologies, cat# 15250)

Certified class II biological safety cabinet

37° C CO₂ incubator (Forma Scientific, Model 3110)

Centrifuge (Thermo Scientific; Sorval Legend RT+)

Serological pipet filler (Thermo Scientific, cat# 9531)

10, 20, 100, 200, and 1000 µL single or multi-channel pipets (Rainin)

5 and 10 mL serological pipettes (Costar, cat# 4051 and 4101)

10 – 1000µL pipet tips (Rainin, cat# SR-L10S,-250S, and -L1000S)

50 mL conical tubes (BD Falcon, cat# 352098)

Hemocytometer (Improved Neubauer 0.1mm deep)

Inverted light microscope (Nikkon TMS and Nikkon LBOPHOT)

50 mL reservoir troughs (Costar, cat# 4870)

Flat-bottom 96-well tissue culture plates (Costar, cat# 3596)

–80°C freezer (Forma Scientific, Model 8517)

15 mL conical tubes (Corning, cat# 430055)

Conical-bottom, deep-well, 96-well plate (eg, 0.5 – 2 mL; VWR, cat# 40002-022, –011, or –014)

Depending on the number of test-wells, different size plates can be employed

Round-bottom 96-well tissue culture plates (Costar, cat# 3788)

V-bottom 96-well plates (Costar, cat# 3357)

Seal plate films (Thomas Scientific, cat# 6980A03)

Cold storage adhesive sealing foil (VWR, cat# 89049-034)

Ziva[®] Ultra SEAP Plus Detection Kit (Jaden BioScience Inc., cat# CM025) (see *Background Information*)

Plate shaker (eg, Thermo Scientific titer shaker model 4625)

White opaque 96-well microplate/OptiPlate-96 (Perkin Elmer, cat# 6005290)

Oven capable of reaching 65–73° C (e.g., Thermo Scientific, Model Heratherm OMH100)

Aluminum foil

Plate reader capable of measuring luminescence (e.g., Molecular Devices, SpectraMax M5)

Computer and appropriate software to operate the plate reader

Preparation of cell line, LoVoT

1. Take out a flask of LoVoT cells from a 37°C, 5% CO₂ incubator (see *Critical Parameters*).

Note: Media used to maintain the cell line is the same as the one used for the Assay Media (see recipe in Reagents and Solutions). However, DMEM with phenol red (Life Technologies; cat# 11965) can be used. As phenol red serves as a pH indicator, it is useful in gauging the general health of the cells in culture.

2. Aspirate the culture media with a 10 mL serological pipet (see *Biosafety*).
3. Gently dispense 10 mL of sterile PBS into the flask, so as not to touch the cells that are attached.
4. Gently swirl the solution of PBS inside the flask with the cap on, and then, aspirate PBS as in Step 2.
5. To detach and collect cells, add 2 mL of 0.05% trypsin/EDTA solution, gently spread it over the cells, put the cap back on, and incubate 3 – 5 minutes in a 37°C, 5% CO₂ incubator.

Note: LoVoT cells adhere very well to plastic, hence examine under an inverted light microscope to ensure that the cells have completely detached. Taps on the side of the flask will help detach the cells.

6. Add 10 mL of Assay Media into the flask to neutralize trypsin/ EDTA, and transfer the cells into a 50 mL tube, and bring the volume to 15–20 mL with Assay Media.
7. Centrifuge for 5 minutes at 300× g at room temperature.
8. Aspirate the supernatant into the waste reservoir, and wash the cells one more time with 10 mL Assay Media to remove all phenol red.
9. Repeat Step 7.
10. Aspirate the supernatant, and resuspend the cell pellet well to achieve single-cell suspension, and add 3– 5 mL of Assay Media.
11. Count the cell number using trypan blue stain, and determine the cell number.
12. Determine how many cells are needed for an experiment, and transfer approximately 20–30% more than needed for your experiment to a different conical tube.
13. Adjust the cell concentration to the predetermined number with Assay Media.

Note: Optimal cell numbers to be used per well should be determined by performing infection of LoVoT cells with fcPsV particles of your interest prior to performing the neutralization assay. $0.15 - 0.3 \times 10^6$ / mL range will generally achieve an optimal signal-to-noise ratio.

14. Place the cells in a 50 mL reservoir, and dispense 50 μ L of cells into all the wells in a flat-bottom 96-well plate using a multi-channel pipet.
15. From a different reservoir that contains Assay Media, pipet 50 μ L into all the wells.

Note: Alternatively, one could adjust the cell concentration in Step 13 to allow one to pipet 100 μ L of cells.

16. Incubate the plate in a 37°C, 5% CO₂ incubator to allow cells to settle for 2–4 hours to allow the cells to settle.

Note: While the cells are being incubated, prepare 1:1 mixture of sample and fcPsV particles. In the end, 100 μ L of the sample/fcPsV mixture will be transferred to the plate that contains LoVoT cells.

Titration of serum and addition of SEAP reporter fcPsVs

17. Thaw serum samples and vials of fcPsV particles that had been diluted to 1:10 in Assay Media on ice inside a biological safety cabinet (see *Critical Parameters*).

Note: If there is a control antibody or a control sample, it should also be taken out. Other controls to consider are no-virus [LoVoT cells only] and infectivity [LoVoT cells plus the fsPsV] controls. Keep vials on ice after the reagents have thawed.

18. Once the serum samples have thawed, prepare 4 $\times$ dilution in Assay Media.

Note: If the final dilution factor of the sample after its addition to the LoVoT cell seeded-plate (Step 15) is 1:100, prepare 1:25 dilution. If the same sample is being tested against multiple different fcPsV HPV types or in multiple plates, prepare large enough volume according to the needs. This can be done in a 15 mL conical tube and then dispensed into a 96-well plate. Depending on a number of test plates being setup, a regular size or a 1 – 2 mL deep well 96-well plate may be used.

19. Dispense 140 μ L of the 4 $\times$ samples into appropriate wells in appropriate sized 96-well plate. For the no-virus and infectivity controls, dispense 140 μ L of Assay Media.
20. Dispense 70 μ L of Assay Media to the rest of the wells, and perform 2-fold serial dilutions by transferring 70 μ L of the 4x sample to the next wells. Mix the samples, and repeat the process.

*Note: Number of serial dilutions and the magnitude at which these dilutions are performed must be estimated (see *Critical Parameters*). Discard 70 μ L from the last wells after mixing.*

21. Put a plate sealer film on the plate, and put it aside while preparing dilution of fcPsV particles.
22. In a 15 mL conical tube, prepare 4× fcPsV dilution in Assay Media.
- Note: The optimal dilution factor to be used for different fcPsV types must have been determined prior to the experiment. We recommend using a dilution factor of fcPsV that would lead to approximately 100-fold increase in signal-to-noise ratio (infectivity divided by no-virus control).*
23. Place the 4× fcPsV particles into a reservoir trough, and dispense 60 µL into appropriate wells in a new round-bottom 96-well plate with a multichannel pipet.
- Note: Layout of the fcPsV particles should match that of the sample plate. For no-virus control, dispense Assay Media.*
24. Peel off the plate sealer from the sample plate prepared in Steps 20 and 21, transfer 60 µL of the diluted samples to the appropriate wells to the round-bottom plate containing fcPsV particles.
25. Put the lid on and incubate the round-bottom 96-well plate that now contains the mixture of fcPsV particles and diluted serum samples in a 37°C, 5% CO₂ culture incubator for 2 hours.
- Note: Each well should contain 120 µL of solution*
26. After 2 hours, take out both the flat-bottom 96-well plate (LoVoT cell plate from Step 16), and the round-bottom 96-well plate (fcPsV/sample plate). Mix by pipetting, and transfer 100 µL of the fcPsV/sample mixture to the plate of LoVoT cells.
- Note: Each well should have 200 µL*
27. Incubate the plate (LoVoT cells/fcPsV/sample) in a 37° C, 5% CO₂ incubator for 3 days (70–74 hours), (e.g., if the assay was performed on Monday, then, Thursday will be the day to harvest the samples).

Preparation of SEAP assay supernatant samples for detection of SEAP

28. After 3 days, take out the plate, transfer 150 µL of the supernatant to a new V-bottom 96-well plate (keep the same layout of the plate).
29. Centrifuge the V-bottom plate for 5 minutes at 300 × g (room temperature) to pellet down cells that might have carried over from pipetting.
30. Transfer 100 – 120 µL of the supernatant to a new round-bottom 96-well plate, seal with cold storage adhesive foil seal, put the lid on top, and store the plate at –20° C or –80° C until ready to perform SEAP detection assay.
- Optional: One may proceed immediately to performing SEAP assay (see below). However, regardless, the supernatant samples should be frozen for storage. We recommend testing the samples within 1 week of the storage.*

Detection of SEAP in the supernatant samples (SEAP assay)

This section describes the detection of SEAP by using a commercially available chemiluminescence-based SEAP detection kit. We describe the use of Ziva® Ultra SEAP Plus Detection Kit from Jaden Bioscience Inc. It is recommended that all procedures for setting up the assay be performed inside a certified class II biological safety cabinet.

31. Bring out the SEAP detection kit from a refrigerator, and equilibrate to room temperature.
32. Take out the plate that was frozen in Step 30 of the previous section, and thaw supernatant samples at room temperature.
33. Once thawed, mix on a shaker at approximately 250 rpm for 1 minute.
Optional: To pellet down any potential debris that may interfere with the assay, centrifuge the plate for 5 minutes at $1700 \times g$.
34. Using a multichannel pipet, dispense 75 μL SEAP Sample Preparation Solution (SSPS) into an OptiPlate-96 white opaque 96-well plate.
Note: Determine how many plates will be tested and calculate how much buffer will be required before dispensing SSPS into a reservoir. As a rule of thumb, approximately 7.2 mL is required per plate.
35. Using a multichannel pipet, transfer 5 μL of the thawed supernatant samples into SSPS-containing plate (remember to keep the same layout when dispensing samples).
Note: The plate of supernatant samples can be refrozen. However, keep your samples at 4°C until the assay is finished and any repeats identified, such that the experiment does not need to be repeated using the same samples. It is recommended that the number of freeze-thaw be limited.
36. Cover the plate with a plate sealer film, mix on a shaker for 1 minute at approximately 250 rpm.
37. Place the plate in an oven to inactivate heat-unstable (endogenous) alkaline phosphatases (see *Critical Parameters*).
Note: The temperature of the oven should be between $65 - 73^{\circ}\text{C}$, and the length of incubation will generally range between 30 – 45 minutes. We heat inactivate at 73°C for 45 minutes. However, these conditions should be optimized for each experimental system with the equipment being used. Also, a water bath may be used instead of an oven. We use an oven because more plates can be placed in an oven than in a water bath, and the placement and removal of plates are easier.
38. After heat-inactivation, cool the plate on ice for 5 – 10 minutes to bring to room temperature.
39. Centrifuge the plate at $1700 \times g$ for 1 minute to bring down liquid that may have formed on the walls of the wells because of condensation.

40. Peel-off the plate sealer, and add 25 μ L of substrate.

Note: Care should be taken when handling substrate, as it is light sensitive. Calculate how much is needed for experiment, and then dispense enough volume to a reservoir. 2.4 mL of substrate is required per plate.

41. Put a plate sealer on, and mix on a shaker at approximately 250 rpm for 1 minute while protecting from light by covering with a piece of aluminum foil.
42. Incubate the plate for 30 minutes in dark by placing it inside a drawer with aluminum foil on top.
43. Measure the level of luminescence using a plate reader.

Note: The integration time should be optimized for your system. With Molecular Devices' SpectraMax M5, we use integration time of 0.2 second. The results will be expressed in relative-light-unit (RLU).

ALTERNATE PROTOCOL 1 PERFORMING FC-PBNA WITH FIREFLY LUCIFERASE

This protocol details the use of HPV fcPsV that deliver a firefly luciferase reporter plasmid. Like the SEAP method (*Basic Protocol 2*), prior to performing the FC-PBNA, fcPsV particles should have been tested for the dilution to achieve a ~ 100 fold signal-to-noise ratio. The utility of using fcPsV with luciferase reporters is that these viruses can also be used for *in vivo* challenge studies.

Addition materials

Clear plastic 96 well plates
 Black 96 well optiplate (#6005290, PerkinElmer)
 Multichannel pipette
 Microplate Luminometer with an injector
 Dual-Luciferase[®] Reporter Assay System Promega (Promega, ca# E1910 or # E1960)

Preparation of LoVoT cells

1. Prepare LoVoT cells as described in *Basic Protocol 2*, Steps 1–12.
Note: unlike the SEAP assay, the presence of phenol red does not interfere with the reading of luciferase reporter. Hence, the same culture media as described in Basic Protocol 2 can be used.
2. Adjust the LoVoT cell concentration to 0.015×10^6 / mL using the culture media. Transfer the cells to a reservoir, and dispense 100 μ L (0.015×10^6) to each well in a flat-bottom 96-well plate.
3. Incubate plates in a 37° C, 5% CO₂ tissue culture incubator for 4 hours to allow cells to seed and settle.

4. Proceed to titrate serum and add HPV fcPsV with luciferase reporter as per the steps indicated in *Basic Protocol 2*, steps 17–27.

Cell lysis and the measurement of luciferase activity

This section discusses the Firefly Luciferase assay using a Luminometer with an injector (e.g. ProMega, GloMax-Multi+Detection System).

Note: Depending on the machine utilized, the method to read the luciferase readings vary and will depend on the manufacturer's recommendation on optimal use of the device.

5. Prior to starting the cell lysis and analysis step, remove the required amounts of luciferase and thaw it as the reporter assay kit is stored at -20°C .
6. Take out the assay plates from the 37°C , 5% CO_2 tissue culture incubator, and aspirate the media manually with a multi-channel aspirator. Place the tips at the side of the well walls so as to not aspirate out the cells.
7. Add 30 μL of 1X Cell Culture Lysis Buffer to each well on the plate using a multichannel pipette.

Note: Lysis Buffer is provided as 6X; therefore, you will need to dilute with water to make it 1X from 6X stock. Following the addition of Cell Culture Lysis Buffer, one may proceed immediately to performing the luciferase assay (see below). However, samples can be stored at -20°C following addition of Lysis Buffer (prior to shaking). However, it is recommended that the luciferase assay be performed within 1 week of the storage.

8. Put the plates on a shaker, and shake for 15 min at a moderate speed.
9. After shaking, use a multichannel pipette and collect all the cell lysate from each well and gently pipette it into a 96 well black plate.
10. Place the plate in a luminometer to measure luciferase activity

Note: The described luminometer utilized here has an injector (Promega, Glomax[®]+Multi-detection System). The integration time should be optimized for your system. With this system, an integration time of 0.5 seconds, injector speed 200 $\mu\text{L}/\text{s}$, substrate volume 50 μL . The results are expressed in relative-light-unit (RLU).

REAGENTS AND SOLUTIONS

Preparations of reagents used during the production of fcPsV particles

DPBS-10mM MgCl_2 with 1% antibiotics—

To make 100 mL:

98 mL of Dulbecco's phosphate-buffered saline with Mg^{++} and Ca^{++} (Invitrogen[™]/Life Technologies, cat# 14287-080),

1mL of 1M MgCl_2 (KD Medical, cat# PMS-0630),

1 mL of antibiotic, anti-mycotic (Invitrogen™/Life Technologies, cat# 15240).

Store at 4° C and can be used up to 1 month.

Lysis Buffer (prepare fresh for each experiment)—

To make 5 mL:

4.75 mL of

DPBS-10mL MgCl₂ solution,

250 µL of 10% Brij58,

5 µL of Ambion® RNase cocktail™ enzyme mix (Invitrogen™/Life Technologies, cat#AM2286).

10% Brij58 (prepare fresh for each experiment)—

Dissolve 10g of solid Brij58 (polyethylene glycol hexadecyl ether) (Sigma Aldrich, cat# P-5884) in 100 mL of

DPBS (Invitrogen™/Life Technologies, cat#14040).

DPBS-0.8M NaCl—

153.5 mL distilled water (Invitrogen™/Life Technologies, cat#15230),

20 mL of 10× DPBS, contains NaCl (Invitrogen™/Life Technologies, cat# 14200-075),

25 mL of 5 M NaCl (KD Medical, cat# RGF-3270),

180µL of 1M CaCl₂ (KD Medical, cat# PMS-0614),

100 µL of 1M MgCl₂(KD Medical, cat# PMS-0630),

420 µL of 1M KCl (KD Medical, cat# PMB-0730).

Filter through 0.2 µM PES filter bottle (Thermoscientific, cat# 568-0020).

Alternatively, add 0.15 volume of sterile 5 M NaCl to DPBS (Invitrogen™/Life Technologies, cat#14040-141)

Store at 4° C and can be used up to 2 months.

Assay Media for use in FC-PBNA with SEAP or Firefly luciferase

Phenol red-free DMEM (Life Technologies, cat# 21063)

10% heat inactivated FBS (Hyclone, cat# SH30070.03)

1% anti-biotic/anti-mycotic (Invitrogen™/Life Technologies, cat# 15240 [100×])

2 mM L-glutamine (Invitrogen™/Life Technologies, cat# 25030, 200 mM [100×])

1 mM sodium Pyruvate (Invitrogen™/Life Technologies, cat# 11360, [100 mM / 100×])

1X MEM non-essential amino acids (Invitrogen™/Life Technologies, cat # 11140, [100×])

200 µg / mL hygromycin B (Invitrogen™/Life Technologies, cat# 10687, [50 mg/mL])

Note: Use a filter bottle (Nalgene 0.2 µm PES membrane; Thermo Scientific) to filter all the components except for Hygromycin B. The volume of Assay Media to be prepared varies depending on the needs of the experiments. Assay Media and culture media are prepared on a weekly basis. For the luciferase assay system, DMEM with phenol red can be used (Life Technologies; cat# 11965) (see below).

Culture media for 293TTF and LoVoT cells

Dubecco's Modified Eagle's Medium (DMEM) (Invitrogen™/Life Technologies, cat #11965),

10% heat inactivated FBS (Hyclone, cat# SH30070.03)

1% anti-biotic/anti-mycotic (Invitrogen™/Life Technologies, cat# 15240 [100×])

2 mM L-glutamine (Invitrogen™/Life Technologies, cat# 25030, 200 mM [100×])

1 mM sodium Pyruvate (Invitrogen™/Life Technologies, cat# 11360, [100 mM / 100x])

1X MEM non-essential amino acids (Invitrogen™/Life Technologies, cat # 11140, [100×])

200 µg / mL hygromycin B (Invitrogen™/Life Technologies, cat# 10687, [50 mg/mL])

2 µg / mL puromycin (for 293TTF cells; Sigma-Aldrich, P8833) **[For 293TTF only]**

Note: Use a filter bottle (Nalgene 0.2 µm PES membrane; Thermo Scientific) to filter all the components except for Hygromycin B. The volume of culture media to be prepared varies depending on the needs of the experiments. Culture media is prepared on a weekly basis. 293TTF cells should also be maintained with 2µg / mL of puromycin additionally.

Freezing media for LoVoT or 293TTF cells

90% Heat-inactivated FBS (Hyclone, cat# SH30070.03)

10% DMSO (Sigma-Aldrich, cat# D2650)

L2-antibody positive control reagents for FC-PBNAs

Depending on the vaccinated serum tested, and the species it originated from, there are several monoclonal antibodies of different origins which can be utilized as positive controls and recommends the following (Table 4). The Roden laboratory has recently sequenced the cDNA sequences of all these mono-clonal antibodies and has successfully cloned them into mammalian expression vectors for large scale monoclonal antibody production and purification *in vitro*. The expression of these respective cDNAs and subsequent purification result in the generation of these antibodies *in vitro* which retain their neutralizing capabilities and other previously characterized traits. These cDNAs are readily available at http://www.addgene.org/Richard_Roden.

It is worth noting that this list of L2 monoclonal antibodies is not exhaustive as several L2 antibodies generated from other laboratories have also been described (Reviewed in (Wang and Roden, 2013)). For L1-based studies, we recommend antisera to VLP preparations of the appropriate HPV type (or commercial vaccine) as a positive control.

COMMENTARY

Background Information

Variations in the current protocol from the standardized HPV PsV production protocol—Over the development of PsV, Buck *et al* have developed several iterations of the purification methodology, from a “Standardized” protocol to an updated method known as the “RIPCORDER” protocol. The key difference between these two protocols occurs during the lysis of the transfected cells. Briefly, in the “Standardized” HPV PsV production protocol, the Lysis Buffer contains the nuclease benzonase (Buck et al., 2004; Pastrana et al., 2004). However, this lysis step was subsequently modified to omit DNase from the Lysis Buffer. It is hypothesized that the changes in the protocol removes or reduces HPV viral capsids that contain cellular DNA fragments rather than reporter genome. This in turn would increase the yield of correctly packaged HPV PsVs and limit the promiscuous encapsidation of cellular DNA. Indeed, an improvement of up to 2-fold higher infectious titer was observed (<http://home.ccr.cancer.gov/LCO/riporder.htm>) (Buck and Thompson, 2007). The current fcPsV protocol here for the production of fcPsV follows this “Riporder” method for lysis.

293TTF cells—The 293TTF cells originate from 293TT cells, which are human embryonic kidney cells that were transfected with the SV40 Large-T antigen (Pastrana et al., 2004). This cell line was stably transfected with the plasmid, FURIN-pIRESpuo2 (Wang et al., 2014a). This is a vector that expresses a bicistronic mRNA consisting of the gene of interest to be cloned in (human furin in this case) and the puromycin resistance gene that is controlled by an IRES. While our personal observations showed that there is no difference in furin production in the presence or absence of puromycin (Wang et al., 2014a), the maintenance of antibiotic selection in the culture medium ensures the expression of the enzyme furin. This cell line was originally created in Dr. Richard Roden’s laboratory at Johns Hopkins University (Wang et al., 2014a) and is available on request. The plasmid (FURIN-pIRESpuo2) is available from Addgene.

LoVoT cells—The LoVoT cell line originates from the LoVo cells (human epithelial cells from metastasized adenocarcinoma of colon; ATCC® CCL-229™) that were stably transfected with the SV40 Large-T antigen. This cell line was originally created in Dr. Richard Roden’s laboratory at the Johns Hopkins University (Wang et al., 2014a) and is available on request. The plasmid for SV40 Large-T antigen is available from Dr. Christopher Buck at NCI.

Broadly reactive monoclonal antibodies of mouse, rat, and human origin to papillomavirus minor capsid protein L2—The mouse monoclonal antibody RG-1 was generated from mice vaccinated with full length HPV16 L2. Subsequently, this monoclonal

antibody was mapped to amino acid regions 17–36 of HPV16 L2 (Gambhira et al., 2007b). An important distinction to emphasize is that while vaccination of animals with L2-based vaccines containing the RG-1 epitope region results in broad neutralization of several HPV genotypes, we have found that the monoclonal antibody, RG-1's range of neutralization is limited to HPV16 and HPV18 (DiGiuseppe et al., 2014). The rat monoclonal antibody, WW1 was thus generated from rats vaccinated with a fusion protein consisting of eight L2 N-terminus (AA 11 to 88) fused together into a single peptide (known as L2 α (11–88)x8) (Jagu et al., 2013). We have found that WW1 neutralizes a greater range of HPV genotypes compared to the mouse mAb RG-1 beyond HPV16/18 such as HPV26, 45, 58 (Wang et al., 2014a). As several HPV L2-based vaccines have finished the pre-clinical testing phase, a human positive control is required for subsequently phase 1 trial analysis to complete the FC-PBNA. While anti-L1 standards for HPV16 (WHO International Standard 05/134) and HPV18 (WHO International Standard 10/140) have been developed by the WHO, no existing human standard exists for HPV L2. To this end, we developed two chimeric human antibodies, JWW1 and JWW2, which recognize different neutralizing epitopes on PV L2. This may be useful for the functional assessment of HPV L2 antibodies in human clinical trials (See Table 4).

SEAP detection kit—There are a number of SEAP detection kits available from different vendors. The two mostly used kits have been Clontech's Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (cat# 631738) and Roche's SEAP Reporter Gene Assay, chemiluminescent (cat# 11779842001). In addition, Life Technologies also offers a kit (cat # N10578), and a relatively more recent product from Jaden Bioscience (cat# CM025) is also available. Clontech's detection kit in the past has been used to measure SEAP in the original 293TT cell-based neutralization assay (now known as L1-PBNA) in our studies (Kemp et al., 2008; Kemp et al., 2011; Pastrana et al., 2005). This L1-PBNA system measures HPV anti-L1 antibody titers using PsV particles where little or no L2 proteins on the virion have been cleaved. Using this system, we compared the Clontech's Great EscAPe™ SEAP Chemiluminescence Kit 2.0 and Jaden Bioscience's Ziva® Ultra SEAP Plus Detection Kit (Kemp et al., 2015). When we compared these 2 kits, we found that Ziva® was able to provide higher signals than those of the Clontech's kit. Also, the amount of HPV16 and HPV18 PsV particles required to infect 293TT cells to achieve 100-fold induction in signal over the background was at least 10-fold less with Ziva®. Neutralizing anti-L1 antibody titers we measured by Ziva® and Great EscAPe™ in sera of Cervarix® (GlaxoSmithKline) vaccinated individuals were virtually identical, suggesting that Ziva® can be a suitable alternative to the well-established Great EscAPe (Kemp et al., 2015)

Critical Parameters

Stability of HPV fcPsV particles—We suspect that because furin cleaved virions represent an intermediate state of the true infectious form of papillomaviruses, their stability is less than that of the regular capsids. Indeed our observations showed that 48 hours of maturation generally resulted in one-fold lower amount of encapsidated virions (based on CT values using qPCR to calculate viral genome equivalents) compared to regular pseudoviruses. Interestingly, however, the encapsidation amounts of 293TTF made fcPsV using a maturation period of only 24 hours (instead of 48 hours) with 5mM calcium has

roughly the same encapsidated amount of virions as per regular PsV at 24 hours of maturation time. However, if maturation in 293TTF cells is done for only 24 hours, we found that the L2 cleavage is only about 50–60 %, rather than 80–90% after 48 hours of maturation (Wang et al., 2014a). Taken together, our personal observations suggest that the more cleaved the virus is, the less stable it seems to be. Therefore, it is important that repeated cycle of freeze-thaw of the fcPsV particles is not recommended, and aliquots should be made, and stored at -80°C .

Choice of Ultra-centrifugation equipment—We have successfully performed the above mentioned protocols utilizing both SW40Ti as well as SW55Ti rotor. Nonetheless, the choice of ultra-centrifugation equipment utilized is still an important consideration/parameter as it determines the amount of density gradient solution to be made and the amount of clarified lysate that should be obtained following the cell lysis and maturation steps. It is important to note that the SW55Ti rotor uses 5mL tubes while SW40Ti rotor uses 15mL tubes.

Choice of reporter genes—Different reporter genes, such as Firefly luciferase, enhanced green fluorescent protein (GFP), and secreted alkaline phosphatase (SEAP), can be encapsidated during fcPsV production (These same reporters are also utilized in regular HPV PsV production). These different reporter gene products may effectively be used, depending on the purpose of the experiments. For example, GFP can be useful in quantitative analysis of target cells that are infected. However, it will require a flow cytometer to measure, and cells will need to be harvested from micro-titer plates, and transferred to flow cytometry tubes for measurement, unless the cytometer is capable for reading samples in the plate format (Day et al., 2012). While it may be possible to measure the GFP signals from infected cells in the 96-well plate format with a plate reader that is capable of detecting fluorescence signal (e.g., Molecular Devices' SpectraMax[®] M5 or i3 etc.), it will not be able to determine percentages of cells that are infected, nor the homogeneity / heterogeneity of the signal level in a population of cells.

The use of firefly luciferase provides a very sensitive measure of infectivity *in vitro* and it can be visualized during *in vivo* infection in murine models of HPV infection (Roberts et al., 2007). For the *in vitro* system, similar to the GFP system, detection of luciferase will require harvesting of cells; however, in addition, cells will need to be lysed. If one chooses to employ Gaussia luciferase rather than firefly luciferase, then, culture supernatants can be collected to measure the level of the reporter gene product. Signals generated from luciferase are more sensitive than that of GFP. Some of the factors to consider are that if a plate-format is desired during a measurement of luciferase activity, then a luminometer should be equipped with an injector. The activity of luciferase is short-lived, as the signal begins to decay within a few seconds to a minute of interaction with its substrate.

Secreted alkaline phosphatase (SEAP) is another widely used marker. With the chemiluminescent-based system, it provides a sensitive and convenient way of measuring the level of infection / neutralization in *in vitro* system. One of the advantages of employing SEAP as a reporter gene is its relative ease in performing high-throughput assays to detect the enzyme. As the enzyme is secreted into the culture supernatant from infected cells, it

does not require collection and lysis of cells. Because the culture supernatants can be used, with a proper plate reader, samples can be measured in a 96-well plate format, increasing the throughput capability. Furthermore, signals generated during the enzyme / substrate interaction are generally stable for hours, and the supernatant samples can be stored at -20° or -80° C without significantly loss in activity. We have subjected supernatant samples to multiple freeze thaw cycles, measured SEAP activity using Ziva[®], and compared the fold-induction levels from each cycle, and found that the levels were not affected.

Assessment of peak fraction—In general, following viral purification utilizing ultracentrifugation, the fractions collected are subjected to SDS-PAGE analysis followed by Commassie blue staining. These experiments determine purity of the sample as well as the peak fraction. Generally, the fractions with the highest amount of HPV L1 and L2 capsids, which will show strong protein bands at 50kDa and 75kDa, respectively, indicate the fractions with the highest amount of infectious particles. However, given that there is the possibility that a proportion of particles within the fraction is empty, these fractions must still be analyzed for their ability to infect target cells by performing infectivity assays. Histones are also normally observed at the bottom of the Commassie gel, indicating successful reporter encapsidation.

Assessment of furin-cleavage—It has been shown that furin-cleavage is required for HPV infection (Day and Schiller, 2009). Importantly, un-cleaved HPV pseudovirions are unable to infect furin-deficient lines, thereby further emphasizing the importance of this step during early infection (Day et al., 2008b). Given that the protocol (see *Basic Protocol 1*) highlights the production of fcPsVs particles, these fcPsVs should be able to infect furin-deficient cell lines such as FD11 cells (a furin knockout cell line derived from Chinese hamster ovary cells) and LoVoT cells (see Background Information). Alternatively, if these cells lines are not available, infection can be assessed with the use of a furin inhibitor. Infect both PsV and fcPsV into 293TT cells with and without 20 μ M of Furin-inhibitor 1 (Calbiochem #344930). In the absence of the furin inhibitor, the infectivity of both virions should be similar (or with the fcPsV slightly better); however, in the presence of the inhibitor, only fcPsV should retain its infectivity (Wang et al., 2014a).

Assessment of optimal viral titer via Infectivity Assays—We recommend a dose response study of each fcPsV preparation (different fractions collected during elution or different HPV fcPsV types). The assay can be setup as described in *Basic Protocol 2* using LoVoT cells. However, in this assay, fcPsV particles are titrated, instead of serum samples. The 2-fold serial dilution of fcPsV is performed in a 96-well plate. Subsequently, 100 μ L of the serially diluted fcPsV particles are transferred to the preplated LoVoT cells. The plates are incubated for 3 days before harvesting appropriate samples (supernatant or cell lysate depending on the reporter gene used). There is no need to include serum samples to mix with fcPsV particles as the purpose of this test is to determine the levels of infectivity.

Percentage HPV L2 cleavage—Western blot analysis is required followed by densitometry calculations to specifically calculate the level of L2 cleavage. Because the difference in the molecular weight of the full-length L2 and the size of the cleaved L2 is

small (9–13 amino acid difference), we recommend using a single gradient (7 or 7.5%) polyacrylamide gels for the best separation of these two proteins. The un-cleaved PsV preparations (made via the standard HPV PsV production method, Fig. 1A) can be used as a control. It is also important not to load too much virus sample onto the gel (recommend 1/100–1/1000 sample dilution prior to Western blot) or it will be difficult to observe the separation of cleaved and full length proteins. Likewise, we recommend multiple film exposures to capture the right exposure level (in linear range) as over exposure will underestimate the % cleavage, or simply obscure the small size difference between cleaved and intact L2. Subsequently, densitometry calculations can be performed using free software such as ImageJ, which can be downloaded from <http://imagej.nih.gov/ij/> (Wang et al., 2014a). Typically, when prepared side by side, we have observed >80% L2 cleavage in HPV fcPsV preparations when compared to HPV PsV L2.

Furin-Cleaved HPV Neutralization Assay (FC-PBNA) considerations—Before starting an experiment, it is essential to have designed the experimental layout of the samples and controls. Generally, each sample and controls are tested at least in duplicates; however, this may be changed according to your needs and the robustness and consistency of the system. This becomes important during an experiment when setting up 1:1 mixtures of samples and fcPsV particles in a 96-well plate. By placing the mixtures of samples and fcPsV particles according to the plate map will allow you to transfer the mixture to a plate of LoVoT cells using a multichannel pipet and maintain the same layout. This layout should be kept throughout the entire experiment.

It is important that LoVoT cells have not been overgrown on the day of conducting neutralization assay. If fcPsV particles encapsidating the SEAP gene are used in experiments, then, **it is critical to use DMEM without phenol red**. This is because phenol red will interfere with the reading of luminescence that is generated during the SEAP/substrate interaction. Thawed serum samples on ice should not be left for more than 4 hours, to assure maximum sample integrity. Although immunoglobulins are generally considered to be stable, a care should be taken to minimize the number of repeated freeze-thaw cycles. To minimize, the sera could be aliquotted into multiple vials.

The fcPsV particles should have been diluted to 1:10 in Assay Media, and aliquotted into siliconized 1.5 mL flip-cap tubes (Fisher, cat# 0554131) in a volume required for each experiment to minimize the effects of freeze-thaw cycles. These vials should be kept in a –80° C freezer. Each preparation of fcPsV particles should be tested for the optimal dilution to infect LoVoT cells. Dose response studies should be conducted for each preparation of fcPsV by serially diluting the fcPsV particles, and incubating them with LoVoT cells. Generally, 1:1000 final dilution factor is a good starting number. We recommend the dilution that would lead to approximately 100-fold increase in signal over the background (signal from infected culture divided by signal from the no-virus culture).

Even with a more sensitive L2-based assay, the titers of anti-L2 antibodies are expected to be lower than those of anti-L1 antibodies. Therefore, the number of serial dilutions to be performed, as well as the magnitude of fold-dilution (e.g., 2-, 3-, or 4-fold) need to be

estimated. Also, if the dilution of samples is too low, there may be a non-specific inhibitory effect (serum matrix effect).

For SEAP assay, we prefer the use of an oven over a water bath to heat-inactivate endogenous heat unstable alkaline phosphatases due to the simplicity of use and throughput capability. Although most kits suggest using 65° C for heat inactivation, the recommended temperature used may vary from kit to kit. The optimal temperature in combination with length of time that the samples are subjected to heat-inactivation should be determined for each system. We recommend incubating for 30 – 45 minutes at 65 to 73°C.

Generally, the range of dilution should cover dilution factors that enable one to determine a 50% neutralization titer within linearity. Neutralization titer has been typically defined as the reciprocal of the dilution that causes 50% reduction in reporter activity. There have been a few others that use a more stringent requirement (i.e 80% reduction). There are several ways to calculate the neutralization titer of the sample tested. For the Roden laboratory, calculation is performed via utilizing the non-linear model $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$ with the neutralization data first $\log_{10}$ transformed. We utilize Graphpad Prism 6 to perform these analyses. The estimated EC50 (determined from 3 triplicate studies) is reported as the titer with 95% confidence interval (see http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_classic_dr_variable.htm)

Biosafety

Procedures described in this unit require handling of human tumor cell lines or sera, and HPV PsV, albeit non-replicative. Therefore, precautions should be taken to handle these materials. Personnel should wear lab coats, gloves, and safety glasses during an experiment. We recommend the use of absorbent sheets (VWR, cat# 51138-504) to cover the surface of biological safety cabinets in case of spillage. Also inside the safety cabinet, waste containers for pipet tips and serological pipets, and for biological waste should be present (waste boxes VWR [Whitney Product, Inc.], cat# BH2003 and BH2006). For the biological waste, a sufficient volume of water containing 10% proportion of sodium hypochlorite should be present, and the waste container should have a lid that tightens securely onto the container. Biological waste and pipettes should be immersed in the 10% sodium hypochlorite for at least 30 minutes, and liquid waste can be then disposed into a sink. When full, the solid waste containers should be placed inside an autoclavable bag (tufpak, 30"x 36", cat# 1112-3036) and be autoclaved or burned.

Although fcPsV particles are not replicative, and they lack genetic materials of HPV, caution should still be taken when handling these reagents during experiments. If these materials are transported to your laboratory, or where the experiments are performed in different locations, use of a bio-transporter is recommended. The transporter should be placed inside a box (e.g., Styrofoam), and use of a cart is recommended. This will help prevent accidental loss of vials, as well as accidental spillage of reagents onto the floor.

Troubleshooting

Low yields of certain HPV fcPsV—In general, we have observed that we were able to produce milligram quantities of HPV fcPsV (measured using Commassie blue, using L1 intensity versus known BSA standards), although yields and infectious titer varies dramatically by genotype. If such yields are unattainable, it is important to check the quality of the 293TTF cells. In general, it is good to create a large batch of early passage cells (also for LoVoT) and freeze it to further reproducibility. Beyond cell-related issues, we also have previously documented that despite transfecting the same amount of HPV capsid DNA for each of the 34 different HPV types tested, the expression in 293TT cells differs for each HPV type, with certain types always producing lower yields, for example HPV 11 and 18 and certain beta-papillomavirus types. (Kwak et al., 2014). Pooling of several production batches may be required for some types to achieve sufficient yield.

Neutralization read-out results—For the SEAP assay, if the results of the read-out do not appear to make sense, and that they seem random, be sure that you have used phenol red-free DMEM to setup the neutralization assay. Alternatively, if an oven is used to heat inactivate samples, it is important that your oven's temperature is even throughout the chamber. Make sure there is no air leakage from incomplete closure of the door. Fluctuation of chamber temperature, and/or uneven distribution of the heat inside the oven could lead to ineffective heat-inactivation depending on where the assay plates were placed. It is recommended that serum samples be heat-inactivated for 30 minutes at 56° C before using them in the neutralization assay. If you observe an inhibitory effect from samples that are not expected to neutralize fcPsV-mediated infections, it is possible that the dilution is too low and you may be observing serum matrix effect. If the serum matrix effect does not appear to be the cause, it is possible that the individuals may have been exposed to HPV and developed high enough titers against L1 protein. Alternatively, the individual might have been immunized with a HPV vaccine. LoVoT neutralization assay will detect anti-L1 antibodies, as fcPsV particles contain L1 proteins.

Anticipated Results

Ability to infect Furin-deficient cell lines—A key distinction between regular HPV PsV and HPV fcPsV is the latter's ability to infect furin-deficient cell lines (see *Assessment of furin cleavage, Critical Parameters*).

Variability in infectivity of different HPV fcPsV genotypes—Different lots of fcPsV particles of the same HPV type may differ in their ability to infect LoVoT cells. Also, the ability of fcPsV of different HPV types could differ with some requiring a low dilution to achieve a reasonable level of infection. This phenomenon, while not well understood, has been hypothesized to be due to a poor expression of the L2 gene (Kwak et al., 2014). In particular, infectivity titers for HPV11 and 18 fcPsV can particularly be low (see *Troubleshooting, Low yields of certain HPV fcPsV*).

Improved L2 titers but still lower than L1-VLP vaccine serum titers—Even with a more sensitive L2-based assay, the titers of anti-L2 antibodies are potentially expected to be lower than those of anti-L1 antibodies. This is because most current L2 vaccines are

peptide-based and thus induce lower titer and lower avidity responses compared to L1 VLP. The licensed HPV vaccines are based on L1-VLP immunogens which have a close-packed and ordered array of conformational neutralizing epitopes that facilitate both B cell receptor cross-linking and bivalent antibody binding. Hence, given the difference in potency, the number of serial dilutions to be performed, as well as the magnitude of fold-dilution (e.g., 2-, 3-, or 4-fold) need to be considered carefully with respect to the vaccine type. Generally, the range of dilution should cover dilution factors that enable one to determine a 50% neutralization titer (i.e., the titer that leads to 50% inhibition of infection by fcPsV particles) within linearity. It is important however to not dilute the samples too low i.e. <1:29 as non-specific inhibitory effect (serum matrix effect) have been reported (Pastrana et al., 2005).

Time Considerations

The production of fcPsV particles will take one week from the time of transfection. However, 293TTF cells will need to have been in culture prior to the start of the production. Therefore, the total time required for fcPsV production including preparation of cells for production should be about 10–14 days. For most of the quality checks, experiments for fcPsV particles, half a day should be set aside. For FC-PBNAs, regardless of the nature of the reporter, the entire process from the start of the experiment to harvest of the cell or supernatants will be 3 days. On the day of setting up the neutralization assay could take up to 5 hours, depending the number of samples and plates being tested. SEAP assay with Ziva[®] will generally take approximately 3 – 4 hours, including the warm-up time for reagents, for 8–10 plates. A similar time frame should also be allocated for luciferase-based FC-PBNAs.

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Figure 1.

Schematic of the differences between standard HPV pseudovirus (PsV) production (A) and HPV furin-cleaved PsV (fcPsV) using 293TTF combined with an extended maturation protocol (B).

Figure 2. A representative image of a 15mL Beckman Ultra-clear polyallomer tubes following 16 hour ultra-centrifugation

The letter A in yellow indicates the interface between 27% and 33% whereas the letter B indicates the gradient interface between 33% and 39% of the gradient. Optimal HPV fcPsV is often found at B even when an opaque layer is not observed.

Figure 3. An overview of L1-PBNA and the newly developed FC-PBNA assays

The basic format of these two assays is similar in that serum or antigen-specific antibodies are incubated with either uncleaved PsV or fcPsV particles prior to the incubating the mixture with the respective target cells. In L1-PBNA, 293TT cells are used as target cells, whereas LoVoT cells are used in FC-PBNA.

Table 1

Preparation of transfection complex mixture.

Tissue Culture Flask	T150cm ²
Total Culture Medium required	40 mL
Serum-free Medium (OptiMEM-Free)	3.8 mL
Codon-optimized HPV L1 and L2, and a reporter plasmids	38 µg (19 µg of L1/L2 vector, and 19 µg of the reporter plasmid)
MirusTransIT-2020	109 µL

Table 2

Preparation of gradient solutions.

OptiPrep Gradient	Vol. of 46% OptiPrep	Vol of DPBS-0.8M NaCl	Total Vol.
39%	16.96 mL	3.04 mL	20 mL
33%	14.34 mL	5.66 mL	20 mL
27%	11.74 mL	8.26 mL	20 mL

Table 3

Preparation of master mix for qPCR for samples and/or standards (e.g., the reporter plasmid used for the fcPsV production).

Mastermix	1X reaction per sample	2.2X reaction per sample of Standard	3.3X reaction per sample of Standard
EVAgreen Supermix (Bio-Rad, cat# 172-5200)	10 µL	22 µL	33 µL
Reporter gene's Forward & Reverse Primers (500nM)	1 µL	2.2 µL	3.3 µL
Sterilized dH₂O	6 µL	13.2 µL	19.6 µL
Reporter DNA template	3 µL	6.6 µL	9.9 µL
Total Volume	20 µL	44 µL	66 µL

Table 4

List of suggested L2-specific monoclonal antibodies as controls.

Monoclonal Antibody	Species origin	L2 site recognized
RG-1	Mouse	17–36
WW1	Rat	17–36
JWW1	Rat-Human Chimeric	17–36
JWW2	Mouse-Human Chimeric	58–64