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# eGFP Marker for Production of Type 6 and 11 Human Papilloma Virus Pseudovirus

Aroem Naroeni[1,2] *, Devia Natalicka[2]

[1]Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia
[2]Virology and Cancer Pathobiology Research Center, Faculty of Medicine, Universitas Indonesia
*Corresponding Author: aroem.naroeni@gmail.com

## ABSTRACT

Human Papillomavirus (HPV) capsid proteins L1 and L2 possess the remarkable ability to self-assemble into virus-like particles (VLPs). This self-assembly property is important in developing pseudovirus (PsV), which are invaluable tools for vaccine research and immunological assay. One prominent application of PsV is in the Pseudovirus Neutralization Test (PVNT), a laboratory assay designed to assess whether immunization induces neutralizing antibodies capable of preventing HPV infection. PVNTs are widely regarded as the gold standard for evaluating the immunogenicity of HPV vaccines. In this study, PsV was developed to support the evaluation of Gardasil 4, a quadrivalent HPV vaccine targeting types 6, 11, 16, and 18. Specifically, PsV for HPV types 6 and 11 were generated using the plasmids pVITRO HPV6 L1L2 and pVITRO HPV11 L1L2, respectively. To facilitate detection, a co-transfection with the pcDNA3.1-eGFP plasmid was performed, allowing the incorporation of a fluorescent marker into the PsV. Pseudovirus for HPV types 16 and 18 are currently also being developed concurrently to complete the evaluation of Gardasil 4. HEK293T cells were transfected using Lipofectamine, and following maturation, the presence of PsVs was confirmed through increased fluorescence intensity, as measured by a Tali Cytometer. Talicytometer is an equipment designed for rapid, image-based analysis of cells in suspension. It combines bright-field and fluorescence imaging to perform various cellular assays. The fluorescence indicates successful PsV formation and the potential for these particles to be used in PVNTs. While the current production of PsVs demonstrates promise, further optimization is necessary to enhance yield and scalability for industrial applications.

**Keywords**: eGFP, HPV, NAb, Pseudovirus, PVNT

## INTRODUCTION

Human Papilloma Virus is a small, double-stranded DNA virus and is responsible for anogenital and cutaneous warts, throat cancer and anogenital cancers, including cervical, anal, vulvar and penile cancers. HPV is classified into two categories: low-risk and high-risk types. High-risk HPV types are detected in over 90% of cervical cancer cases, making cervical cancer the fourth leading cause of cancer-related deaths among women worldwide [1,4,6,7,8]. The HPV repartition data show that HPV infection and related diseases are more prevalent in developing countries. Various efforts have been undertaken to prevent the spread of these diseases. One of them is by providing vaccines, such as the 2-, 4-, and 9-valent vaccines based on virus-like particles. Therefore, the efficacity evaluation of these vaccines is required like the evaluation of the effectiveness vaccine in producing antibodies by using Neutralization Antibody Assay (NAb) [13]. It is required to evaluate vaccines and to accelerate the approval of new vaccines in pre-clinical models and/or small clinical trials [9,18]. The Gold Standard for measuring Nab titre are live virus neutralization assay. However, it usually requires high containment like the BSL-3 facility [2].

Pseudovirus neutralization test (PVNT) therefore presents a safer and more cost-effective system. So, this research can resolve the problem.

PsV HPV types 6,11, 16, and 18 can serve for PVNT to evaluate Gardasil 4, a quadrivalent HPV vaccine targeting types 6, 11, 16, and 18. The use of enhanced green fluorescent protein (eGFP) serves as a marker for easily detecting pseudovirus formation following transfection, maturation, and infection.

## METHOD

### Cell Culture

PsV was produced in HEK 293 T. Cells were cultured in High Glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) as described previously [17]. This media was added with 1% Penicillin-Streptomycine and 10% Foetal Bovine Serum (FBS). Cells were seeded on 25cm$^2$ and incubated in 5% $CO_2$ at 37ºC until 80-90% confluent. Cells then were subcultured to flask 75cm$^2$.

### Transfection, Maturation, and Infection

Cells were transfected with pVITRO-HPV6L1L2 and pVTRO HPV11L1L2 which contain HPV 6 and HPV 11 genes, respectively. Plasmids were obtained from Addgene (Addgene, Massachusetts, USA). They were transfected into HEK 293T, an epithelial-like cell isolated from the kidney of a patient (ATCC, USA) by using Lipofectamin and co-transfected with pcDNA 3.1. eGFP. eGFP is a fluorescence protein served as a biomarker as described in figure 1. After transfection, cells were lyzed using 10% Triton X 1:20 v/v. Maturation was performed at 37ºC for 24h DNAse was added to eliminate residual plasmid. The presence of PsV was confirmed by PCR of eGFP. PsVs were then infected into HEK 293T cells. HEK 293 T cells were seeded in plate 48 wells. After more than 50% of cells were confluent, PsVs were prepared and serially diluted to 1/225, 1/50,1/100,1/200. 1/400 and 1/400. They were infected into cells. After 4h infection, cells were placed in the growth medium for 48h. Finally, cells were trypsinized for analysis.

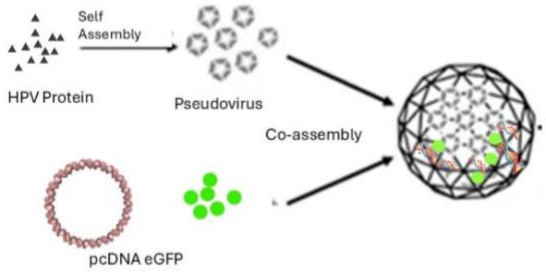


Figure 1. Co-transfection pVITRO HPV and pcDNA eGFP.

### Cells identification based on eGFP marker

Identification cells containing PsV were done using a Talicytometer. This machine can analyze cell size, number of cells, and cell fluorescent in Green and Red. eGFP was analyzed in green fluorescent.

## RESULTS

### HEK 293T cells.

The initial step in preparing for pseudovirus production is culturing HEK 293T cells. Pseudovirus was produced in HEK 293T cells, and the initial preparation involved propagation of HEK 293T cells and preparing a stock culture. HEK 293T cells stored in liquid nitrogen are thawed for cell culture in the laboratory. The expanded HEK 293T cells are at passage 9. After being grown in a T25 flask, daily cell observations are conducted. Once the HEK 293T cells reach approximately 80–90% confluency, they are subcultured and grown in a T75 flask (Figure 1).

Figure 2. Culture of HEK 293T in DMEM medium. Cells reach 60% confluency. Microscope magnification: 200x

**Transfection**

After the cells reached >60% confluency, they were transfected with the pVITRO-HPV11 L1L2 and pVITRO-HPV6 L1L2 plasmids. After 48 hours, the next steps include cell harvesting, lysis, and maturation of HPV pseudovirus (PsV HPV). The cell culture condition after 48 hours of transfection shows confluency reaching >90% (Figure 3).

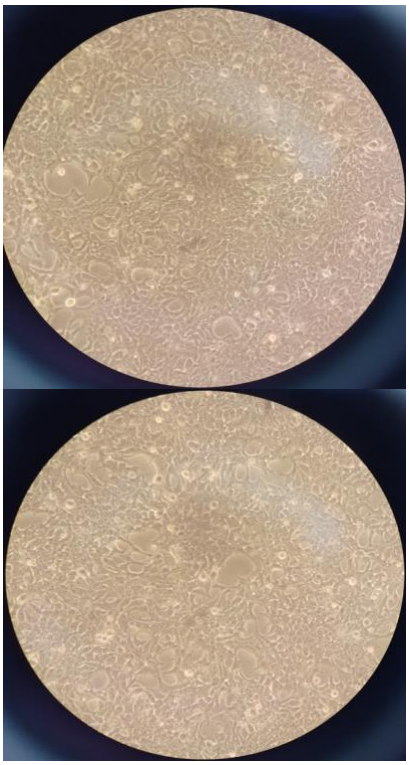


Figure 3. Cells after 24 hours of transfection. They reach > 90% confluence. Microscope Magnification: 200x.

After harvesting and maturation of the PsV, the lysate was treated with DNase I and analyzed using PCR. The PCR results for eGFP showed no remaining DNA in the lysate, as no band was detected except in the positive control. This indicates that there was no residual plasmid DNA after DNase I treatment (Figure 4).

Lysat then was extracted for PCR to confirm eGFP of PsV. The PCR results showed a band corresponding to the size of the positive control (Figure 5), indicating that the DNA contained eGFP genes. However, a weak band was still observed in the cell control (Figure 5).

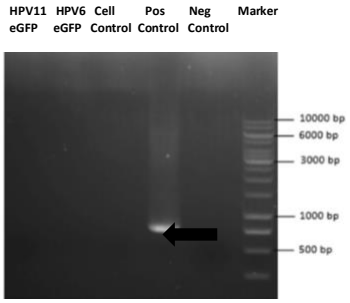


Figure 4. Electrophoresis gel analysis of HEK 293T cells after lysis and treatment with DNAse I. PCR of eGFP showed no remaining DNA in the lysate after treatment.

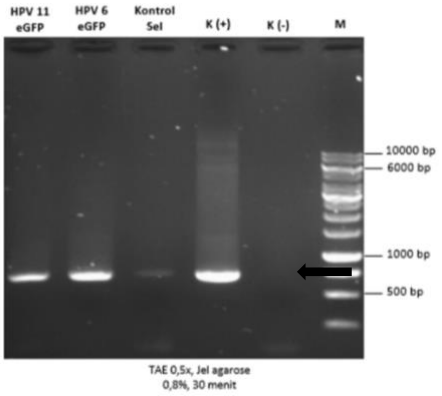


Figure 5. Electrophoresis gel analysis of PsV PCR

**Infection**

HEK 293T cells were seeded in a 48-well plate. Once the cells reached >50% confluence, they were infected with HPV 11 eGFP and HPV 6 eGFP. The obtained PsV was serially diluted at ratios of 1/25, 1/50, 1/100, 1/200, 1/400, and 1/800 using complete DMEM as the diluent. This dilution is used to obtain a rate number for effective infection. The PsV and control were then added to the cells. PsV were infected to HEK 293 T cells. After 4h incubation, media were replaced with growth medium and incubated 4h in 5% $CO_2$ at 37ºC. After infection, cells morphological change was observed in a microscope. They formed an aggregate and became giant cells (Figure 6)

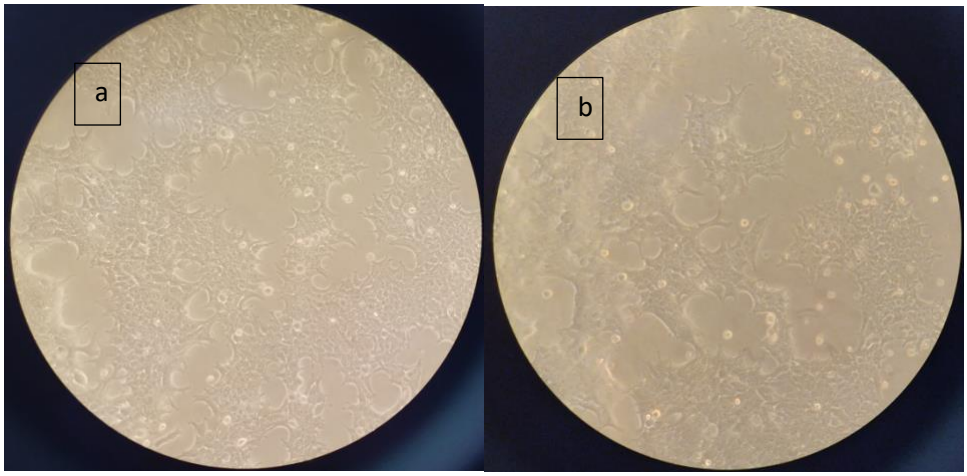


Figure 6. HEK 293T cells after 48h infection PsV HPV11 (a) and HPV 6 (b). Microscope magnification 200x

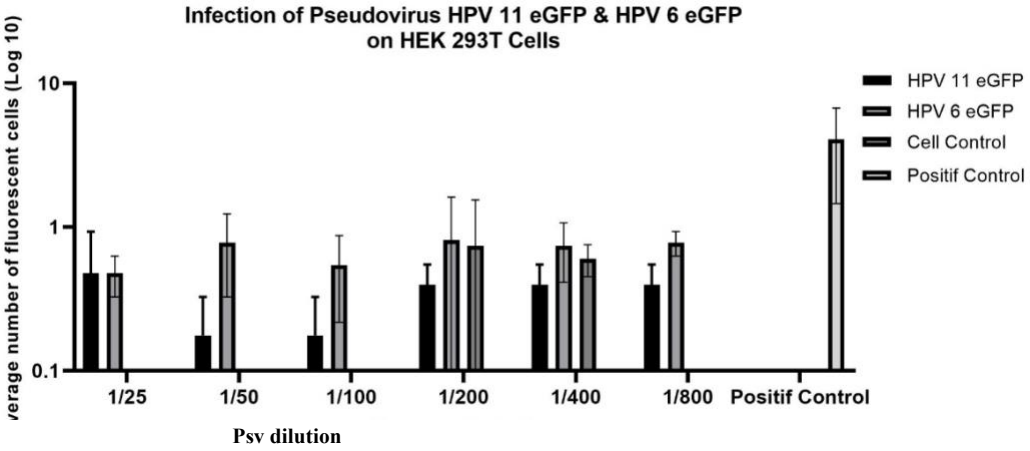


Figure 7. Cells fluorescent count after infection with HPV PsV

**eGFP Detection**

Cells infected with HPV 11 eGFP and HPV 6 eGFP were trypsinized to detach them from the wells and then analyzed using Talicytometry to detect green fluorescence from the eGFP reporter. The positive control used was the pCDNA 3.1 eGFP plasmid, which was transfected into HEK 293T cells alongside the infection. The results of the analysis are shown in Figure 7.

The number of fluorescent cells was increased after HPV PsV infection compared to control cells. Nevertheless, some control cells had some fluorescent.

## DISCUSSION

PsV production is aimed at developing PVNT to replace VNT because this technique needs a high containment facility for high pathogen viruses. Consequently, VNT is expensive and has limited throughput [2]. Several works concerning PsV HPV have been performed. Lamprecht et al produced PsV by expressing L1 and L2 HPV Capsid genes in plants [14] while Viral-like particle L1 HPV 52 was produced in *Escherichia coli* BL21 DE3 [16]. Moreover, HPV type 16 L1 and L2 coding plasmid transfected along with pEGFP-N1 into HEK 293T resulted in self-assembly of 45-55nm pseudovirus [3]. PcDNA eGFP is a plasmid expressing GFP protein. It is a protein composed of 238 amino acids with molecular masses of around 26.9 kD. This pcDNA can be produced and utilized for different purposes. [10,15]

Currently, HEK 293T and HEK 293TT are common cell lines for producing PsV HPV. HEK 293T expresses the SV40 large T antigen that enhances significantly its capability of transfection and protein production. However, this capability is

still less efficient compared to HEK 293TT for PsV production because it expresses a higher level of SV40 large T antigen [5,11].

In this research, pCDNA eGFP was utilized to serve as a marker for the formation of PsV. L1L2 HPV protein naturally self-assembles to form viral-like proteins, sometimes also known as Pseudovirus. This HPV capsid protein can co-assemble with a complex of DNA and fluorogen where VLPs of HPV encapsulate the complex via electrostatic interactions [12]. Our results show the formation of PsV HPVL1L2 type 6 and type 16 detected via the presence of eGFP fluorescence by using a Talicytometer machine. This research showed that eGFP produced by co-transfection plasmid expressing L1L2 HPV and pcDNA.3. 1.eGFP served as a marker for easily identifying the formation of PsV in the cells. However, the presence of repeatable weak bands and fluorescent in control cells remains to be elucidated. Moreover, the production of this PsV for industrial purposes needs more optimization to achieve productiveness.

## CONCLUSION

Co-transfection plasmid expressing HPV L1L2 protein and pcDNA 3.1. eGFP produces PsVs and facilitates easy identification through the detection of fluorescence generated by eGFP. The production of this PsV still needs more improvement to achieve productiveness for industrial purposes.

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