Neutralization Assay

Papillomavirus Neutralization Assay

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Abstract

It has recently become possible to generate high titer papillomavirus-based gene transfer vectors. The vectors, also known as papillomavirus pseudoviruses (PsV), have been useful for studying papillomavirus assembly, cellular entry and neutralization and may have future utility as laboratory gene transfer tools or vaccine vehicles. In this protocol, PsV encapsidating a secreted alkaline phosphatase (SEAP) reporter plasmid are used for a high throughput in vitro neutralization assay in a 96 well plate format. Infection of 293TT cells is monitored by SEAP activity in the culture supernatant using a highly sensitive chemiluminescent reporter system. Antibody-mediated PsV neutralization is detected by a reduction in SEAP activity. The neutralization assay has similar analytic sensitivity and higher specificity compared to a standard virus-like particle (VLP) ELISA.

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 capsomeres in the presence of plasmid DNA. However, none of these strategies efficiently produces high infectious titers. Because high-titer PsV carrying an easily scored marker gene were unavailable, papillomavirus neutralization assays have been laborious, both in terms of production of the infectious capsids and in the conduct of the neutralization assays. At our website we provide a procedure for generating purified PsV with titers in excess of one billion transducing units per ml. We have used PsV produced by this method to develop a simple high throughput assay for detecting papillomavirus-neutralizing antibodies.

PsV encapsidating a secreted alkaline phosphatase (SEAP) reporter plasmid were used to develop the in vitro neutralization assay presented below. PsV transduction of 293TT cells is monitored by SEAP activity in the culture supernatant using a highly sensitive chemiluminescent reporter system. Antibody-mediated PsV neutralization is detected by a reduction in SEAP activity. This is the first papillomavirus neutralization assay to be adapted to a high-throughput 96 well plate format. A single 75 cm² flask can produce sufficient SEAP PsV for conducting thousands of neutralization assays. The neutralization assay appears to be as sensitive as, but more specific than, a standard VLP-based ELISA, and requires similar operator effort as an ELISA. The assay should have utility in both vaccine and sero-epidemiology studies.

2. Materials

1. 293TT cells. Recently, Invitrogen has developed a conceptually similar cell line, 293FT (cat# R700-07). It has been reported to us that 293FT work well in the neutralization assay.
2. DMEM-10: DMEM, 10% 56°C inactivated fetal calf serum, 1% essential amino acids, 1% Glutamax-I (Invitrogen)
3. Methods

3.1 Culture of 293TT Cells

3.1.1 Thawing 293TT Cells

293TT cells are cultured in DMEM-10. To thaw 293TT cells, place the thawed cells directly into a 150 cm² flask with 25 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 25 ml of DMEM-10 and plate in a 75 cm² flask.

3.1.2 Passaging 293TT Cells

Split 293TT cells 1:5 to 1:20 when they reach 80-90% confluence. Allowing 293TT cells to become super-confluent can irreversibly reduce their performance. Detach cells by gently rinsing the flask once with several milliliters of trypsin, followed by a 5-10 minute incubation in 2 ml of fresh trypsin in a humidified 37°C incubator. It is important to trypsinize the cells thoroughly since insufficient trypsinization can lead to 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 it inactivated by the fetal calf serum in DMEM-10.

After the cells have fully recovered from thawing, DMEM-10 can be supplemented with 400 µg/ml hygromycin B 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.

To freeze 293TT cells, reserve several ml of supernatant (conditioned medium) from a sub-confluent flask of cells. Trypsinize cells as described above, then resuspend in the reserved conditioned medium. Mix the cell suspension 1:1 with freeze medium (fetal calf serum + 20% DMSO). Freeze in 1ml aliquots of a few million cells per aliquot. Place aliquots in a “Mr. Frosty” isopropanol bath pre-cooled to 4°C. Place Mr. Frosty at -80°C overnight, then transfer aliquots into liquid nitrogen for long-term storage.

3.2 Neutralization Assay

The methods described below outline (1) titration of the SEAP-PsV stock, (2) luminometry to detect SEAP production, and (3) determination of the neutralization titer of test sera. The PsV used for this assay encapsidates a reporter plasmid, pYSEAP, encoding secreted placental alkaline phosphatase (SEAP). When PsV infect 293TT cells, the pYSEAP reporter plasmid, which carries an SV40 ori, is replicated to high copy number by SV40 T antigen. This leads to high-level production of alkaline phosphatase that is secreted into the culture medium, and so can be easily assayed. Antibody-mediated neutralization of the PsV results in a corresponding reduction in SEAP expression.
3.2.1 Titration of SEAP-PsV Stocks

Before assaying for neutralizing activity of test sera, it is important to titrate the PsV stock to determine the inoculum that will be used in each assay. The goal of the titration is to determine the minimum amount of PsV required to give a robust signal in the SEAP assay (Subheading 3.2.2) that is well above background, but within the linear range of the assay. Typically, this falls in a range between 30 and 100 Relative Light units (RLUs) in the absence of neutralizing antibodies, with a background of no more than 1 RLU when the PsV is maximally neutralized with the positive control antibody or heparin. The method to titrate the stock follows.

1. Calculate the number of plates needed to titer the PsV.
2. Trypsinize 293TT cells and suspend in neutralization/growth media.
3. Count the cells and preplate 2-5 hours before the PsV are added (see Note 2).
4. Dilute the cells to 300,000/ml in the neutralization/growth media, place in a sterile reservoir.
5. Using a multichannel pipettor deliver 100 µl of cell suspension to each of the internal wells of a 96-well tissue culture treated plate. To avoid evaporation, do not use external wells and instead fill surrounding wells with 120-150 µl of medium with phenol red (Fig. 1).
6. Replace cover and place cells at 37°C in an incubator until ready to add the PsV.
7. Using siliconized tubes and tips, make serial dilutions of the PsV. Each dilution is tested in triplicate and enough should be diluted for at least 6 wells; three with and three without positive neutralization control. Depending on the papillomavirus type, appropriate dilution ranges between 1:250 and 1:20,000.
8. Place 80 µl of the diluted PsV into the wells of the dilution plates.
9. To the wells that will have untreated PsV, add 20 µl of neutralization/growth media.

10. Dilute the positive neutralization antibody (or heparin) such that it is 5-fold more concentrated than its known 95% neutralizing dilution. For example:
   - V5 (anti-HPV16 monoclonal) at 1:250,000
   - 5B6 (anti-BPV monoclonal) at 1:25,000
   - Rabbit anti-VLP polyclonal sera at 1:10,000 to 1:1,000,000
   - Heparin H-4784 at 1 mg/ml
   Dilute the antibody another 5-fold by adding 20 µl of diluted antibody (above) to triplicate wells containing 80 µl of diluted PsV.

11. Once the PsV and positive neutralization control(s) are combined, place on ice for 1 hour
12. Add the whole mixture to the preplated cells.
13. Return to the incubator for 72 hours.
14. The media should not be replaced during these 72 hours (see Note 3).

3.2.2 Chemiluminescent Detection of Secreted Alkaline Phosphatase

For this section of the protocol use a multi-channel pipettor when transferring liquids from one plate to the other. Make up kit reagents as indicated below and transfer to a reservoir so you can also use a multi-channel pipettor for those steps. Although SEAP activity can be detected colorimetrically (see Note 4), chemiluminescent methods, such as the one described here, are generally preferable since they offer a much higher signal to noise ratio.

1. After the 72 hour incubation, lightly shake plates to obtain a homogeneous sample of the supernatant.
2. Transfer 50 µl of supernatant to the corresponding well of a sterile 96 well polystyrene plate
3. Spin the plate at 1,000 x g for 5 min.
4. Use Great Escape SEAP Chemiluminescence Detection Kit 2 reagents according to manufacturer's instructions. Clontech has replaced the Chemiluminescent Great Escape SEAP detection kit with Great ESCAPE SEAP Chemiluminescence Detection Kit 2. There are only two reagents in the new kit instead of the three reagents in the old kit.
5. Add 45 µl of 1x Dilution buffer directly into wells of a white optiplate-96 assay plate
6. Transfer 15 µl of clarified supernatant to the plate, cover with plastic coverfoil and incubate 30 min at 65°C.
7. Incubate plates on ice 2-5 min.
8. Add 60 µl of room temperature Chemiluminescence substrate to each well
9. Incubate at room temperature for 60 min

10. Read on MLX Microplate Luminometer (Dynex Technologies) set at Glow-Endpoint 0.20 sec/well RAW Data Handling Average readings at 20 min after adding substrate.

The relative light units (RLUs) obtained from triplicate samples should not vary by more than 10 or 15%. If they vary more than that check the notes section to try to troubleshoot the problem.
3.2.3 Neutralization Assay

Once the PsV has been titrated, test sera (see Notes 5 and 6) can be assayed to determine endpoint neutralization titers. To monitor inter-assay variability, the following controls should be included for each plate: (1) at least two wells of cells in neutralization/growth media without PsV or serum, (2) at least four wells of PsV-infected cells to which no antibody was added, (3) cells treated with PsV pre-incubated with a known serum with at least 4 dilutions that span the 50% neutralizing titer that has been recorded in other experiments; and (4) cells treated with PsV pre-incubated with at least 1 dilution of a known non-neutralizing serum. See Fig. 2 for a typical arrangement of samples.

1. Preplate cells as described in Subheading 3.2.1.
2. Perform serial dilutions of the unknown sera in sterile polystyrene plates. Be sure to take into account the 1:5 dilution of the serum once 20 µl of diluted serum are added to 80 µl of the PsV.
3. Pre-wet a sterile multichannel pipet reservoir with neutralization/growth media to prevent sticking of PsV to the reservoir.
4. Dilute PsV in neutralization/growth medium to the concentration determined in Subheading 3.2.1 in a 15 or 50 ml polystyrene centrifuge tube.
5. Vortex briefly and decant into the pre-wetted reservoir.
6. Using siliconized tips and a multichannel pipettor, transfer 80 µl of the diluted PsV to each well of a 96-well dilution plate. If siliconized tips are unavailable, pipette diluted PsV up and down five times before delivering to the first row of the plates, then use the same tips for all other rows.
7. Add 20 µl of the test or control sera to the PsV and incubate on ice for 1 hour.
8. Add the entire volume of the virus-antibody mixture to the corresponding wells of the plate using a multi-channel pipettor.
9. Return the plate to the incubator for 72 hours.

10. The medium should not be changed.

11. The supernatant is then assayed for presence of SEAP (Subheading 3.2.2). The titer is defined as the reciprocal of the highest dilution of serum that reduces the SEAP activity by at least 50% in comparison to the reactivity in the wells that received PsV but no antibody.

If the same PsV stock is used to repeat the neutralization, then the 50% neutralizing titer would should be the same, or vary by 3 or 4 fold. If the results vary by more than 4 fold, the assay should be repeated a third time. Report the geometric mean titer of all assays performed.

4. Notes

1. The presence of phenol red tends to give a higher background in the chemiluminescent (and colorimetric) detection assay; therefore medium without phenol red is preferred for the assays.
2. The timing of plating of cells in the neutralization assay is important. Preplating overnight, or just before adding PsV results in suboptimal transduction.
3. Since 293TT cells detach very easily, replacement of the media once the procedure has been started is not recommended.
4. Although the chemiluminescent assay is more sensitive for detecting SEAP compared to colorimetric substrates, chemiluminescent methods require access to a microplate luminometer and are substantially more expensive. Because of the lower sensitivity of colorimetric substrates, 3- to 10-fold more PsV must be used if a colorimetric readout is to be performed. With many PsV / antibody combinations, the neutralization assay probably operates under conditions of antibody excess (for an excellent review of this important concept, see Klasse and Sattentau). For this reason, modest changes in the amount of input PsV typically do not alter the 50% neutralization cutoff (Ratish Gambhira, Yuk-Ying S. Pang and Richard B.S. Roden, unpublished observation). Thus, for some types of experiments, colorimetric reagents may be an acceptable substitute for chemiluminescent methods. A colorimetric protocol developed by Richard Roden’s group is outlined here.
5. Heparin and its biochemical relatives interfere with papillomavirus infection. Therefore, do not use plasma collected in heparinated tubes.
6. Sera frequently have non-specific interfering activity at dilutions of 1:20 or lower, so 1:40 is a typical starting dilution. Although highly neutralizing anti-VLP sera may need to be diluted one to a few million to encompass the 50% neutralization cutoff, titers of sera from naturally HPV-infected women typically range between 1:40 and 1:10,000. BPV1 pseudovirions should be used as a control for non-specific neutralizing activity in anti-HPV sera. Serum is considered positive in an HPV neutralization assay only if it neutralizes HPV PsV at a 4-fold higher dilution than it neutralizes BPV PsV.

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Fig. 1 Schematic drawing for a 96-well plate for titering PsV. Shaded cells should be filled with 120µl of medium with phenol red to avoid evaporation from inner wells.

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Fig. 2: Schematic drawing for a typical set up for determining neutralizing titer of unknown sera with HPV16 PsV. Shaded wells should be filled with 120µl of medium with phenol red to avoid evaporation from inner wells.

References
