Chapter 7 – HPV serology

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7.1 INTRODUCTION

Antibodies against human papillomavirus (HPV) are a marker of prior exposure to viral antigen via natural infection or vaccination. In pre-vaccination settings, serology, or the detection of antibodies in serum, can be used to study the epidemiology of HPV. Currently the most important use of serology is to evaluate the immunogenicity of new vaccines, reduced or altered dosing schedules, and novel methods of administration. This section outlines the principles of a binding enzyme-linked immunoabsorbent assay (ELISA), both single and multiplex formats, and HPV neutralization assay, along with example methods for each assay format. To date, commercial reagents are not available. Therefore, laboratories are required to prepare and quality control all reagents.

The results of varying assay types will vary somewhat as explained in review by Schiller et al, and Pinto et al.[1,2] Neutralization assays generally detect antibodies of all classes that prevent binding of pseudovirions. These assays (described below) are often time consuming. Binding assays generally detect neutralizing and non-neutralizing antibodies of a specific IG class. As neutralizing antibodies predominate and immunoglobulin G (IgG) is the dominant class, the results are generally in agreement, so ELISAs are frequently employed because they are faster. Adding a competitive monoclonal antibody directed to neutralizing epitope to a chemiluminescent Immunoassay (CIA)-based binding assay could help mimic a neutralization assay more closely. The assay formats are described in more detail in subsequent sections. **Table 7-1** shows the advantages and disadvantages of the main assay types.

Table7-1 Main serology assays

	Advantages	Disadvantages
Neutralization assay	• Measures function closest to presumed mechanism of protection • All immunoglobulin classes are detected	• Requires pseudovirions for each type • Requires cell culture and time for cells to grow • Time-consuming and labor intensive • Limited ability to multiplex • Higher coefficients of variation
Competitive immunoassay	• Detects neutralizing antibodies • Easily multiplexed with bead arrays (e.g. Luminex) • Rapid, high throughput • All immunoglobulin classes detected	• Only a subset of total neutralizing antibodies detected • Requires type-specific neutralizing monoclonal antibodies • Mulitplexing requires compromise between selecting dominant epitope and retaining type specificity
Enzyme linked immunosorbent assay	• Fast, high throughput • Familiar assay format • Amenable to multiplexing (bead arrays or multispot wells)	• Detects one immunoglobulin class (IgG or IgA), determined by secondary antibody • Non-neutralizing binding antibodies can be detected

Table adapted and used with permission from Pinto et al, 2018[2]

7.2 VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) are non-infectious, composed of viral protein subunits, L1 or L1 and L2, that self-assemble into particles that resemble intact virions and present conformational epitopes. Protection from infection is associated with antibodies against conformational epitopes, therefore using conformationally intact VLPs of high quality are essential for adequate performance of ELISAs. VLP proteins can be expressed in a variety of expressions systems such as insect yeast and mammalian cells. Appendix 1 includes an example of a standardized protocol for preparation and qualification of VLPs. This involves several steps:

- Bacterial Transformation
- Bacterial plasmid culture
- Preparation of bacteria for plasmid purification
- Plasmid purification using the Zymo Research Kit
- HEK-293TT cell culture
- Transfection of HEK-293TT cells with VLP plasmids
- VLP purification and analysis
- VLP ELISA for specificity

7. 3 ENZYME-LINKED IMMUNOSORBENT ASSAY

An ELISA is used to detect type-specific antibodies. There are several variations of the ELISA method (**Figure 7-1**), such as direct assays that coat HPV VLPs directly on the microplate, indirect assays that bind HPV VLPs to the microplate via heparin or via anti-HPV VLP antibodies, and competitive assay formats that use monoclonal antibodies to block epitope specific antibody binding to an HPV type specific VLP (e.g., clia). The nature of the secondary antibody determines the kind or kinds of serum immunoglobulins detected. HPV ELISAs are commonly directed to serum IgG, often a single isotype but in some situations total IgG or IgA may be the focus. ELISAs also differ in the detection method, again depending on the label on the secondary antibody (e.g., Fluorescent tagged, enzyme linked with varying enzyme-chromogen combinations).

International standards (IS) are available for all nine HPV types included in current vaccine formulations. Use of a reference standard in each assay that is calibrated to IS allows results ELISA results to be reported in IU/ml. This improves assay standardization and allows comparisons across ELISA platforms.

The example protocol provided below is the single-plex ELISA that the Frederick National Laboratory for Cancer Research HPV Serology Laboratory uses for HPV type-specific IgG testing. Laboratories may choose to perform another serological method or alternative procedure that performs well and is validated according to established quality indicators, if so desired.

Figure 7.1 ELISA

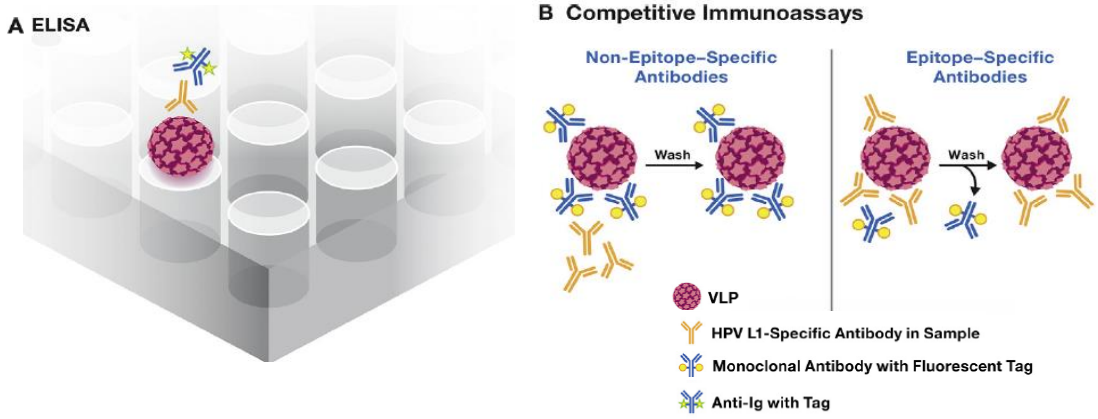


Figure used with permission from Pinto et al.[2]

VLPs, self-assembled HPV L1 or L1/L2 protein capsids that resemble intact virions, are used as antigen in the ELISA assay. These VLPs present type-specific conformational epitopes and are a critical reagent in determining assay sensitivity and specificity. Human serum samples are allowed to react with the antigen. If HPV type-specific antibodies are present in the serum, the antibodies will bind to the VLPs adsorbed onto the surface of the well. Bound human IgG is detected with a secondary antibody conjugated to horseradish peroxidase (HRP). A colorimetric substrate allows detection of HPV antibodies in the samples through an optical density readout.

Each serum sample can be tested against one or several different HPV types. There are a few important controls to consider when developing and running an ELISA on clinical samples. Each ELISA plate should include at least one negative control sample, one positive control sample, and an internal standard (reference) control sample to monitor the suitability of each plate run. It is preferred to run multiple serial dilutions of each serum sample and control such as 2-fold dilutions (1/100, 1/200, 1/400, 1/800, …) or half-log dilutions (1/10, 1/31.6,1/100, …), along with appropriate serial dilutions of the reference serum sample is tested on each plate.

The amount of antibodies in the sample is estimated relative to the reference serum using the parallel-line method or appropriate curve-fit model such as five parameter logistic curve fit model. Samples are also scored as positive or negative using a predetermined “cut-off” level. This system has good reproducibility (e.g. coefficient of variation below 15%) for samples tested on different plates and at different times.

7.3.1 Equipment, supplies, and reagents

Equipment

- Biosafety cabinet
- Freezer at -65°C to -90°C
- Incubator
- Microplate reader with a 450 and 620 nm filter (e.g., Molecular Devices)
- Pipettes
- Plate shaker
- Microplate Washer (Optional)
- Refrigerator at 2°C to 8°C
- Serological Pipettor
- Timer
- Vortexer
- Computer with Microsoft Excel® software

Supplies

- 1.5 mL tube, polypropylene (VWR, Cat #87003-294 or equivalent)
- 10 mL serological pipette
- 15 mL conical tube
- 150 mL bottle, media storage (Thomas Scientific, Cat #8600B13 or equivalent)
- L bottle, media storage – Corning Polystyrene Roller Bottle (VWR, Cat 89184-640 or equivalent)
- 25 mL serological pipette
- 250 mL bottle, media storage (Thomas Scientific, Cat #1743D05 or equivalent)
- Assorted 10-100mL serological pipettes
- 50 mL conical tube
- 50 mL serological pipette
- Alcohol resistant lab marker
- Ice pan
- Kimwipes
- Pipette Tips
- Plate Sealers (Thomas Scientific, Cat #6980A01 or equivalent)
- Plate, Maxisorp, Flat bottom (Thomas Scientific, Cat #6925A00)
- Wet ice
- Low-lint paper towels
- Bleach, Concentrated
- Primary Disinfectant (Cavicide, or equivalent)
- Secondary Disinfectant (Ster-ahol, VWR, Cat # 14003-358 or equivalent)

Reagents

In order to provide a detailed SOP, catalogue numbers of materials found to perform the ELISA are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

- 0.36N Sulfuric acid (H_2SO_4)(VWR, Cat # JT4700-1)
- 0.5 mg goat anti-human IgG antibody conjugated to peroxidase (seracare, Cat # 5220-0390)
- 1X Dublecco's phosphate-buffered saline (DPBS), sterile (Life Technologies, Cat #14190-136 or equivalent)
- 1X Wash Buffer
- Blocking Buffer
- HPV ELISA coating buffer
- HPV type specific negative control
- HPV type specific positive control
- HPV virus-like particles (VLPs) types 16 and 18 (stored at -65°C to -90°C) (see section 7.4)
- Skim milk powder (BD Biosciences Cat # 232100)
- 3,3′,5,5′-Tetramethylbenzidine (TMB) (seracare, Cat # 50-76-03)
- TWEEN® 20 (VWR, Cat # EM-PX1296-1 or equivalent)
- Proclin 300 (Sigma-Aldrich, Cat # 48914-U)
- Sodium Chloride (nacl) (VWR, Cat # EM1.06404.5000)
- Potassium Phosphate, Monobasic (KH_2PO_4) (VWR, Cat # PX1565-1)
- Sodium Phosphate Dibasic Anhydrous (Na2HPO4) (VWR, Cat # 97061-584)
- Type I water- ultrapure/Reagent Grade/critical applications (Resistivity > 18 MΩ-cm and TOC ≤ 50 ppb)

7.3.2 Preparation of reagents

- 5X Wash Buffer
    - For 4 liters (4L), weigh out 404±0.4 g of Sodium Chloride and add it to an appropriately sized container.
    - Weigh out 4±0.2 g of Potassium Phosphate, Monobasic and add it to the container.
    - Weigh out 18.34±0.2 g of Sodium phosphate dibasic anhydrous and add it to the container.
    - Add approximately 3 L of Type I water to dissolve the chemicals.
    - **Note**: May add stir bar to the container and mix without heat or use an overhead stirrer to mix contents. Chemicals can take approximately 1-2 hours to dissolve.
    - Once chemicals are dissolved, add 10 mL of TWEEN® 20 using a serological pipette. Rinse pipette well in the solution.
    - QS reagent to 4 L using Type I water.
    - Reagent expires 2 months from date of preparation and must be stored at 2-8°C.
- 1X Wash Buffer
    - Mix 1 L of 5X Wash Buffer with 4 L of Type I water.
    - Reagent may be prepared and used same day at room temperature or stored up to 1 month from date of preparation at 2-8°C.
- Blocking Buffer, 4% Skim Milk with 0.2% TWEEN® 20 in DPBS.
    - For 6 plates, weigh out 16±0.4 g of Skim Milk and add it to an appropriately sized container.
    - Add 400 mL of 1X DPBS to the container.
    - Mix vigorously until Skim Milk is fully dissolved.

    - Once solution is homogenous, add 800 µL of TWEEN® 20. Invert slowly to avoid producing excessive bubbles in the solution.
    - Allow Blocking Buffer to sit at room temperature for at least 30 minutes before use.
    - Reagent expires 24 hours from date of preparation and must be stored at 2-8°C overnight.
- HPV ELISA coating buffer
    - Combine 2 mL of Proclin 300 with 998 mL DPBS (Gibco, Cat # 14190-136) in a 1 L container.
    - Swirl until mixed.
    - Reagent expires 1 month from date of preparation and must be stored at 2-8°C.

7.3.3 Safety

Blood products are potentially infectious and should be handled under Biosafety level 2 (BSL-2) conditions. Gloves, gowns, and protective eyewear should be worn during collection and processing.

7.3.4 Procedure recommendations

To avoid problems with ELISA, observe the following.

- Use only thoroughly cleaned glassware, particularly for preparing substrate solutions.
- All pipetting steps should be performed with the utmost care and accuracy.
- To avoid contamination, do not touch the top of the plates or strips with your gloved fingers, and do not touch the walls or bottom of the wells with the pipette tips when adding conjugate or substrate.
- Check for air bubbles after all pipetting steps; if present, remove by gentle tapping.
- Check efficacy of your washing procedure by visually inspecting that there is no residual liquid in the wells, especially after incubation with the conjugate.

7.3.5 Test Procedure

The protocol described is for the use of high binding Nunc Maxisorp 96-well microplates. Other types of plates and manufacturers may be used after evaluating and validating the performance characteristics.

7.3.5.1 Procedure Summary

- Plate coating
- Plate blocking

- Standard and control preparation
- Sample plating
- ELISA procedure
- Plate reading
- System Suitability
- Data analysis
- Well masking

7.3.5.2 Controls

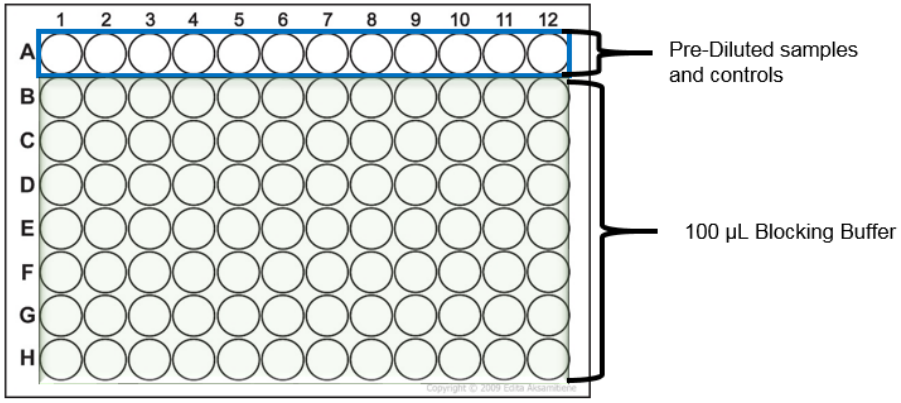
- International Standards (IS) for antibodies to HPV-16, HPV-18, HPV-6, HPV-11, HPV31, HPV-33, HPV-45, HPV-52, HPV-58 have been established by the World Health Organization (WHO) Experts Committee on Biological Standardization (ECBS) and are available from the National Institute for Biological Standards and Control (NIBSC). The IS are not used as controls in each assay. They are used for establishing secondary standard.
    - IS for HPV-16 antibodies - assigned 5 international units (IU)/ampoule (10 IU/ml)
    - IS for HPV-18 antibodies - assigned 8 IU/ampoule (16 IU/ml)
    - IS for HPV-6 antibodies - assigned 7 IU/ampoule (28 IU/ml)
    - IS for HPV-11 antibodies - assigned 6 IU/ampoule (24 IU/ml)
    - IS for HPV-31 antibodies - assigned 3 IU/ampoule (12 IU/ml)
    - IS for HPV-33 antibodies - assigned 8 IU/ampoule (32 IU/ml)
    - IS for HPV-45 antibodies - assigned 2 IU/ampoule (8 IU/ml)
    - IS for HPV-52 antibodies - assigned 14 IU/ampoule (56 IU/ml)
    - IS for HPV-58 antibodies - assigned 20 IU/ampoule (80 IU/ml)
- Each laboratory may use its own secondary or tertiary standard (reference) serum to calculate units. The serum should have been tested in parallel with the IS serum to enable traceability to the same IU. The standard serum may be tested in 2-fold dilutions (1/100, 1/200, 1/400, 1/800, …) or half-log dilutions (1/10, 1/31.6, and 1/100, …) on each plate depending on the testing format that is chosen for running the assay. Standard pre-dilution must be established prior to running clinical samples.
- Please refer to the "WHO manual for the establishment of national and other secondary standards for antibodies against infectious agents focusing on SARS-cov2" for guidance on calibrating assay reference standards to the WHO International Standards. Every assay used to report HPV serology results must be calibrated to the appropriate WHO International Standards and must report results in International Units (IU)/ml. The WHO International Standards may be obtained from the following link.
- To monitor daily plate run suitability as well as assay drift or shift in antibody binding, the inclusion of a seropositive and seronegative control is important. Quantitative ranges and pre-dilution for the seropositive control must be established prior to running clinical samples.

7.3.5.3 Plate Layouts

An example of initial plate and plate map can be found in **Figure 7-2**

Figure 7-2 Plate Layouts

A. Initial Plate Set Up Layout



B. Plate Map Example

Plate	1	2	3	4	5	6	7	8	9	10	11	12
	Sample 1	STD	NEG	CP1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	CP2
A	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
B	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
C	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
D	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
E	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5
F	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6
G	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7
H	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8

A. For the initial loading of the plate, load pre-diluted samples and controls to row A, and 100 µL Blocking Buffer to all other rows. B. Plate layout after dilution protocol. STD- Internal standard (reference) sample; NEG- seronegative control sample; CP1 and CP2- seropositive control sample.

7.3.5.4 Plate Coating

- Calculate total volume needed using the following formula:
- # of plates x 96 wells/plate x 100 µL/well ÷ 1000 µL/mL + Overage. Overage can be scaled as needed. Round volume (ml) to the nearest whole number.
- For example, to coat 6 plates:
    - 6 plates x 96 wells/plate x 100 µL/well ÷ 1000 µL/mL + 4 mL overage = 61.6 ml, round up to 62 ml.
- Calculate the volume of VLP needed using the following formula:
    - V1 = C2V2 / C1, where C1 is the starting VLP concentration, C2 is the final VLP coating concentration, and V2 is the total volume calculated above. Then, multiply the value by 1000 (to convert volume from mL to µl, and round volume (µl) to the nearest whole number.
- For example, to coat 6 plates at a final VLP concentration of 2.7 µg/ml, with a starting VLP concentration of 3850 µg/ml:
    - V1 = ((2.7 µg/mL x 62 ml) / 3850 µg/ml) x 1000 µg/mL = 43 µL of VLP needed.
- In a Biological Safety Cabinet (BSC), add the total calculated volume of coating buffer to a container. Remove the calculated volume of VLP needed from the coating buffer.
- Add the calculated volume of VLP to the coating buffer. Mix by inversion.
- Using a multichannel pipette, add 100 µL of coating solution to each well of the 96-well plate. Cover plate with a plate sealer.
- **Note**: Plates are stored at 2-8°C and must be used between Day 3 and 5 from the coating date.

7.3.5.5 Plate Blocking

Note: Before starting the assay procedure, warm all reagents to room temperature (RT) (20ºC±5) prior to use.

- Discard the VLP solution and wash the plate three times by adding 350 µL 1X Wash Buffer to each well using a multi-channel pipette or in a plate washer. After the final wash step, tap the plate thoroughly against paper towels.
- Add 300 µL/well of Blocking Buffer and incubate for 90±10 minutes at RT (20ºC±5) without agitation.
- Discard the Blocking Buffer.
- Wash the plate three times by adding 350 µL 1X Wash Buffer to each well using a multi-channel pipette or in a microplate washer. After the final wash step, tap the plate thoroughly against paper towels.

7.3.5.6 Serum

- Thaw serum samples on ice or in the refrigerator.
- Note: a minimum of 10 µL of sample is required.

- Prepare 3 mL of pre-diluted (D1) (see section 7.3.6.2 Controls) seropositive control and then split into two tubes.
- Seronegative control is pre-diluted 1:100.
- Samples are pre-diluted at a minimum of 1:100.
- Standard are pre-diluted according to section 7.3.6.2 Controls)
- Standard, controls, and samples are serially diluted 1:2 from their starting volume.
- Transfer D1 Standard, controls, and samples into Row A at a volume of 200 µL per well. Use Figure 7.2A as a template for Standard, controls, and sample placement.
- Note: Vortex the Standard, controls, and samples for approximately 3-10 seconds at speed settings ≥6 prior to adding to the plate.
- Using a multichannel pipette, aspirate 100 µL from Row A. Without touching the bottom of the plate, put the tips into the diluent in Row B and mix 10 times. Discard empty tips.
- Continue the serial dilution through to Row H, mixing each dilution 10 times and switching tips between each row. Discard the extra 100 µL from row H.
- Seal each plate with a plate seal and incubate at room temperature for 60±5 minutes with gentle shaking between 200-300 RPM.
- Remove the plate seal and wash the plate three times by adding 350 µL 1X Wash Buffer to each well using a multi-channel pipette or in a microplate washer. After the final wash step, transfer the plate to a BSC and tap the plate thoroughly against paper towels.

7.3.5.7 Conjugate

- Add 100 µL conjugate (diluted to a concentration of 0.025 µg/ml) to all wells. Cover each plate with a plate seal and incubate at RT for 60±5 minutes with gentle shaking between 200-300 RPM.
  Note: Avoid air bubbles in the wells. Different commercially- available conjugates and manufacturer lots of conjugate may differ in performance. Use of different conjugates may require titration and assessment for gamma- chain-specificity under these conditions, and separate validation of the assay.
- Remove the plate seal and wash the plate three times by adding 350 µL 1X Wash Buffer to each well using a multi-channel pipette or in a microplate washer. After the final wash step, transfer the plate to a BSC and tap the plate thoroughly against paper towels.

7.3.5.8 Substrate

- Approximately 15 minutes prior to use, prepare TMB solution. Mix TMB Reagent A with TMB Reagent B in equal volumes.
  Note: protect solution from light.
- Example: To prepare TMB solution for 6 plates:
  - Total Volume Calculation: 6 plates * 12 mL = 72 ml
  - TMB Preparation: 36 mL TMB A + 36 mL TMB B

- Add 100 µL per well of TMB solution to all wells.
  Note: It is recommended to pipette from lowest concentration (Row H) to highest concentration (Row A). Avoid air bubbles. This is especially important at this step, since bubbles will cause an incorrect absorbance reading.
- Cover each plate with a plate seal and incubate at RT for 25±2 minutes protected from light, with no agitation.
- After the TMB incubation, remove the plate seal and add 100 µL of 0.36 N Sulfuric Acid (H_2SO_4) to each well to stop the reaction.
- Read plates within 10 minutes at 450 nm (λ1) and 620 nm (λ2) in a microplate reader.
  Note: If possible, use wavelength subtraction (λ1- λ2).

7.3.5.9 System Suitability

System suitability for Standard Curve and Controls can be seen in **Table 7-2** and **Table 7-3**, respectively.

Table. 7-2 System Suitability for Standard Curve

Standard curve		
Parameter	Acceptance Criteria	Action Upon Failure
R^2	≥ 0.990	Repeat Plate
Optical density (OD) of First Std Dil (A2)	2.0 OD – 4.0 OD	Repeat Plate if negative and positive controls failed
OD of Last Std Dil (H2)	≤ 0.25 OD	Repeat Plate if negative and positive controls failed
Ods for Std Dilutions 1-7	The difference observed between the ods of the n and the n+1 serial dilutions is ≥ 30% ((Delta(OD "Std"))/(OD "Std" x 100)) >=30%	Exclude raw results from analysis.
CV determined on the serial Standard values adjusted for Dilution	≤ 30%	If after masking no more than one well and the plate fails, then repeat plate.
Back-fit dose, Std Curve Dilution 2	Recalculation must be within 90-110% of the theoretical concentration of the Standard	If after masking no more than one well and the plate fails, then repeat plate.

Table. 7.3. System Suitability for Controls

Controls		
Parameter	Acceptance Criteria	Action Upon Failure
Calculated concentration of the Negative control	Lower than the technical cut-off	If after masking no more than one well and the plate fails, then repeat plate.
Calculated concentration of the Positive control	Within the range	If after masking no more than one well of the CP1 and CP2 and the plate fails, then repeat plate.
CV on the serial dilution of the Positive control adjusted for Dilution	≤ 30%	If after masking no more than one well of the CP1 and CP2 and the plate fails, then repeat plate.
CV between the two replicates of the Positive control	≤ 16%	If after masking no more than one well of the CP1 and CP2 and the plate fails, then repeat plate.
Ods of the Positive Control Dilutions 1-7	The difference observed between the ods of the n and the n+1 serial dilutions is ≥ 30% ((Delta(OD "Std"))/(OD "Std" x 100)) ≥ 30%	Exclude raw results from analysis.

7.3.5.10 Data Analysis

Note: Data Analysis is for serum samples only.

Antibody levels, expressed as IU/ml, are calculated by interpolation of optical density (OD) values from the standard curve (five parameter logistic curve fit) by averaging the calculated concentrations from all dilutions that fall within the working range of the standard curve.

For antibody concentration > [assay cutoff * 3], serum antibody concentration is accepted if the percent coefficient of variance (CV) is ≤ 30% based on the calculated serial sample values adjusted for dilution.

Note: Assay cutoff is predetermined by running > 40 sera samples collected from HPV naïve individuals (e.g., unvaccinated children under the age of 14 years or pre-sexual debut) and calculating the lower limit of quantification.

For antibody concentration between [0.5 * assay cutoff] and [assay cutoff * 3] the serum sample is typically retested to verify seropositivity. If the sample first tested seropositive and retests as seropositive, it is reported as the first result. If the sample first tested seronegative and retests seronegative then the sample is reported as seronegative. If the sample first tested seronegative and retests seropositive, it is tested a third time and then reported according to which finding was found twice.

Note: The Frederick National Laboratory for Cancer Research HPV Serology Laboratory has developed a protocol file for SoftMax Pro 7.0.3 GXP (Molecular Devices) that incorporates the analysis parameters outlined in this manual and is available upon request.

Masking of Wells

Wells can be masked when results are erroneous due to pipetting error and/or well contamination.

- Standard:
    - If the delta percentage for any of the sample dilutions is <30% or >70%, one of the eight (1/8) serially diluted wells may be masked for the Standard.
    - If the CV determined on the serial dilution values adjusted for dilution is >30%, one of the eight (1/8) serially diluted wells may be masked for the Standard.
    - If the second serial diluted well (B2) of the Standard needs to be masked, the back-fit dose system suitability criteria must be transferred to the third serially diluted well (B3).
    - Note: At most one well can be masked for the Standard.
- Positive Controls:
    - If the delta percentage for any of the sample dilutions is <30% or greater than 70%, one of the eight (1/8) serially diluted wells may be masked for a single positive control sample.
    - If the CV of the final concentration between the two positive control samples (control positive (CP) 1 and CP2) is >16%, one of the eight (1/8) serially diluted wells may be masked for only one of the positive control samples.
    - If the CV calculated on average valid concentration is >30%, one of the eight (1/8) serially diluted wells may be masked for a single positive control sample.
    - Note: At most one well of each positive control can be masked.
- Negative Control:
    - If erroneous OD in a well due to pipetting error and/or well contamination, then one of the eight (1/8) serially diluted wells may be masked for the negative control sample.
    - Note: At most one well can be masked for the negative control.

- Samples:
    - If the delta percentage for any of the sample dilutions is <30% or >70%, one of the eight (1/8) serially diluted wells may be masked for a single sample.
    - If the CV calculated on average valid concentration is >30%, one of the eight (1/8) serially diluted wells may be masked for a single sample.
    - Note: At most, one well of each sample can be masked.

Alternative Analysis Tool for Calculating Serology Results

How to calculate the results using parallel line (PLL) shareware programme

- Export absorbance values to a spreadsheet in Microsoft Excel®.
- Calculate final absorbance value for each sample dilution by subtracting the absorbance of the serum on the control plate from that on the test plate. Most sera should have absorbance <0.1 on the control plate, but occasional “sticky” sera may have high values.
- Access the PLL programme and enter the final mean absorbance values of the three dilutions of reference serum in the field indicated in yellow in columns B, C and D of the spreadsheet. Write the sample ID in column A and the final absorbance values of each dilution in corresponding columns B, C and D. As the PLL shareware can only use integers, multiply the mean absorbance values by 1000. The programme will calculate the PLL value and the correlation between data points.

How to interpret your results using PLL shareware programme

- Cut-off values for PLL values/IU units should be known prior to testing. Pre-assigned cut-off values for HPV serology should be based on ELISA units calculated by the PLL method.
- PLL values above the assigned cut-off, if the serum has a correlation >0.9, are considered positive.
- Continue interpretation by evaluating correlation, slope, slope ratio and data points (see definitions above). If a sample does not fulfill the quality criteria it has to be retested.
- If the reference serum has a correlation and/or slope that does not fulfill the criteria, the entire plate has to be retested.
- All samples with a correlation less than 0.9 are retested.
- If the absorbance values of the second or third dilution are higher than the OD value of the first dilution (pro-zone phenomenon), the sample should be diluted further in the retest.
- Samples that have a data point outside cut-off level should also be retested.
- Samples with all absorbance values below 0.1 are not retested.

7.4 MULTIPLEX ELISA

While individual ELISAs can be performed to detect antibodies to each HPV type of interest, increasing valency of vaccines could require running as many as 9 assays for each sample. This takes time and increases reagent and serum sample use. The purpose of the multiplex assay is to detect multiple HPV type specific L1/L2 VLP and antibody interactions within the same reaction well for increased assay efficiency. Multiplex assays generally use less VLP antigen and conserves serum and reagents. The disadvantage is specialized expensive instruments are required. The principles of two different multiplex assays are described, one using Luminex xmap® technology and the other mesoscale Discovery platform. Detailed protocols for the Luminex assay are included in the appendix. References and contacts for more information about the M9ELISA (using mesoscale Discovery platform) are found below.

7.4.1 HPV Multiplex assay using Luminex xmap® technology

The Luminex bead-based system is a proven technology for multiplex serological analysis. Specifically, the Luminex bead-based multiplex immunoassay uses the same principles as in an ELISA method: any antibodies in the sample specific to the target antigen will bind to the HPV type-specific L1/L2 VLPs attached to the assay beads, allowing the quantitation of anti-antigen antibodies in the sera.

Each Luminex bead set contains a unique fluorescent signature, and Luminex manufactures 500 different bead sets that may be distinctively recognized by their instruments (i.e., FLEXMAP 3D). Moreover, the Luminex instrumentation also detects R-Phycoerythrin in a separate channel, which allows for the detection/quantitation of antigen-antibody interactions with a secondary antibody tagged with R-Phycoerythrin. HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58 L1L2 VLPs were coupled to separate Luminex bead sets, so antibody interactions are uniquely identified.

A goat anti-human IgG antibody tagged with R-Phycoerythrin is used to detect IgG antibody interactions with each HPV type-specific VLP. The fluorescent signal is proportional to the concentration of the HPV type-specific IgG antibodies within the sera. An internal reference standard (contains a specific concentration of anti-HPV type-specific antibodies), two levels of positive control (each contain a specific concentration of anti-HPV type-specific antibodies), and negative control (lack anti-HPV type-specific antibodies) are tested on each plate to assess plate acceptability and quantitate a relative amount of HPV type-specific IgG antibodies within the sera.

Note: A detailed description of the method can be found in Appendix 2.

7.4.2 Multiplex HPV Virus-Like Particle based IgG ELISA on the Meso Scale Discovery Platform (M9ELISA)

This method is a plate-based multiplex ELISA using self-assembled 9-valent HPV L1/L2 VLPs as antigen in the assay. HPV-type specific antibodies present in test samples will bind to type-specific conformational epitopes on the VLPs adsorbed onto the surface of the microwell in a 10-spot MULTI-SPOT® plate. Bound anti-HPV human IgG is detected with a Anti Human IgG conjugated to MSD-SULFO-TAG™ using electrochemiluminescent detection on the Meso Scale Discovery (MSD) plate reader that generates relative light units. The amount of antibodies in the sample is estimated relative to a in-house standard (calibrated to International Standard) using the parallel line method. Two levels of positive control and negative control are tested on each plate to assess plate acceptability. Samples are scored as positive or negative using a pre-determined “cut-off” level. The assay can be carried out as published in manuscript by Panicker et.al.[3]

Note: For more details or training on the assay, please contact US Centers for Disease Control and Prevention, HPV Laboratory (ADD Link to contacts page for CDC in this manual).

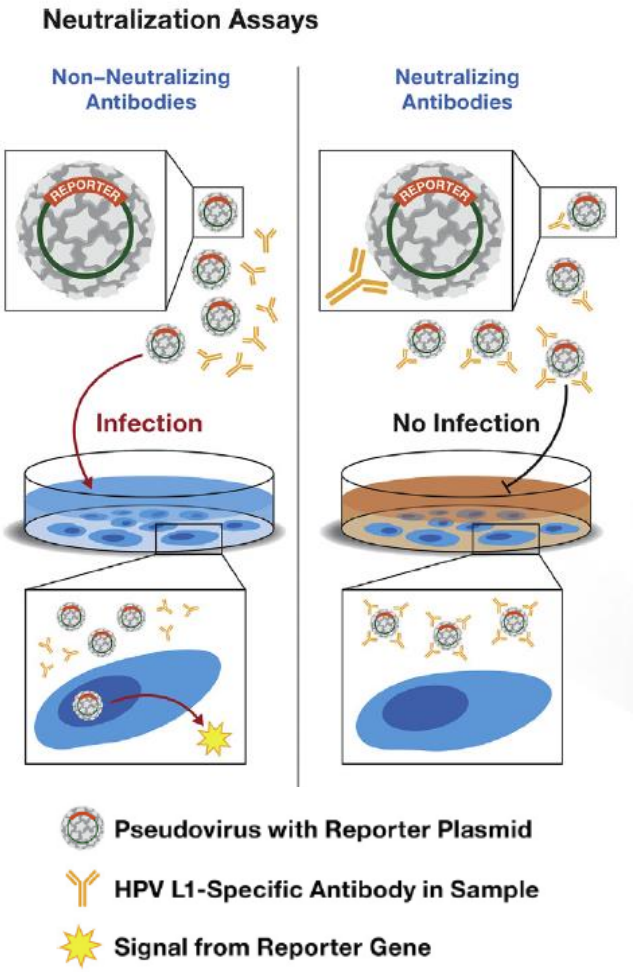
7.5 HPV NEUTRALIZATION ASSAY

Although there is a relatively good correlation between the presence of anti-VLP conformational antibodies found in the classical VLP-based ELISA, and their neutralizing activity, neutralization assays are the gold standard to assess the protective potential of antibodies induced by papillomavirus vaccines in experimental systems. Papillomavirus neutralization assays rely on neutralization of one of the following: authentic virions; pseudotyped virions that are capsids carrying a reporter gene on their surface, or pseudovirions (PsVs) that have encapsidated reporter genes. A schematic representation of neutralizing assays can be found in **Figure 7-3**.

PsVs are used in the neutralization assay described below, as a selected example of a commonly-used neutralization assay. The assay was developed by a group led by Dr John Schiller at the National Cancer Institute in the United States of America.[4] Of note, the neutralization detects capsid-binding antibodies of all immunoglobulin classes, whereas most ELISAs only measure IgG.

The neutralization assay described here is performed in 96-well plates with type-specific HPV PsVs that have encapsidated a reporter DNA encoding for secreted alkaline phosphatase (SEAP) under an SV40 promoter.[4] When PsVs bind to 293TT cells that stably express SV40 large tumour antigen, the reporter SEAP DNA is expressed and alkaline phosphatase is secreted into the media.[4] Assaying the media for alkaline phosphatase activity measures the PsV infection. Neutralizing antibodies bind to PsV and prevent the PsV from infecting the target cells (note that some antibodies can neutralize even after the virion has bound the cell).[4] PsV neutralization is indicated by a decrease in alkaline phosphatase in the media.[4] The titer of neutralizing antibody is calculated as the reciprocal of the highest dilution of sample to cause 50% reduction in SEAP activity.[4]

Figure 7-3 Schematic of neutralizing assay



7.5.1 Equipment, supplies, and reagents

In order to provide a detailed SOP, catalogue numbers of materials found to perform well by the FNLCR HPV Serology Laboratory are provided. A laboratory may use alternative products if they can be validated to yield equivalent results.

Equipment

- Centrifuges (Microcentrifuge, Bench Top)
- Class II Biosafety Cabinet (BSC)
- CO_2 Incubator
- Convection Incubator

- Freezers (-20°C, -80°C)
- Inverted Microscope
- Microplate Reader (Molecular Devices M5 or equivalent)
- Microplate Shaker
- Pipettes/Pipette Systems
- Refrigerator (2-8°C)
- Serological Pipettor
- Timer
- Vortex

Supplies

- Alumaseal CS Foil (Thomas Scientific, Cat # 1230Y24 or equivalent)
- Ice Pan (Thomas Scientific, Cat # 1200R42 or equivalent)
- Marker, Lab, Alcohol Resistant
- Pipette Tips
- Plate, 2.2 mL Deep Well, D96 (VWR, Cat# 10755-248 or equivalent)
- Plate, 96-well, Flat Bottom, Tissue Culture, F96 (Corning Costar, Cat # 3596 or equivalent)
- Plate, 96-well, White Opaque, O96 (Perkin Elmer, Cat # 6005290)
- Plate, 96-well Round Bottom, R96 (Corning Costar, Cat # 3788 or equivalent)
- Plate, 96-well V Bottom, V96 (Corning Costar, Cat# 3894 or equivalent)
- Plate Sealers (Thomas Scientific, Cat# 6980A01 or equivalent)
- 50 mL Reagent Reservoir
- 300 mL Nalgene Disposable Polypropylene Robotic Reservoirs (Thermo Fisher Scientific, Cat#: 1200-2301 or equivalent)
- 5 mL Serological Pipette
- 10 mL Serological Pipette
- 25 mL Serological Pipette
- 50 mL Serological Pipette
- 50 mL Tubes, Conical
- 1.7 mL Tube, Microcentrifuge, Polypropylene, Sterile (VWR, Cat # 87003-294 or equivalent)
- Waste Container, White, HDPE Plastic, with Screw Top Lid (Amphorea, Cat # 250MLPHARMA or equivalent)
- Wet Ice
- Wipe, Low-Lint, Wypalls

Reagents

- Bleach, Clorox, Concentrated
- Control, Negative (Anti-HPV-16 (V5) mouse monoclonal antibody (Gift from John Schiller, NCI), Anti-HPV-18 (5074) rabbit polyclonal antibody (Gift from John Schiller, NCI), or HPV Seronegative sample)
- Control, Positive [Heparin (Sigma, Cat # H1784-250MG) or Gardasil9 Serum sample]

- 1X Dulbecco's Phosphate-Buffered Saline (DPBS), Sterile (Life Technologies, Cat # 14190-136 or equivalent)
- Great escape SEAP Chemiluminescence Kit (Takara, Cat# 631738)
- HEK 293TT Cells
- Neutralization Buffer, 293TT Pseudovirion Based
- PsV: BPV, HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-45, HPV-52, and HPV-58. Reagents can be requested here.
- Primary Disinfectant (Cavicide)
- Secondary Disinfectant (Ster-ahol, VWR, Cat # 14003-358 or equivalent)
- Trypsin-EDTA (0.05%), Phenol Red (Life Technologies, Cat # 25300-054 or equivalent)
- Viastain™ AOPI Staining Solutions (Nexcelom, Cat # CS2-0106-5ml)
- Water, Distilled (Life Technologies, Cat # 15230204 or equivalent)

7.5.2 Preparation of reagents

7.5.2.1 293TT Pseudovirion Based Neutralization Assay Media (PBNA_M)

- Combine the following reagents according to **Table 7-4**. Scale volumes as needed.

Table 7-4 293TT PBNA_M

Chemical/Consumable	Source / Cat #	Amount for 200 ml	Amount for 500 ml
Phenol Red-Free Dulbecco's Modified Eagle Medium (DMEM-PF)	Invitrogen, Cat # 21063-029	172 ml	430 ml
Heat Inactivated Fetal Bovine Serum (FBS)	Hyclone, Cat # SH30070.03HI	20 ml	50 ml
Glutamax I (Glut)	Invitrogen, Cat# 35-050-061	2 ml	5 ml
Antibiotic-Antimycotic (A/A)	Invitrogen, Cat # 15240-062	2 ml	5 ml
HEPES	Invitrogen, Cat # 15630-080	2 ml	5 ml
MEM, Non-Essential Amino Acids (NEAA)	Invitrogen, Cat # 11-140-050	2 ml	5 ml
0.2 µm PES Filter	VWR, Cat # 73520-988	1 unit	

Note: New stock media should be made for each neutralization assay and not shared between experiments or cell maintenance.

- Combine Phenol Red-Free DMEM-PF, FBS, Glutamax I, Anti-Anti, HEPES, and MEM NEAA together into the top of a filter unit.

- Filter media using a 0.2 µm PES Filter.
- If preparing 500 ml, store in two separate aliquots to prevent accidental contamination if 500 mL is not used in a single batch run.
- Label a 5-15 mL tube with the media type, lot number, date, and analyst initials and place the tube in an appropriately sized tube rack.
- Aliquot 2 mL of PBNA_M into the tube. Close cap ensuring to leave it slightly loose for proper ventilation during incubation.
- Place tube rack in CO_2 incubator.
- Incubate for a minimum of 18 hours.
- After incubation, check to see if there is any growth in the tube.
- If growth is observed, discard the lot of media.
- Reagent expires 2 weeks from date of preparation and must be stored at 2-8°C.

7.5.2.2 Considerations

- All procedural processes are performed in BSC.
- Pseudovirion (PsV)-Based Neutralization Assay (PBNA) uses PsV particles that contain the SEAP reporter gene encoded in a plasmid.
- Dispense and work with one PsV type at a time.
- Incubation start and end times are based on last plate, except for substrate incubation prior to plate read, which are based on first plate.
- The assay requires 0.3 x 106 cells/ml.
- Label plate skirt with Plate Number, Data Reference, HPV Type, Analyst Initials and Date.
- Do not use cells if they have been passaged greater than 30 passages.
- Do not use cells if they appear to be contaminated.
- Use alcohol resistant lab markers when labeling plates by hand.
- PBNA_M reagent should be stored on wet ice between sample, control and PsV preparation steps.
- Quality Controls run on each plate:
    - HPV Type Specific Positive Control (PC): Human serum from HPV immunized subjects or subject previously infected with HPV type specific DNA. The positive control serum is diluted according to the HPV-Type. The positive control serum is included in Column 9 and 10 Rows A-D of every plate.
    - HPV Type Specific Negative Control (CN): Human serum that is negative for HPV Type specific antibodies. The negative control serum is included in Column 9 and 10 Rows E-H of every plate and starting dilution is 1:10 and 4-fold serial dilutions thereafter.
    - No PsV / No Sera Control (NV/NS): Column 11 and 12 Rows A-D contain cells only and show the background signal without PsV.
    - PsV + Neutralization Buffer Control (PsV+NB): Column 11 and 12 Rows E-H contain cells and PsV with no sera, and show the maximum SEAP signal from PsV.

7.5.3 Procedure

Note: PsVs are produced by transfection of 293TT cells with HPV-type-specific plasmids that are available from Dr John Schiller (2). Each purified PsV lot must be tested to evaluate SEAP production.

7.5.3.1 Day one: PsV titration

7.5.3.1.1 Titration of PsV

Only required upon startup of a new series of PBNA assays to confirm the activity and proper dilution factor of PsV that has been stored at -65°C to -90°C.

Note: For testing of human serum samples, proceed to DAY ONE: SAMPLE NEUTRALIZATION ASSAY (7.5.3.2)

7.5.3.1.2 Prepare Cells for F96 Plates.

Note: The number of plates setup depends on the total number of PsV being evaluated. Up to three PsV may be tested in a single F96 plate.

- Examine each cell culture flask of 293TT cells under an inverted light microscope and visually inspect confluency.
  Note: Cells should be 70-95% confluent.
- Discard media from flask into waste container.
- Add 10±1 mL of sterile DPBS to the flask with cells.
- Gently rinse cells with DPBS by slowly rotating flask so that DPBS washes over the cells 3-5 times.
- Discard DPBS into waste container.
- Repeat DPBS rinse one time.
- Add 3-5 mL of 0.05% Trypsin-EDTA solution to the flask with cells, and gently spread over cells.
- Incubate flask for 3-7 minutes in a 37±2°C, 5±2% CO_2 incubator.
- Ensure cells have detached by examining flask under an inverted microscope.
  Note: If cells are not completely detached, gently tap flask to dislodge cells.
- Add 10±1 mL of PBNA_M into flask to neutralize Trypsin-EDTA and transfer cells to a 50 mL conical tube.
- Centrifuge cells at 300±20 x g for 5 minutes at RT.
- Discard supernatant into waste container.
- Tap on the bottom of the tube with an index finger to loosen cell pellet.

- Add 20±1 mL of PBNA_M to cell pellet and gently mix to achieve single-cell suspension.
- Count cells.
- Dilute cells in PBNA_M to a final concentration of 0.3×10^6 cells/ml.
- Note: Prepare at least 12 mL of cell suspension for each plate (100 µL of cells are needed per well.)

  Example:

  $C_1 = 2 \times 10^6$ cells/mL (Cell Stock Concentration - Measured)

  $C_2 = 0.3 \times 10^6$ cells/mL (Desired Cell Concentration)

  $V_2 = 36$ mL or 36,000 µl (Total Volume required for three plates)

  $V_1 = C_2V_2 / C_1$

  $V_1 = (0.3 \times 10^6 \times 36) / (2 \times 10^6)$

  $V_1 = 5{,}400$ µl

  5.4 mL of cell stock would be added to 30.6 mL PBNA_M for a total volume of 36 ml.
- Record calculations.
- Mix cells by gently inverting tube.
- Dispense 100 µL of cells into all wells of each F96 Plate.
- Note: Mix cells with pipette between plates to maintain a homogenous mixture during process.
- Place plate lids on F96 Plates and incubate plates for 120-360 minutes (2-6 hours) in a 37±2°C, 5±2% CO_2 incubator.
- Record incubation start time for last plate.

7.5.3.1.3 PsV particle preparation

- Remove PsVs from -65°C to -90°C freezer and thaw on wet ice inside BSC.
- Prepare an initial dilution for each PsV according to **Table 7-5**.

Table 7-5 Recommended PsV Starting Dilution

HPV Type	HPV-6	HPV-11	HPV-16	HPV-18	HPV-31	HPV-33	HPV-35	HPV-45	HPV-52	HPV-58
Recommended Starting Dilution	1:2,000	1:400	1:40,000	1:4,000	1:40,000	1:40,000	1:1,000	1:10,000	1:2,000	1:40,000

Note: PsV aliquots are typically pre-diluted 1:10. Confirm any pre-dilution factor and recommended dilution factor, if available, prior to following dilution scheme in **Table 7-6.**

Example: 1:10,000 starting dilution

Add 10 µl of PsV (1:10 predilution) to a 15 mL conical tube.

Add 9990 µl of PBNA_M to the tube with PsV and gently mix by inversion (8-10 times).

Store on wet ice or in a refrigerator at 2-8°C until use.

- Prepare two-fold serial dilutions of PsV in D96 plate according to Table 6.
    - Add 450 µl of PBNA_M to Rows B-H, Columns 1-3, 5-7, and 9-12.
    - Add 900 µl of each HPV PsV dilution (see **Table 7-6** for recommended starting dilution) to the appropriate wells of Row A.
    - Dilute PsVs by performing a 1:2 serial dilution from Row B through H following **Table 7-7.**

      Note: Mix each dilution at least 10 times using a multichannel pipette, and discard tips between each dilution.

      Note: Perform procedure with D96 plate incubating on wet ice in BSC.

Table 7-6 D96 PsV Dilution Plate Layout

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
A	HPV-6 1:1	HPV-11 1:1	HPV-16 1:1		HPV-18 1:1	HPV-31 1:1	HPV-33 1:1		HPV-35 1:1	HPV-45 1:1	HPV-52 1:1	HPV-58 1:1
B	1:2	1:2	1:2		1:2	1:2	1:2		1:2	1:2	1:2	1:2
C	1:4	1:4	1:4		1:4	1:4	1:4		1:4	1:4	1:4	1:4
D	1:8	1:8	1:8		1:8	1:8	1:8		1:8	1:8	1:8	1:8
E	1:16	1:16	1:16		1:16	1:16	1:16		1:16	1:16	1:16	1:16
F	1:32	1:32	1:32		1:32	1:32	1:32		1:32	1:32	1:32	1:32
G	1:64	1:64	1:64		1:64	1:64	1:64		1:64	1:64	1:64	1:64
H	1:128	1:128	1:128		1:128	1:128	1:128		1:128	1:128	1:128	1:128

Table 7-7 Serial Dilutions of PsV for Testing in Triplicate

Dilution	PsV Volume (µl)	PBNA_M Volume (µl)	Dilution Factor*
1	900 µl of pre-diluted PsV	NA	1:1
2	450 µl from row A	450	1:2
3	450 µl from row B	450	1:4
4	450 µl from row C	450	1:8
5	450 µl from row D	450	1:16
6	450 µl from row E	450	1:32
7	450 µl from row F	450	1:64
8	450 µl from row G	450	1:128

Note: Pre-dilution of any PsV needs to be accounted for in this dilution factor. See Table 7.26 for Recommended Starting Dilution for each HPV PsV.

- Label R96 plates.
- Add 25 µl of PBNA_M into Columns 1-9, Rows A-H of R96 plates.
- Add 125 µl of PBNA_M to Columns 10, 11 and 12, Rows A-H for “No PsV” Controls.
- Transfer 100 µl of serially diluted PsVs from D96 plate into corresponding columns and rows of R96 plate.
    - Dispense by columns, one PsV at a time.
    - Change pipette tips for each PsV transfer.
- Cover R96 plates with plate lid and incubate for 60±20 minutes at 2-8°C.
- Record incubation start time for last plate.
- After incubation of the PsV, remove F96 plates containing 293TT cells prepared earlier and incubated R96 plates. Place plates in BSC and record time.
- Gently transfer 100 µL of PsV solutions to corresponding wells on F96 plates containing 293TT cells; do not disturb cells. Plates now referred to as Assay Plates. Return plate lid to plates.

    Note: Dispense speed setting of 4 out of 10 is recommended when using electronic pipettes.
- Incubate Assay Plates in a 37±2°C, 5±2% CO_2 incubator for at least 70-74 hours.
- Record start date and time of 3-day incubation.

    Note: The time will start when final plate is placed into incubator.
- Proceed to DAY 2: HARVEST

7.5.3.2 Day one: sample neutralization assay

7.5.3.2.1 Prepare Cells in F96 plates

- Examine each cell culture flask under an inverted microscope and visually inspect confluency.

    Note: Cells should be 70-95% confluent.
- Discard media from flask into waste container.
- Add 10±1 mL of sterile DPBS to flask without disturbing cells.
- Rotate flask slowly 3-5 times to gently rinse cells.
- Discard DPBS into waste container.
- Repeat DPBS wash one time.
- Add 3-5 mL of 0.05% Trypsin-EDTA solution to the flask with cells without disturbing cells, then gently spread over cells.
- Incubate flask for 3-7 minutes in a 37±2°C, 5±2% CO_2 incubator.
- Ensure cells have detached by examining under an inverted microscope. If cells are not completely detached, gently tap flask to dislodge cells.
- Add 10±1 mL of PBNA_M to the flask to neutralize the Trypsin-EDTA.
- Transfer cells to 50 mL conical tube.
- Centrifuge cells at 300±20 x g for 5 minutes at RT.
- Discard supernatant into waste container.

- Tap on bottom of tube to loosen cell pellet.
- Add 20±1 mL of PBNA_M to the conical tube with cells and gently mix cells to achieve single-cell suspension.
- Count cells.
- Dilute cells in PBNA_M to a final concentration of 0.3×10^6 cells/mL and record calculations.

  Note: Prepare at least 12 mL of cell suspension for each plate (100 µL of cells are needed per well.)

  Example:

  $C_1 = 4 \times 10^6$ cells/mL (Cell stock concentration)

  $C_2 = 0.3 \times 10^6$ cells/mL (Desired cell Concentration)

  $V_2 = 12$ mL or 12,000 µl (Total Volume required)

  $V_1 = C_2V_2 / C_1$

  $V_1 = (0.3 \times 10^6 \times 12) / (4 \times 10^6)$

  $V_1 = 900$ µl

  900 µl of the cell stock would be added to 11.1 mL PBNA_M for a final volume of 12 ml.
- Mix cells by gently inverting tube (8-10 times).
- Dispense 100 µL of cells into all wells of each F96 Plate.
- Note: Mix cell suspension after coating 2-3 plates (if using Rainin multichannel pipette) to maintain a homogenous mixture of cells during the process. If using a 96-well head multidispenser such as epmotion, then cell suspension should be mixed prior to each aspiration of cell suspension.
- Place plate lids on F96 Plates after each plate is coated with cells and incubate plates for 120-360 minutes (2-6 hours) in a 37±2°C, 5±2% CO_2 incubator.
- Record incubation start time for last plate.

7.5.3.2.2 Sample preparation

- Remove serum samples and assay controls from -65°C to -90°C freezer, and thaw on wet ice inside BSC. Alternatively, samples can be placed in a rack and thawed in a refrigerator at 2-8°C.
- Note: Ideally, samples should be diluted and prepared for addition to the PsV within 4 hours of starting the sample thaw.
- Once thawed, mix tubes by inversion (8-10 times) or vortex (5-10 seconds).

  Note: If samples present with significant precipitant or debris, the samples and controls may be centrifuged at 10,000±1000 x g for 5 minutes at 2-8°C.
- Prepare 1:4 serial dilutions in R96 or D96 plate.
- Dilute samples at 1/10 starting dilution using volumes listed in **Table 7-7**.

- Dilute positive and negative controls starting at the dilution of 1/10. Some PsV types require a 1:160 starting dilution of PC.

  Note: Mix each dilution 10 times using a multichannel pipette, and discard tips between each dilution.

Note: **Table 7-8** is presented as an example and volumes may be increased or decreased accordingly.

Table 7-8 Serial Dilutions of Samples (Testing in Duplicate)

Dilution	Sample Volume (µl)	PBNA_M Volume (µl)	Dilution Factor
1	10 µl from sample vial	90	1:10
2	12.5 µl from row A	37.5	1:40
3	12.5 µl from row B	37.5	1:160
4	12.5 µl from row C	37.5	1:640
5	12.5 µl from row D	37.5	1:2560
6	12.5 µl from row E	37.5	1:10240
7	12.5 µl from row F	37.5	1:40960
8	12.5 µl from row G	37.5	1:163840

- Cover plates with a clear plate sealer and set aside in BSC on wet ice or place in 2-8°C refrigerator while preparing PsV dilutions.

7.5.3.2.3 PsV particle preparation

Note: Ensure proper study specific PsVs are used.

- Prepare 12 mL per plate of Working Dilution of each PsV with PBNA_M per **Table 7-8**.
  Note: A sterile polystyrene tube is preferred to store the working dilution into; however, a polypropylene tube or bottle may also be used.

- Example: If PsV final concentration required is 1:60,000, and PsV is pre-diluted at 1:1000, then a dilution of 1:60 is needed.
  Note: Pre-dilution of any PsV (usually 1:10) needs to be accounted for in this dilution factor.
  Note: PsV titration assay will determine Final Dilution Factor, while **Table 7-9** represents historical Final Dilution Factors for guidance only.

Table 7-9 Final Dilution Factors for 10 PsV Types

HPV Type	HPV-6	HPV-11	HPV-16	HPV-18	HPV-31	HPV-33	HPV-35	HPV-45	HPV-52	HPV-58
Recommended Dilution	1:20,000	1:2,000	1:200,000	1:30,000	1:150,000	1:150,000	1:16,000	1:600,000	1:7,000	1:400,000

- Mix by gently inverting tube 8-10 times. Store on wet ice or in a refrigerator at 2-8°C until use.
- Record PsV dilution preparation.
- Use R96 plates to combine diluted samples and controls and PsV.

  Note: Refer to **Figure 7-4** for Assay Plate Map.

Figure 7-4 Day One Assay Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 1/10		Sample 2 1/10		Sample 3 1/10		Sample 4 1/10		PC-R7O2 1/160 or 1/10		No PsV/No Sera	No PsV/No Sera
B	1/40		1/40		1/40		1/40		1/640 or 1/40			
C	1/160		1/160		1/160		1/160		1/2560 or 1/160			
D	1/640		1/640		1/640		1/640		1/10240 or 1/640			
E	1/2560		1/2560		1/2560		1/2560		NC 1:10		PsV + NB	PsV + NB
F	1/10240		1/10240		1/10240		1/10240		NC 1:40			
G	1/40960		1/40960		1/40960		1/40960		NC 1:160			
H	1/163840		1/163840		1/163840		1/163840		NC 1.640			

* See **Table 7-10** for guidelines on positive control starting dilutions.

- Transfer Diluted Samples
  - Using serially diluted samples from R96 or D96 plate, transfer 25 µl of each sample into corresponding columns and rows of R96 plate (May use multi-dispense feature on electronic pipette). See Figure 7.4 or alternate plate map.
  - Change pipette tips between each sample transfer.
- Transfer Diluted Controls

  Note: Use Initial PC Dilution factor listed in Table 7.10 for each PsV to define the appropriate four serial dilutions to transfer to the plate. (See **Figure 7-4**) Transfer 1:1, 1:4, 1:16, and 1:64 dilutions.

Table 7-10 Initial Dilutions of Positive Controls for 10 PsV Types

PsV Type	HPV-6	HPV-11	HPV-16	HPV-18	HPV-31	HPV-33	HPV-35	HPV-45	HPV-52	HPV-58
Dilution Factor*	1:160	1:160	1:160	1:10	1:160	1:160	1:10	1:10	1:160	1:160

  - Using serially diluted controls from R96 or D96 plate, transfer 25 µl of each control into corresponding columns and rows of R96 plate (May use multi-dispense feature on electronic pipette). See Figure 7.4 or alternate plate map.
  - Change pipette tips between each sample transfer.

- Signal and Noise
    - Columns 11-12, Wells A-D: add 125 µl/well of PBNA_M “No PsV/No Sera” Controls.
    - Columns 11-12, Wells E-H: add 25 µl/well of PBNA_M “PsV + NB” Controls.
- Add PsV – Working Dilution.
    - Column 11 and 12
        - Rows A-D: Do NOT add Working Dilution of PsV.
        - Rows E-H: Add 100 µl/well of Working Dilution of PsV.
    - Columns 1-10
        - Add 100 µl of Working Dilution of PsV to each well.

  Note: When pipetting Working Dilution of PsV to R96 plate (Figure 7.4) start with Row H (lowest concentration) moving to Row A (highest concentration). Do not touch pipette tips to liquid (samples) in plate. If pipette tips touch liquid in plate, discard tips and any volume remaining in tips.
- Cover R96 plates with plate lid after the addition of PsV and incubate for 60±20 minutes at 2-8°C.
- Record incubation start time for last plate.

7.5.3.2.4 Sample addition to cells

- After incubation of the PsV+Samples, remove F96 plates containing 293TT cells and R96 plates. Place plates in BSC and record time.
- Gently transfer 100 µL of sample/PsV solutions to corresponding wells on F96 plates containing 293TT cells; do not disturb cells. Plates now referred to as Assay Plates. Return plate lid to plates.

  Note: Dispense speed setting of 4 out of 10 is recommended when using electronic pipettes.
- Incubate Assay Plates in a 37±2°C, 5±2.0% CO_2 incubator for 72h ±2 hours.
- Record start date and time of 3-day incubation.

  Note: The time will start when final plate is placed into incubator.

7.5.3.3 Day 2: harvest

- Label one V96 plate and one R96 plate per Assay Plate (For PsV Titration and For Sample Titration) with the corresponding unique identifier that is on the Assay Plate, with analyst initials and date.
- Remove Assay Plates from incubator.
- Record incubation end date and time.
- Check and record cell confluency by visually scanning a subsection of each plate on an inverted microscope. Record results.
- Transfer 100 µL of supernatant from Assay Plate to corresponding wells of V96 plate.

  Note: Avoid touching the bottom of the plate with pipette tips.
- Place plate lid from Assay Plate onto V96 plate.

- Cover Assay Plates with clear plate sealer and store in BSC until harvesting is complete. Discard at end of harvest.
- Centrifuge V96 plates at 300±20 x g for 5 minutes at RT to pellet down cells that might have carried over from pipetting.
- Being careful not to touch the bottom of V96 plate, transfer 80 µL of supernatants to corresponding wells in R96 plate.
- Seal R96 plate with adhesive foil plate sealer and transfer plate lid from the V96 plate to the R96 plate over the foil sealer.
- Keep V96 in BSC until harvesting is complete. Discard at end of harvest.
- Place R96 plates on dry ice until all wells are visibly frozen. To help freeze the supernatant quicker, place dry ice on top of plates as well.
- Store R96 plates in -10°C to -30°C freezer for at least 12 hours.

  Note: R96 plates must go through one freeze/thaw cycle prior to use.
- Record start date and time of the last plate.

7.5.3.4 Day 3: chemiluminescence - SEAP substrate development

- Turn on convection incubator and set it to 65-70°C.
- Remove R96 plates containing supernatants, kit with SEAP Substrate and 5X Buffer from -10 to -30°C freezer, and thaw at RT.

  Note: Approximately 12 mL SEAP Substrate is needed per plate.
- Record removal date and time of the plates
- Label one O96 plate per R96 plate
- Prepare approximately 8 mL of 1X Buffer per O96 plate by making a 1:5 dilution using 5X Buffer and Distilled Water.
  - Example: 1.6 mL of 5X Buffer plus 6.4 mL of Distilled Water is 8 ml.
- Record dilution.
- Once R96 plates thawed, mix for 1 minute at RT on microplate shaker at 300-400 rpm.
- Centrifuge R96 plates at 1700±50 x g for 5 minutes at RT.
- Add 75 µL of 1X Buffer to each well of O96 plates.
- Carefully transfer 25 µL/well of supernatants from R96 plates to corresponding wells in O96 plates.

  Note: Retain R96 plate in BSC in case retesting is needed. If retesting is needed, re-seal R96 plate containing supernatant with foil seal and plate lid, place R96 plates on dry ice until all wells are visibly frozen, and store at -10°C to -30°C. To help freeze quicker, place dry ice on top of plates as well.
- Cover O96 plates with a clear plate sealer. Verify O96 plate is completely sealed; otherwise, sample may evaporate in incubator.
- Mix O96 plates for 1 minute at RT on microplate shaker at 300-400 rpm.
- Incubate O96 plates in convection incubator for 45±2 minutes at 65-70°C.
- Record start and end time of the last plate.

- Carefully remove O96 plates from incubator and incubate for 5–10 minutes at 2-8°C using wet ice or refrigerator.
- Record start and end time for last plate.
- Centrifuge O96 plates at 1700±50 x g for 1 minute at RT to pull down condensation.
    Note: Condensation may still be visible on plate sealer but should no longer be on walls of O96 plate wells.
- Turn off light in the BSC.
- Add 100 µL of SEAP substrate to each well, starting at Row H and progressing to Row A.
- Cover O96 plates with new clear plate sealer and mix plate on plate shaker for 1 minute at 300-400 rpm while protecting from light (e.g., put a piece of aluminum foil to cover the plates during shaking).
- Incubate for 20-25 minutes at RT, while protecting plate from light.
    Note: May place O96 plates in a lab bench drawer during incubation. Be sure to label drawer so plates are not disrupted during incubation or utilize ice tray and cover with foil.
- Record incubation start time for First Plate.
- During incubation, set up Microplate Reader
- Read plate on plate reader.
- Record read time/incubation end time of first plate.
- Save file.

7.5.3.5 System suitability

Note: Each plate is assessed individually for system suitability.

7.5.3.5.1 System Suitability for Sample Titration Neutralization Assay testing.

Note: Use “Max/Min Summary” section (Signal to Noise Ratio) for assessing.

- PsV + NB S:N Ratio: must be ≥ 50, which is indicated by “PASS.”
- No PsV/No Sera (Mean NS/NV): must be ≤ 2000 RLU for assay plate to pass.
    - If RLU is > 5000, repeat chemiluminescent assay (See Day 3.)
    - If RLU is > 5000 on retest, PsV and samples need to be retested (See Day 1: Sample Neutralization Assay.)
- PsV + NB RLU (Mean PsV + NB): must be ≥ 80,000.
    - If Mean RLU is < 80,000, repeat chemiluminescent assay (See Day 3.)
    - If Mean RLU is < 80,000 on retest, PsV and samples need to be retested (See Day 1: Sample Neutralization Assay.)
- “PsV + NB” CV (%CV PsV + NB): must be below 50% to pass.
    - If “PsV + NB” CV ≥ 50%, repeat chemiluminescent assay (See Day 3.)
    - If “PsV + NB” CV ≥ 50% on retest, PsV and samples need to be retested (See Day 1: Sample Neutralization Assay.)

7.5.3.5.2 System Suitability for PsV Titration

- No PsV/No Sera (Mean NS/NV): must be ≤ 2000 RLU for assay plate to pass.
    - If Mean NS/NV RLU is > 5000, repeat chemiluminescent assay (See Day 3.)
    - If Mean NS/NV RLU is > 5000 on retest, then repeat Titration of PsV assay (See Day 1: PsV titration.)
- PsV + NB RLU: dilution series must have a dilution determined for 200K titer, 150K titer, and 100K titer and the R^2 value must be greater than or equal to 0.98 for the dilution series.
    - If the dilution cannot be determined for each titer and the R^2 value is less than 0.98, repeat chemiluminescent assay (See Day 3.)
    - If the retest of the chemiluminescent assay fails, then the PsV Titration assay must be repeated.
      Note: Typically, the PsV Titration assay passes the criteria, and an acceptable PsV dilution is selected, when the PsV chemiluminescent value falls between 150,000 and 100,000 RLU.

7.5.3.6 Sample acceptance and reporting

Sample Titer CV (%CV): must be below 50% to pass, except if one of the sample titer values is below the cutoff value for the type-specific HPV.

- Example: The reported HPV-18 Titer#1 value is 8 and Titer#2 value is 22. The cutoff value for HPV-18 is 16. CV is calculated based on a value of 16 for titer#1 and 22 for titer#2. CV= 22.3%, NOT 66.0%.
    - If Sample Titer CV ≥ 50%, repeat chemiluminescent assay (See Day 3.)
    - If Sample Titer CV ≥ 50% on retest, PsV and samples need to be retested (See Day 1: Sample Neutralization Assay.)

Sample Titer results below the assay cutoff value are reported as one-half the type-specific HPV cutoff value.

  Example: The cutoff value for HPV-18 is 16. If the calculated sample value for HPV-18 Titer#1 is 12, then the reported Titer#1 value is 8.

- Sample Titers are presented as whole numbers, and the Mean Titer is presented to the tenths digit.

7.5.3.7 Masking of wells

- Wells can be masked when results appear to be erroneous due to pipetting error, or well contamination.
    - When masking wells:
        - PsV + NB (PsV + NB): Only two out of eight (2/8) data points are masked per plate.
        - No PsV/No Sera (NS/NV): Only two out of eight (2/8) data points are masked per plate.

- Samples: If mean RLU for any of the matched sample dilutions has a percent CV > 20%, any two of the serially diluted wells may be masked for a single sample. In total, two wells may be masked for a sample.

7.6 REFERENCES

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