SOP Title: HPV Neutralization Assay for Titer Determination		
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1. PURPOSE

1.1. The purpose of this procedure is to describe the method for the Human Papillomavirus (HPV) Pseudovirus (PsV) Neutralization Assay using SEAP Substrate.

2. SCOPE

2.1. This procedure applies to all Pseudovirus (PsV) Neutralization Assays using SEAP Substrate.

2.2. The HPV Neutralization Assay is used to detect and measure the presence of antibodies in human serum that neutralize infectivity of HPV Pseudovirus of the following 9 types: HPV-6, 11, 16, 18, 31, 33, 45, 52, and 58.

3. REFERENCES

3.1. 10009: General Record Review

3.2. EHS-WM-1: Disposal and Minimization of Chemical Waste

3.3. EHS-WM-2: Biological Waste Handling and Disposal

3.4. 26000: Biosafety Cabinet (BSC) Use and Maintenance

3.5. 26031: Use and Maintenance of the epMotion 96

3.6. 26001: Operation, Use and Maintenance of C02 Incubators

3.7. 26033: Use and Maintenance of the Thermo Fisher Sorvall Legend XTR Centrifuge

3.8. 26003: Use and Maintenance of a Molecular Devices M5 Plate

3.9. 26004: Use and Maintenance of the Cellometer Auto 2000

3.10. 26005: Use and Maintenance of a 2-8°C Refrigerator

3.11. 26030: Use and Maintenance of -80°C Freezers

3.12. 26009: Use and Maintenance of Pipettes

3.13. 26013: Use and Maintenance of -20°C Freezers

3.14. 26014: Use and Maintenance of a Laboratory Convection Oven

3.15. 26015: Use and Maintenance of the Inverted Microscope

3.16. 26017: Use and Maintenance of the Eppendorf Centrifuge

3.17. 26020: Use and Maintenance of a Compact Digital MicroPlate Shaker

3.18. 15000: Waste Disposal at the Advanced Technology Research Facility

3.19. HSL_GL_006: Reagent Preparation for the HPV Serology Laboratory

3.20. 20001: 293TT Cell Culturing and Maintenance

4. RESPONSIBILITIES

4.1. The Research Associate, hereafter referred to as Analyst, is responsible for reviewing and following this procedure, and documenting assay information.

4.2. The Scientific Manager or designee is responsible for training personnel in this procedure and reviewing associated documentation.

4.3. The Quality Assurance Specialist is responsible for quality oversight and approval of this procedure.

4.4. Trained personnel perform assay record review per “10009: General Record Review.”

5. DEFINITIONS

Term	Definition
BPV	Bovine Papillomavirus
CV	Coefficient of Variance
FIO	For Information Only
NB	Neutralization Buffer (PBNA_M)
NC	Negative Control
Noise	Background assay response without stimulus (no PsV or no Sera) (Minimum)
NS	No Sera
NV	No PsV
PBS	Phosphate Buffer Solution
PC	Positive Control
Plate Skirt	Lower protruding outer edge of 96-well plate
PsV	Pseudovirus
RLU	Relative Light Unit
RT	Room Temperature
SeAP	Secreted Alkaline Phosphatase
Signal	Stimulated assay response without enablers (Maximum)
S:N	Signal to Noise Ratio
TBD	To Be Determined
RCF	Relative Centrifugal Force (g-force); Centrifuge display reads x g
D96	Deep 96-Well Plate
F96	Flat Bottom 96-Well Plate
O96	96-Well OptiPlate

R96	Round Bottom 96-Well Plate

6. REAGENTS, MATERIALS, AND EQUIPMENT

6.1. Reagents

6.1.1. Bleach, Clorox, Concentrated (FNLCR Warehouse, Cat # 68100251 or equivalent)

6.1.2. 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 Sero Negative Control)

6.1.3. Control, Positive [Heparin (Sigma, Cat # H1784-250MG) or Gardasil 9 Serum Control]

6.1.4. 1X Dulbecco's Phosphate-Buffered Saline (DPBS), Sterile (Life Technologies, Cat # 14190-136 or equivalent)

6.1.5. Great EscAPe SEAP Chemiluminescence Kit (Takara, Cat# 631738)

6.1.6. HEK 293TT Cells (HSL_LAB_001: 293TT Cell Culturing and Maintenance)

6.1.7. Neutralization Buffer, 293TT Pseudovirion Based (PBNA_M, HSL_GL_006, Section 29)

6.1.8. PsV: BPV, HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58

6.1.9. Primary Disinfectant (Cavicide, FNLCR Warehouse, Cat # 79300360 or equivalent)

6.1.10. Secondary Disinfectant (Ster-ahol, VWR, Cat # 14003-358 or equivalent)

6.1.11. Trypsin-EDTA (0.05%), Phenol Red (Life Technologies, Cat # 25300-054)

6.1.12. ViaStain™ AOPI Staining Solutions (Nexcelom, Cat # CS2-0106-5mL)

6.1.13. Water, Distilled (Life Technologies, Cat # 15230204 or equivalent)

6.2. Consumables

6.2.1. AlumaSeal CS Foil (Thomas Scientific, Cat # 1230Y24)

6.2.2. Ice Pan (Thomas Scientific, Cat # 1200R42 or equivalent)

6.2.3. Marker, Lab, Alcohol Resistant (FNLCR Warehouse, Cat # 66400058)

6.2.4. Pipette Tips (Rainin, EP Motion, Hyda DT Robot)

6.2.5. Plate, 2.2 mL Deep Well, **D96** (VWR, Cat# 10755-248 or equivalent)

6.2.6. Plate, 96-well, Flat Bottom, Tissue Culture, **F96** (Corning Costar, Cat # 3596)

6.2.7. Plate, 96-well, White Opaque, **O96** (Perkin Elmer, Cat # 6005290)

6.2.8. Plate, 96-well Round Bottom, **R96** (Corning Costar, Cat # 3788)

6.2.9. Plate, 96-well V Bottom, **V96** (Corning Costar, Cat# 3894)

6.2.10. Plate Sealers (Thomas Scientific, Cat# 6980A01 or equivalent)

6.2.11. 50 mL Reagent Reservoir (FNLCR Warehouse, Cat # 66401270 or equivalent)

6.2.12. 300mL Nalgene Disposable Polypropylene Robotic Reservoirs (Thermo Fisher Scientific, Cat#: 1200-2301)

6.2.13. 5 mL Serological Pipette (FNLCR FNLCR Warehouse, Cat # 66401365 or equivalent)

6.2.14. 10 mL Serological Pipette (FNLCR Warehouse, Cat # 66401370 or equivalent)

6.2.15. 25 mL Serological Pipette (FNLCR Warehouse, Cat # 66401361 or equivalent)

6.2.16. 50 mL Serological Pipette (FNLCR Warehouse, Cat # 66401363 or equivalent)

6.2.17. 50 mL Tubes, Conical (FNLCR Warehouse, Cat # 66401486 or equivalent)

6.2.18. 1.7 mL Tube, Microcentrifuge, Polypropylene, Sterile (VWR, Cat # 87003-294 or equivalent)

6.2.19. Waste Container, White, HDPE Plastic, with Screw Top Lid (Amphorea, Cat # 250MLPHARMA or equivalent)

6.2.20. Wet Ice

6.2.21. Wipe, Low-Lint, Wypalls (FNLCR Warehouse, Cat # 79300335 or equivalent)

6.3. Equipment

6.3.1. Centrifuges (Microcentrifuge, Bench Top)

6.3.2. Class II Biosafety Cabinet (BSC)

6.3.3. CO_2 Incubator

6.3.4. Convection Oven

6.3.5. Freezers (-20°C, -80°C)

6.3.6. Inverted Microscope

6.3.7. Microplate Reader (Molecular Devices M5 or equivalent)

6.3.8. Microplate Shaker

6.3.9. Pipettes/Pipette Systems (Rainin, EP Motion, Hyda DT Robot)

6.3.10. Refrigerator (2-8°C)

6.3.11. Serological Pipettor

6.3.12. Timer

6.3.13. Vortex

7. HEALTH AND SAFETY CONSIDERATIONS

7.1. Proper safety precautions should be taken while working in a laboratory setting. This includes, but is not limited to, proper protective equipment such as lab coats, safety glasses, closed-toe shoes, and non-latex gloves.

7.2. Refer to the respective Safety Data Sheets (SDS) when working with any chemicals.

7.3. Refer to “15000: Waste Disposal at the Advanced Technology Research Facility,” “EHS-WM-1: Disposal and Minimization of Chemical Waste,” and “EHS-WM-2: Biological Waste Handling and Disposal” for waste disposal processes.

8. PROCEDURE PRINCIPLES

8.1. All procedural processes are performed in BSC.

8.2. L1 Pseudovirion (PsV)-Based Neutralization Assay (L1 PBNA) uses PsV particles that have encapsidated SEAP gene plasmid as a reporter gene.

8.3. Dispense and work on one PsV type at a time.

8.4. Incubation start and end times are based on last plate, except for substrate incubation (Step 12.17) prior to plate read, which are based on first plate.

8.5. Cell culture is prepared per 20001 prior to start of assay. The assay requires **0.3×10^6 cells/mL**. Refer to 20001 for cell culture techniques.

8.6. The Data Reference number is the Logbook number and Page number. For example, Logbook Reference number (*LAB2017003*) and Page number (*001 for page 1*) are combined for final Data Reference number *LAB2017003001*.

8.7. Label plate skirt with Plate Number, Data Reference, HPV Type, Analyst Initials and Date. See Attachment 1: 96-Well Plate Skirt Label.

8.8. Unused plates in a plate sleeve are sealed with tape (date and initial) before removing from BSC to maintain plate sterility.

8.9. Cell culture can be prepared exclusively for use in the Neutralization Assay or provided from an existing culture.

8.9.1. If using cells prepared exclusively for the Neutralization Assay, perform steps 9.2 to 9.2.10 for PsV Titration or steps 10.1 to 10.1.10 for Neutralization Assay at cell harvest and document on “30000-01: HPV Neutralization Assay, Sample Preparation Form.”

8.9.2. If using cells from an existing culture, disregard steps 9.2 to 9.2.10 or 10.1 to 10.1.10; these steps are performed and documented as part of the on-going cell culture maintenance in 20001. The required number of cells are provided at passage. N/A cell passage reagents (i.e., DPBS and Trypsin-EDTA).

8.10. Do not use cells if they have been passaged greater than 30 passages, unless approved by Scientific Manager or designee. Record comment on 30000-01.

8.11. Do not use cells if they appear to be contaminated.

8.12. Use alcohol resistant lab markers when labeling plates by hand.

8.13. PBNA_M reagent should be stored on wet ice between steps.

8.14. Quality Controls run on each plate:

8.14.1. HPV Type Specific Positive Control (PC): Human serum from HPV immunized subjects. The positive control serum is diluted according to the HPV-Type Specific Attachment. The positive control serum is included in Column 12 Rows A-D of every plate.

8.14.2. HPV Type Specific Negative Control (CN): Human serum that is negative for HPV Type specific antibodies. The negative control serum is included in Column 12 Rows E-H of every plate.

8.14.3. No PsV / No Sera Control (NS/NV): Column 11 Rows A-D contain cells only and show the background signal without PsV.

8.14.4. PsV + NB Control (PsV/NB): Column 11 Rows E-H contain cells and PsV with no sera, and show the maximum SeAP signal from PsV.

9. TITRATION OF PsV

9.1. Purpose

9.1.1. Titration of PsV is 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 -80°C.

Note: For testing of human serum samples, proceed to Section 10 for DAY ONE: NEUTRALIZATION ASSAY

9.2. Prepare Cells for Three F96 Plates.

9.2.1. Examine each cell culture flask of 293TT cells under an inverted light microscope per “26015: Use and Maintenance of the Inverted Microscope” and visually inspect confluency.

Note: Cells should be 70-95% confluent. If not, seek Scientific Manager or designee for approval. Record comment on 30000-01.

9.2.2. Discard media from flask into waste container.

9.2.3. Add 10 mL of sterile DPBS.

9.2.4. Gently rinse cells with DPBS by slowly rotating flask so that DPBS washes over the cells 3-5 times.

9.2.5. Discard DPBS into waste container.

9.2.6. Repeat steps 9.2.4 to 9.2.5 one time.

9.2.7. Add 3 mL of 0.05% Trypsin-EDTA solution, and gently spread over cells.

9.2.8. Incubate flask for 3-7 minutes in a 37˚C, 5% CO_2 incubator per “26001: Operation, Use and Maintenance of C02 Incubators.”

9.2.9. Ensure cells have detached by examining flask under an inverted microscope per 26015.

Note: If cells are not completely detached, gently tap flask to dislodge cells.

9.2.10. Add 10 mL of PBNA_M into flask to neutralize Trypsin-EDTA and transfer cells to a 50 mL conical tube.

9.2.11. Centrifuge cells at 300 x g for 5 minutes at RT per “26033: Use and Maintenance of the Thermo Fisher Sorvall Legend XTR Centrifuge.”

9.2.12. Discard supernatant into waste container.

9.2.13. Tap on bottom of tube to loosen cell pellet.

9.2.14. Add 20 mL of PBNA_M to cell pellet and mix by pipetting up and down 5 times with a serological pipette to achieve single-cell suspension.

9.2.15. Count cells per "26004: Use and Maintenance of the Cellometer Auto 2000" or per Attachment 1 of HSL_LAB_001.

9.2.16. Dilute cells in PBNA_M to a final volume 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 3 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.

9.2.17. Record calculations in 30000-01.

9.2.18. Mix cell suspension by gently rotating or inverting tube with cells.

9.2.19. Dispense 100 µL of cells into all wells of all F96 Plates.

Note: Mix cell suspension with pipette between plates to maintain a homogenous mixture of cells during process.

9.2.20. Place plate lids on F96 Plates and incubate plates for 120-360 minutes in a 37°C, 5% CO_2 incubator.

9.2.21. Record incubation start time for last plate on 30000-01.

9.3. PsV Particle Preparation

9.3.1. Remove PsVs from -80°C freezer per "26030: Use and Maintenance of -80°C Freezers." and thaw on wet ice inside BSC.

9.3.2. Prepare an initial dilution for each PsV according to Table 1.

Table 1. Recommended PsV Starting Dilution

HPV Type	HPV-6	HPV-11	HPV-16	HPV-18	HPV-31	HPV-33	HPV-45	HPV-52	HPV-58
Recommended Starting Dilution	1:2000	1:400	1:40000	1:4000	1:40000	1:40000	1:100000	1:2000	1:40000

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 1.

Example: 1:10000 starting dilution

Add 10 mL of PBNA_M to a 15 mL conical tube.

Add 10 µL of PsV (1:10 predilution) to the tube with PBNA-M and gently mix by inverting 5-10 times.

Store on wet ice or in a refrigerator per “26005: Use and Maintenance of a 2-8°C Refrigerator” at 2-8°C until use.

9.3.3. Prepare two-fold serial dilutions of samples in D96 plate according to Diagram 1.

9.3.3.1. Add 450 µL of PBNA_M to Rows B-H, Columns 1-3, 5-7, and 9-11.

9.3.3.2. Add 900 µL of each HPV PsV dilution (see table 1 for recommended starting dilution) to the appropriate wells of Row A.

9.3.3.3. Dilute PsVs by performing a 1:2 serial dilution from Row A through H following Table 2.

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

Diagram 1: D96 Sample 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-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	
C	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	
E	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	
G	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	

Table 2: Serial Dilutions of PsV for Running in Triplicate

Dilution	PsV Volume (µL)	PBNA_M Volume (µL)	Dilution Factor*
Dil 1	900 µL of pre-diluted PsV	NA	1:1
Dil 2	450 µL from row A	450	1:2
Dil 3	450 µL from row B	450	1:4
Dil 4	450 µL from row C	450	1:8
Dil 5	450 µL from row D	450	1:16
Dil 6	450 µL from row E	450	1:32
Dil 7	450 µL from row F	450	1:64
Dil 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 1 for Recommended Starting Dilution for each HPV PsV.

9.3.4. Label three R96 plates.

Note: These plates will be used to combine diluted PsVs and PBNA_M to mimic incubation with samples in Day One Neutralization Assay (Section 10.)

Note: Refer to Attachment 2: Plate Layouts for Titrating PsV.

9.3.5. Add 25 µL of PBNA_M into Columns 1-9, Rows A-H of R96 plates.

9.3.6. Add 125 µL of PBNA_M to Columns 10, 11 and 12, Rows A-H for “No PsV” Controls.

9.3.7. Transfer 100 µL of serially diluted PsVs from D96 plate (Step 9.3.3.3) into corresponding columns and rows of R96 plate.

9.3.7.1. Dispense by columns, one PsV at a time.

9.3.7.2. Change pipette tips for each PsV transfer.

9.3.8. Cover R96 plates with plate lid and incubate for 60 ± 20 minutes at 2-8°C.

9.3.9. Record incubation start time for last plate on 30000-01.

9.4. Continue to section 10.4 for SAMPLE ADDITION TO CELLS

10. DAY ONE: NEUTRALIZATION ASSAY

Note: Use sterilized pipette tips throughout processing of Day One due to cell culture.

10.1. Prepare Cells in F96 plates

10.1.1. Examine each cell culture flask under an inverted microscope per 26015 and visually inspect confluency.

Note: Cells should be 70-95% confluent. If not, seek Scientific Manager or designee for approval. Record comment on 30000-01.

10.1.2. Discard media from flask into waste container.

10.1.3. Add 10 mL of sterile DPBS to flask without disturbing cells.

10.1.4. Rotate flask slowly 3-5 times to gently rinse cells.

10.1.5. Discard DPBS into waste container.

10.1.6. Repeat Steps 10.1.3 to 10.1.5 one time.

10.1.7. Add 3 mL of 0.05% Trypsin-EDTA solution without disturbing cells, then gently spread over cells.

10.1.8. Incubate flask for 3-7 minutes in a 37˚C, 5% CO_2 incubator.

10.1.9. Ensure cells have detached by examining under an inverted microscope per 26015. If cells are not completely detached, gently tap flask to dislodge cells.

10.1.10. Add 10 mL of PBNA_M to flask to neutralize Trypsin-EDTA.

10.1.11. Transfer cells to 50 mL conical tube.

10.1.12. Centrifuge cells at 300 x g for 5 minutes at RT per 26033.

10.1.13. Discard supernatant into waste container.

10.1.14. Tap on bottom of tube to loosen cell pellet.

10.1.15. Add 20 mL of PBNA_M and pipette up and down 5 times with a serological pipette to achieve single-cell suspension.

10.1.16. Count cells per 26004.

10.1.17. Dilute cells in PBNA_M to a final volume of **0.3 × 10^6 cells/mL** and record calculations in 30000-01.

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

Example:

C_1 = 4 × 10^6 cells/mL (Cell stock concentration)
C_2 = 0.3 × 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.

10.1.18. Mix cell suspension by gently rotating or inverting tube with cells.

10.1.19. Dispense 100 µL of cells into all wells of F96 Plates.

Note: Mix cell suspension in reservoir with pipette between plates to maintain a homogenous mixture of cells during process.

10.1.20. Place plate lids on F96 Plates and incubate plates for 120-360 minutes in a 37°C, 5% CO_2 incubator.

10.1.21. Record incubation start time for last plate on 30000-01.

10.2. **SAMPLE PREPARATION**

10.2.1. Remove serum samples and assay controls from -80°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, do not keep samples at 4°C for more than 4 hours.

10.2.2. Once thawed, mix tubes by inversion 5 times or vortex at intermediate speed for 10 seconds.

10.2.3. Centrifuge samples and controls at 10,000 x g for 5 minutes at 4°C per "26017: Use and Maintenance of the Eppendorf Centrifuge."

10.2.4. Prepare 1:4 serial dilutions in R96 or D96 plate (see Diagram 2).
Note: Mix each dilution at least 10 times using a multichannel pipette, and discard tips between each dilution.

10.2.4.1. Dilute samples in Columns 1-10 starting at the recommended dilution per Attachment 3, using volumes listed in Table 3.

10.2.4.2. Dilute positive (Pos Ct in Column 11) and negative controls (Neg Ct in Column 12) starting at the recommended dilution per Attachment 3.

10.2.5. Record volumes of samples, controls, and buffer used on 30000-01.

Diagram 2: Sample & Control 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	**Sample 1** 1/10	**Sample 2** 1/10	**Sample 3** 1/10	**Sample 4** 1/10	**Sample 5** 1/10	**Sample 6** 1/10	**Sample 7** 1/10	**Sample 8** 1/10	**Sample 9** 1/10	**Sample 10** 1/10	**Pos Ct** 1/10	**Neg Ct** 1/10
B	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40
C	1/160	1/160	1/160	1/160	1/160	1/160	1/160	1/160	1/160	1/160	1/160	1/160
D	1/640	1/640	1/640	1/640	1/640	1/640	1/640	1/640	1/640	1/640	1/640	1/640
E	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560	1/2560
F	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240	1/10240
G	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960	1/40960
H	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840	1/163840

Table 3: Serial Dilutions of Samples (Testing in Duplicate)

Dilution	Sample Volume (µL)	PBNA_M Volume (µL)	Dilution Factor
Dil 1	55 µL from source vial or pre-diluted controls	495	1:10
Dil 2	150 µL from row A	450	1:40
Dil 3	150 µL from row B	450	1:160
Dil 4	150 µL from row C	450	1:640
Dil 5	150 µL from row D	450	1:2560
Dil 6	150 µL from row E	450	1:10240
Dil 7	150 µL from row F	450	1:40960
Dil 8	150 µL from row G	450	1:163840

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

10.3. **PsV PARTICLE PREPARATION**

Note: Ensure proper study specific PsVs are used.

10.3.1. Prepare 12 mL per plate of Working Dilution of each PsV with PBNA_M per Table 4 (also see Attachment 3 for dilution series).

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.

Table 4: 7 PsV Types with Final Dilution Factors to make Working Dilutions

PsV Type	HPV-6	HPV-11	HPV-31	HPV-33	HPV-45	HPV-52	HPV-58
Dilution Factor*	60,000	3500	400,000	400,000	1,600,000	18,000	800,000

*Note: Pre-dilution of any PsV needs to be accounted for in this dilution factor.

10.3.1.1. Mix by gently inverting closed tube until mixture is homogeneous (5-10 times). Store on wet ice or in a refrigerator at 2-8°C until use.

10.3.1.2. Record PsV dilution preparation on 30000-01.

10.3.2. Use R96 plates to combine diluted samples and controls (Step 10.2.6) and PsV (Step 10.3.1).

Note: Refer to Diagram 3 for Assay Plate Map. If alternative plate map used document in "Other" plate map section of 30000-01.

Diagram 3: Day One Assay Plate Map

	Columns 1 & 2	Columns 3 & 4	Columns 5 & 6	Columns 7 & 8	Columns 9 & 10	Column 11	Column 12
A	**Sample 1** 1/10	**Sample 2** 1/10	**Sample 3** 1/10	**Sample 4** 1/10	**Sample 5** 1/10	No PsV / No Sera **(NS/NV)**	**PC*** 1:1
B	1/40	1/40	1/40	1/40	1/40		1:4
C	1/160	1/160	1/160	1/160	1/160		1:16
D	1/640	1/640	1/640	1/640	1/640		1:64
E	1/2560	1/2560	1/2560	1/2560	1/2560	PsV + NB **(PsV/NB)**	**NC** 1/10
F	1/10240	1/10240	1/10240	1/10240	1/10240		1/40
G	1/40960	1/40960	1/40960	1/40960	1/40960		1/160
H	1/163840	1/163840	1/163840	1/163840	1/163840		1/640

* See Table 4 for guidelines on PsV starting dilutions.

10.3.2.1. Transfer Diluted Samples (Columns 1-10)

10.3.2.1.1. Using serially diluted samples from R96 or D96 plate (Step 10.2.4.1), transfer 25µL of each into corresponding columns and rows of R96 plate (Step 10.3.2). See Diagram 3 or alternate plate map.

10.3.2.1.2. Change pipette tips between each sample transfer.

10.3.2.2. Transfer Diluted Controls (Column 12)

Note: Use Initial Dilution factor listed in Table 4 to determine which PC starting dilutions for each PsV to transfer to plate. (See Diagram 3.) Transfer 1:1, 1:4, 1:16, and 1:64 dilutions.

Table 4: Initial Dilutions of Positive Controls for 7 PsV Types

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

* Select this as starting dilution from Dilution Plate for Row A (1:1 dilution).

10.3.2.2.1. Using serially diluted controls from R96 or D96 plate (Step 10.2.4.2), transfer 25 µL of each into corresponding columns and rows of R96 plate (Step 10.3.2). See Diagram 3 or alternate plate map.

10.3.2.2.2. Change pipette tips between each sample transfer.

10.3.2.3. Signal and Noise (Column 11)

10.3.2.3.1. Wells A-D: add 125 µL/well of PBNA_M “No PsV/No Sera” Controls.

10.3.2.3.2. Wells E-H: add 25 µL/well of PBNA_M “PsV + NB” Controls.

10.3.3. Add PsV – Working Dilution from Step 10.3.1.1.

10.3.3.1. Column 11

10.3.3.1.1. Wells A-D: Do not add Working Dilution.

10.3.3.1.2. Wells E-H: Add 100 µL/well of Working Dilution of PsV.

10.3.3.2. Columns 1-10 & 12

10.3.3.2.1. Add 100 µL of Working Dilution of PsV to each well.

Note: When pipetting Working Dilution to R96 plate (Step 10.3.2) 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 subsequently any volume remaining in tips.

10.3.4. Cover R96 plates with plate lid and incubate for 60 ± 20 minutes at 2-8°C.

10.3.5. Record incubation start time for last plate on 30000-01.

10.4. **SAMPLE ADDITION TO CELLS**

10.4.1. After incubation of the PsV+Samples (Step 10.3.4), remove F96 plates containing 293TT cells prepared from Step 10.1.20 and R96 plates from Step 10.3.5. Place plates in BSC and record time on 30000-01.

10.4.2. Gently transfer 100 µL of sample/PsV solutions (Step 10.3.4) 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 is recommended when using electronic pipettes.

10.4.3. Incubate Assay Plates in a 37°C, 5% CO_2 incubator for 70-74 hours.

10.4.4. Record start date and time of 3-day incubation on 30000-01.

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

11. DAY 2: HARVEST

11.1. Label one V96 plate and one R96 plate per Assay Plate (Step 10.4.3) with the corresponding unique identifier that is on the Assay Plate, with analyst initials and date.

11.2. Remove Assay Plates from incubator.

11.3. Record incubation end date and time on 30000-01.

11.4. Check and record cell confluency by visually scanning a subsection of each plate on an inverted microscope per 26015. Record results on 30000-01.

Note: If confluency of Assay Plates on Harvest Day is less than 70% for any of the sections (4 wells per sample; 1 well per PsV+NB; 1 well per NS/NV; 1 well of Pos Ct; or 1 well of Neg Ct) consult with Scientific Manager or designee to determine if plate may be harvested or discarded, and record a comment on 30000-01.

11.5. Transfer 100 µL of supernatant from Assay Plate to corresponding wells of V96 plate.

11.6. Place plate lid from Assay Plate onto V96 plate.

11.7. Cover Assay Plates with clear plate sealer and store in BSC until harvesting is complete. Discard in autoclave bags at end of harvest.

11.8. Centrifuge V96 plates at 300 x g for 5 minutes at RT per 26033 to pellet down cells that might have carried over from pipetting.

11.9. Being careful not to touch the bottom of V96 plate, transfer 80 µL of supernatants to corresponding wells in R96 plate from step 11.1.

11.10. Seal R96 plate with adhesive foil plate sealer and transfer plate lid from the V96 plate to the R96 plate over the foil sealer.

11.11. Cover V96 plates with clear plate sealer and store in BSC until harvesting is complete. Discard in autoclave bags at end of harvest.

11.12. Store R96 plates in -20°C freezer per “26013: Use and Maintenance of -20°C Freezers” for at least 12 hours.

Note: R96 plates must go through one freeze/thaw cycle prior to use in section 12.

11.13. Record start date and time on 30000-01.

Note: Time will start when final plate is placed into -20°C freezer.

12. DAY 3: CHEMILUMINESCENCE - SEAP SUBSTRATE DEVELOPMENT

12.1. Turn on convection oven and set it to 68°C per "26014: Use and Maintenance of a Laboratory Convection Oven."

12.2. Remove R96 plates containing supernatants from -20°C freezer, and thaw at RT.

12.3. Record removal date and time on "30000-02: HPV Neutralization Assay, Substrate Development Form."

12.4. Label one O96 plate per R96 plate (from Step 12.2.)

12.5. Remove kit with SEAP Substrate and 5X Buffer from -20°C freezer and bring to RT before using.

Note: Approximately 12 mL SEAP Substrate is needed per plate.

12.6. 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.

12.7. Record dilution on 30000-02.

12.8. Once R96 plates thawed (Step 12.2), mix for 1 minute at RT on microplate shaker at 300-400 rpm per "26020: Use and Maintenance of a Compact Digital Microplate Shaker."

12.9. Centrifuge R96 plates at 1700 x g for 5 minutes at RT per 26033.

12.10. Add 75 µL of 1X Buffer to each well of O96 plates.

12.11. 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, and store at -20°C.

12.12. Cover O96 plates with a clear plate sealer. Verify O96 plate is completely sealed; otherwise, sample may evaporate in oven.

12.13. Mix O96 plates for 1 minute at RT on microplate shaker at 300-400 rpm per 26020.

12.14. Incubate O96 plates in convection oven for 45 ± 2 minutes at 65-70°C per 26014.

12.15. Record start and end time on 30000-02.

12.16. Carefully remove O96 plates from oven and incubate for 5 – 10 minutes at 2-8°C using wet ice or refrigerator.

12.17. Record start and end times for last plate on 30000-02.

12.18. Centrifuge O96 plates at 1700 x g for 1 minute at RT per 26033to pull down condensation.

Note: Condensation may still be visible on plate sealer but should no longer be on walls of O96 plate wells.

12.19. Add 100 µL of SEAP substrate to each well, starting at Row H and progressing to Row A.

12.20. Cover O96 plates with new clear plate sealer and mix plate on plate shaker for 1 minute at 300-400 rpm per 26020while protecting from light (e.g., put a piece of aluminum foil to cover the plates during shaking).

12.21. 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.

12.22. Record incubation start time for First Plate on 30000-02.

12.23. During incubation, set up Spectramax M3 or 5 series Microplate Reader using “HPV Neutralization Assay” template in Softmax Pro software. See Attachment 4.

12.24. Read plate on plate reader per “26003: Use and Maintenance of a Molecular Devices M5 Plate Reader.”

12.25. Record read time/incubation end time on 30000-02.

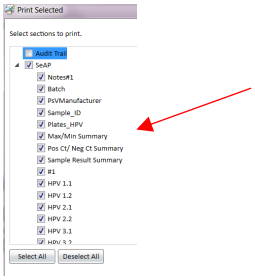
12.26. Save file as follows, using the first page of 30000-02 where the experiment is recorded as the logbook number and Batch number (Bx) in the Data Reference:

“Data Reference_HPVNeut_Bx_DDMMMYYAnalyst Initials”
(LB1234567001_HPVNeut_B1_20May17ABC)

12.27. Record Data File Reference on 30000-02.

12.28. Select “Print Select” option in SoftMax, deselect all boxes, then select to print: Assay Information/Notes, Sample ID Information, all Sample and Quality Control Summaries, Signal to Noise, and all Raw Plate Data. See Figure 1.

Figure 1: Print Select Options



12.29. Initial and date first page of printed raw data file and attach to assay form.

13. ASSAY ACCEPTABILITY GUIDELINES

13.1. If confluency of Assay Plates on Harvest Day is less than 70% for any of the sections (4 wells per sample; 1 well per PsV+NB; 1 well per NS/NV; 1 well of Pos Ct; or 1 well of Neg Ct) consult with Scientific Manager or designee to determine if plate may be harvested or discarded, and record a comment on 30000-01.

13.2. If signal to noise ratio (S:N) result states “Check” then consult with Scientific Manager or designee to determine if any results are valid. Record comment on 30000-02.

14. SYSTEM SUITABILITY

Note: Each plate is assessed individually for system suitability.

Note: Report S:N, percent CV, and RLU to a whole number.

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

14.1. PsV + NB S:N Ratio: must be ≥ 50, which is indicated by “PASS.”

14.2. No PsV/No Sera (Mean NS/NV): must be ≤ 2000 RLU for assay plate to pass.

14.2.1. If RLU is between 2000-5000, discuss results with Scientific Manager or designee prior to acceptance. Write comment on 30000-02.

14.2.2. If RLU is > 5000, repeat chemiluminescent assay (See Section 12.)

14.2.3. If RLU is > 5000 on retest, PsV and samples need to be retested (See Section 10.)

14.3. PsV + NB RLU (Mean NB): must be ≥ 80,000.

14.3.1. If RLU is < 80,000, repeat chemiluminescent assay (See Section 12.)

14.3.2. If RLU is < 80,000 on retest, PsV and samples need to be retested (See Section 10.)

Note: Discuss results with Scientific Manager or designee prior to retest.

14.4. "PsV + NB" CV (%CV NB): must be below 50% to pass.

14.4.1. If "PsV + NB" CV ≤ 50%, repeat chemiluminescent assay (See Section 12.)

14.4.2. If "PsV + NB" CV ≤ 50% on retest, PsV and samples need to be retested (See Section 10.)

15. MASKING OF WELLS

15.1. Wells can be masked when results appear to be erroneous due to pipetting error, well contamination, or other reason as determined by Scientific Manager or designee.

15.2. When masking wells:

15.2.1. PSV + NB (PSV/NB): Only one out of four(1/4) data points are masked per plate.

15.2.2. No PsV/No Sera (NS/NV): Only one out of four(1/4) data points are masked per plate.

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

16. ATTACHMENTS

16.1. Attachment 1: 96-Well Plate Skirt Label

16.2. Attachment 2: Plate Layouts for Titrating PsV

16.3. Attachment 3: HPV PsV Dilutions and Inhibiting Antibody Dilutions

16.4. Attachment 4: Settings for "HPV Neutralization Assay" Template in Softmax Pro Software

16.5. Attachment 5: Neutralization Assay Workflow

16.6. Attachment 6: 30000-01: HPV Neutralization Assay, Sample Preparation Form

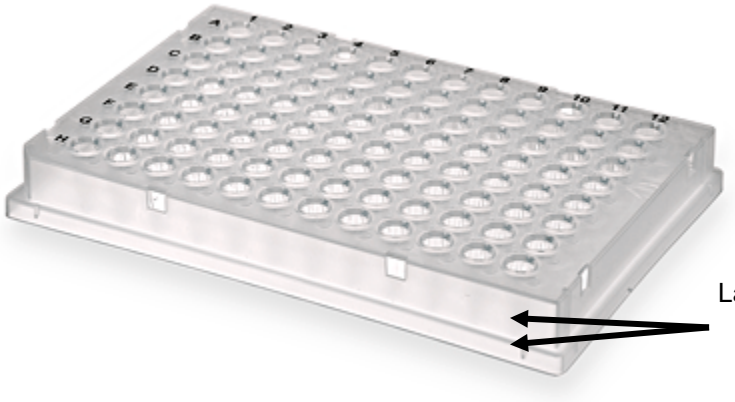
16.7. Attachment 7: 30000-02: HPV Neutralization Assay, Substrate Development Form

17. REVISION HISTORY

Version	Change	Reason
2.0	Update forms.	Correct missing equipment, added sample results section.
	Update Attachment 1.	Include updated dilutions and the dilution schemes for ease of use.
	Update reporting results and masking of wells.	Missing from previous version.
3.0	Added Viastain to Reagents	Used in cell counting procedure.
	Step 9.2, added option to vortex.	Reflect current practice.
	Updated PsV Recommended Dilutions table: HPV-45 changed from 1:300,000 to 1:400,000, HPV-31 and HPV-16 changed from 1:200,000 to 1:300,000. HPV-11 and HPV-33 added to table (TBD and NA). Note added at bottom of table.	Reflect current practice.
	Negative Control Dilutions Table updated: HPV-16 changed to 5074 only. HPV-11 and HPV-33 added.	Reflect current practice.
	HSL_LAB_006.01: Positive Control changed to Gardasil 9 Serum Control; reformatted PsV preparation section.	Reflect current practice, ease of use.
4.0	Changed Version numbers in Revision History (New to 1.0, 1.0 to 2.0). Added 3.0 changes and reasons.	Clarification and ease of tracking. Changes for version 3.0 were not captured prior to release.
	Typo and grammar changes throughout document.	Clarification
	Updated formatting, forms now separate.	Consistency between procedures. Ease of use.
	Added Attachment for 96-Well Plate Skirt Label.	Clarification.

	Removed HSL_GL_002, HSL_GL_003, HSL_GL_004, HSL_GL_007, HSL_GL_008, HSL_GL_009, HSL_GL_010 and HSL_EQ_009 from References section. Added HSL_EQ_020 and HSL_LAB_001 to References section.	Removed procedures not referenced in body of procedure. Added procedure referenced in body of procedure.
5.0	Added centrifuges to Equipment section.	Clarification.
	Added 5X Buffer to Reagents.	Used in procedure.
	Removed e.g., D96, F96, O96, R96, SDS, V96, SOP, PBNA_M from Definitions list. BPV, CV, NB, Plate skirt, RLU, TBD added to Definitions list.	Removed definitions: acronyms used earlier in procedure, not needed in definitions section. Added definitions: acronyms used later in procedure, needed in definitions section.
	Added new Procedural Principles section that includes information about Data Reference, cell culture used, procedures performed in BSC, and handling unused plates.	Clarification.
	Added D96 plate diagram.	Clarification.
	Updated HSL_LAB_006.01: added plate diagrams, formatting, updated control options, added cell count criteria and pass/fail boxes.	Clarification, ease of use, reflect current practice.
	Updated formatting of HSL_LAB_006.02.	Ease of use.

Attachment 1: 96-Well Plate Skirt Label



Attachment 2: Plate Layouts for Titrating PsV

Plate 1

	Columns 1, 2, 3	Columns 4, 5, 6	Columns 7, 8, 9	Columns 10, 11, 12
A	**HPV-6** 1:1	**HPV-11** 1:1	**HPV-16** 1:1	No PsV PBNA_M Only
B	1:2	1:2	1:2	No PsV PBNA_M Only
C	1:4	1:4	1:4	No PsV PBNA_M Only
D	1:8	1:8	1:8	No PsV PBNA_M Only
E	1:16	1:16	1:16	No PsV PBNA_M Only
F	1:32	1:32	1:32	No PsV PBNA_M Only
G	1:64	1:64	1:64	No PsV PBNA_M Only
H	1:128	1:128	1:128	No PsV PBNA_M Only

Plate 2

	Columns 1, 2, 3	Columns 4, 5, 6	Columns 7, 8, 9	Columns 10, 11, 12
A	**HPV-18** 1:1	**HPV-31** 1:1	**HPV-33** 1:1	No PsV PBNA_M Only
B	1:2	1:2	1:2	No PsV PBNA_M Only
C	1:4	1:4	1:4	No PsV PBNA_M Only
D	1:8	1:8	1:8	No PsV PBNA_M Only
E	1:16	1:16	1:16	No PsV PBNA_M Only
F	1:32	1:32	1:32	No PsV PBNA_M Only
G	1:64	1:64	1:64	No PsV PBNA_M Only
H	1:128	1:128	1:128	No PsV PBNA_M Only

Plate 3

	Columns 1, 2, 3	Columns 4, 5, 6	Columns 7, 8, 9	Columns 10, 11, 12
A	**HPV-45** 1:1	**HPV-52** 1:1	**HPV-58** 1:1	No PsV PBNA_M Only
B	1:2	1:2	1:2	No PsV PBNA_M Only
C	1:4	1:4	1:4	No PsV PBNA_M Only
D	1:8	1:8	1:8	No PsV PBNA_M Only
E	1:16	1:16	1:16	No PsV PBNA_M Only
F	1:32	1:32	1:32	No PsV PBNA_M Only
G	1:64	1:64	1:64	No PsV PBNA_M Only
H	1:128	1:128	1:128	No PsV PBNA_M Only

Attachment 3: HPV PsV Dilutions and Inhibiting Antibody Dilutions

PsV Recommended Dilutions

Pseudovirion (PsV)	Final Dilution for PsV	Predilution of Stock *	Recommended Dilution Scheme
HPV-6	1:60,000	10	7 µL of PsV in 39.993 mL of PBNA_M
HPV-11	1:3,500	10	85.7 µL of PsV in 29.9143 mL of PBNA_M
HPV-16	1:300,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 12 µL of 1st Dilution in 35.988 mL of PBNA_M
HPV-18	1:30,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 120 µL of 1st Dilution in 35.880 mL of PBNA_M
HPV-31	1:400,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 90 µL of 1st Dilution in 35.910 mL of PBNA_M
HPV-33	1:400,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 90 µL of 1st Dilution in 35.910 mL of PBNA_M
HPV-45	1:1,600,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 22.5 µL of 1st Dilution in 35.9775 mL of PBNA_M
HPV-52	1:18,000	10	20 µL of PsV in 35.980 mL of PBNA_M
HPV-58	1:800,000	10	1st Dilution: 10 µL of PsV in 990 µL of PBNA_M 2nd Dilution: 45 µL of 1st Dilution in 35.955 mL of PBNA_M

***Note:** PsVs are stored in the -80°C freezer and are typically pre-diluted 1:10. Confirm any pre-dilution factor prior to following dilution scheme above.

Negative Control Recommended Dilutions

Pseudovirion (PsV)	Negative Assay Control (Neg Ct)	Negative Control Starting Dilution Factor	Recommended Dilution Scheme
HPV-6	5074 or V5	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-11	5074 or V5	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-16	5074	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-18	V5	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-31	5074 or V5	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-33	5074 or V5	1000	54 µL of 1:100 diluted negative control in 486 µL of PBNA_M
HPV-45	5074 or V5	1000	54µL of 1:100 diluted negative control in 486µL of PBNA_M
HPV-52	5074 or V5	1000	54µL of 1:100 diluted negative control in 486µL of PBNA_M
HPV-58	5074 or V5	1000	54µL of 1:100 diluted negative control in 486µL of PBNA_M
All Types	Negative response to 9 HPV types included in Gardasil-9 vaccine	10	54µL of negative control in 486µL of PBNA_M

Positive Control Recommended Dilutions

Pseudovirion (PsV)	Positive Assay Control (Pos Ct)	Pos Ct Starting Dilution Factor	Recommended Dilution Scheme
All Types	Gardasil-9 Positive Serum	1:10	32 µL of positive control in 288µL of PBNA_M
All Types	Heparin*	N/A*	10 µL of pre-diluted heparin in 490 µL of PBNA_M
*Can vary depending on stock concentration. Final concentration should be ~1 mg/mL for complete inhibition.			

Note: If available, use of the serum control is advised over heparin.

Positive Control Dilutions Recommended for Starting Dilutions (1:1) on Plate

Initial Dilutions of Positive Controls for All PsV Types

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

* Select this as starting dilution from Dilution Plate for Row A (1:1 dilution).

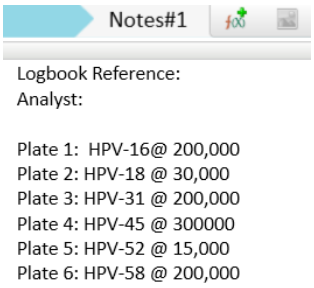
Attachment 4: Settings for "HPV Neutralization Assay" Template in Softmax Pro Software:

Enter the following information into the HPV Neutralization Template:

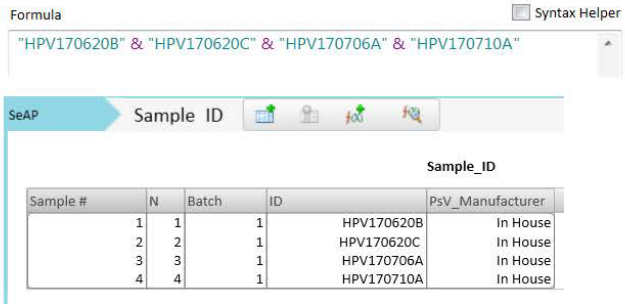
Template Settings:

Endpoint
Luminescence
200 ms Integration Time
Check all wavelengths
Shake 5 seconds before read
Read entire plate
Use 96 well Standard Opaque Plate

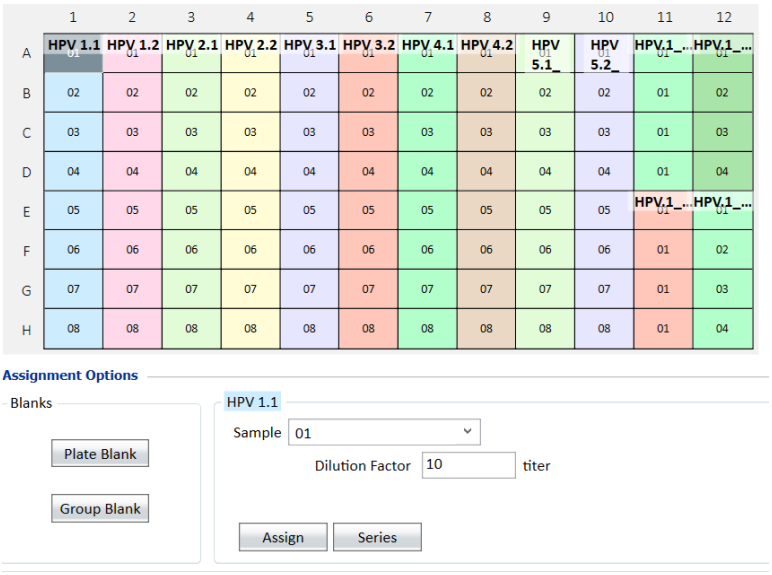
Notes Section: Logbook Reference with page number, Analyst Initials, PsV Type and Dilution Factor.



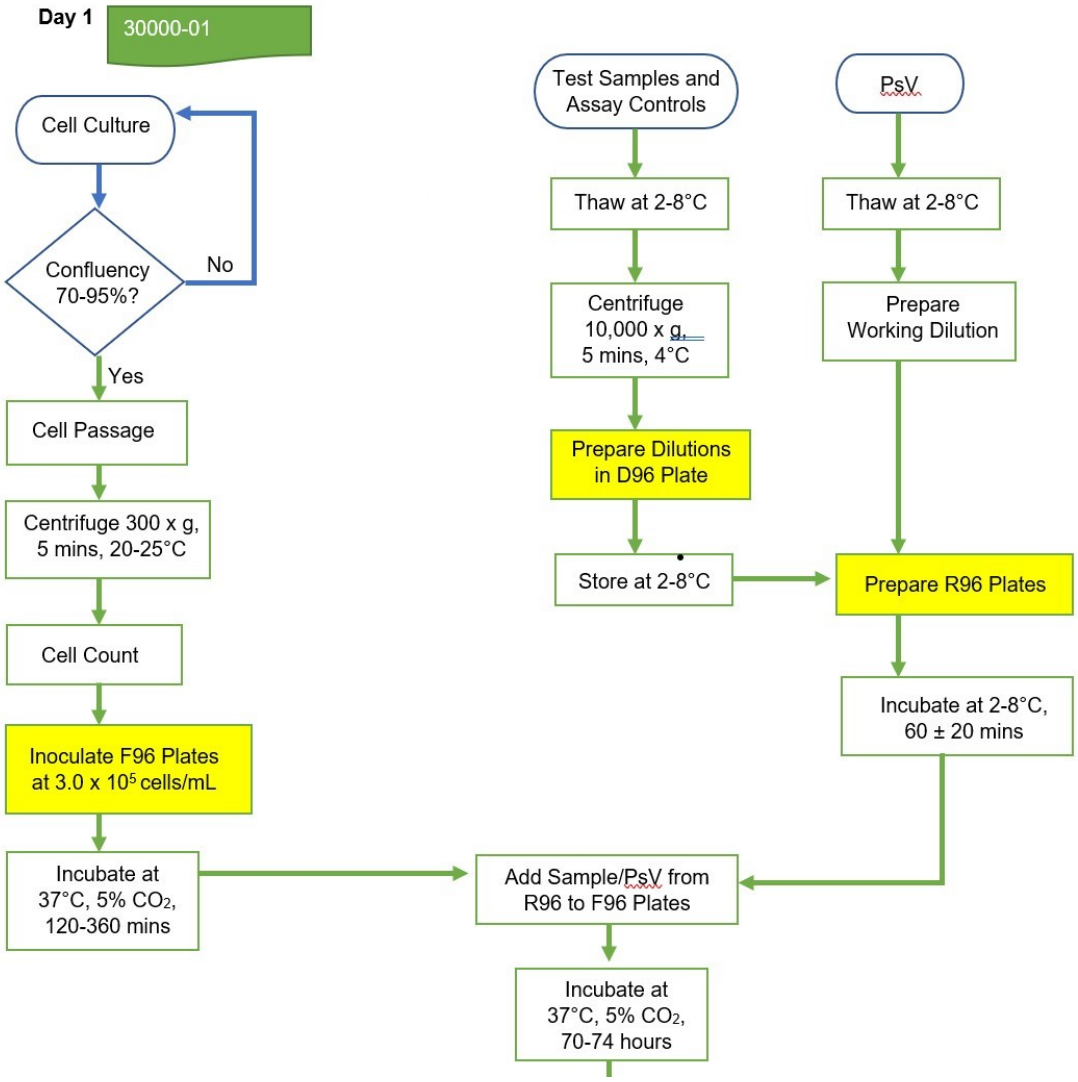
Sample Identification (ID): Click the "ID" column and enter the samples being tested. If less than five samples are being tested, enter "No Sample" into the field. When entering fields, use quotation marks around each sample ID as shown in Figure 2 below.

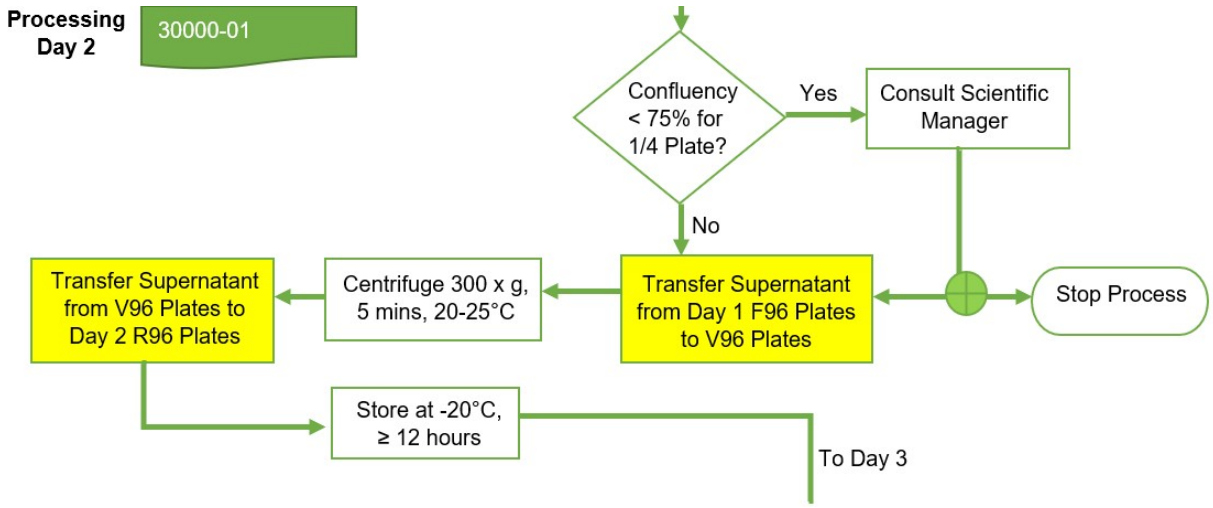


Confirm the dilution factors for samples and controls being tested for each plate being analyzed, adjust series if needed.

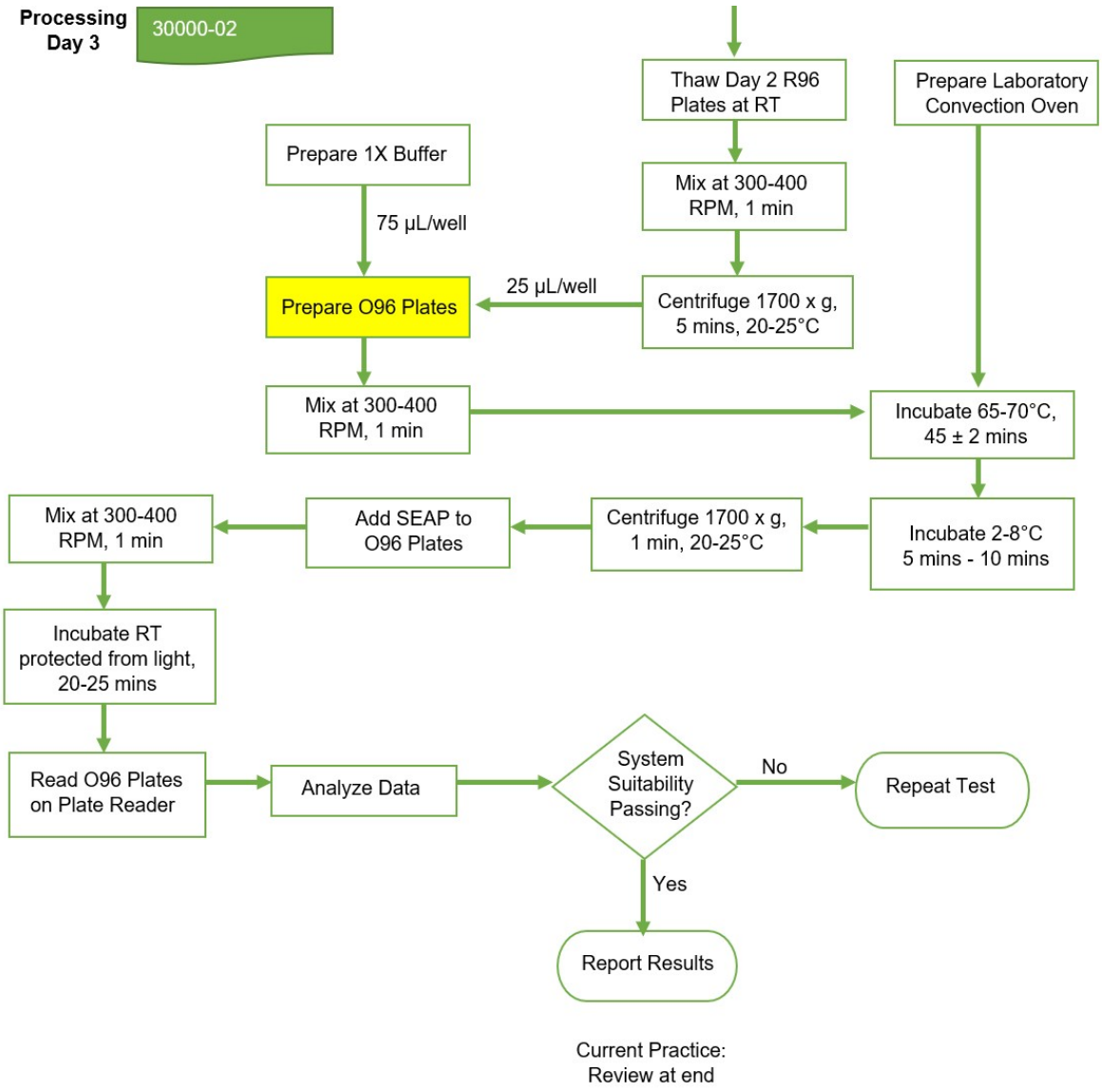


Attachment 5: Neutralization Assay Workflow





5 – 10 mins



Attachment 6: 30000-01: HPV Neutralization Assay, Sample Preparation Form

Frederick National Laboratory for Cancer Research
sponsored by the National Cancer Institute

Vaccine, Immunity and Cancer Directorate
Standard Operating Procedure Form

Form Title: HPV Neutralization Assay, Sample Preparation Form

Document ID: 30000-01		Version:	5.0
Associated SOP: 30000		Effective Date:	02Jul21
Supersedes Version:	4.0	**Page 1 of 4**	

Day 1: HPV Neutralization and Cell Growth

Equipment

Equipment Description	Equipment ID	Calibration Due Date
BSC	☐ HSL_117 ☐ HSL_074 ☐ HSL_075 ☐ HSL_076 ☐ Other:	
CO_2 Incubator	☐HPV_025 ☐HPV_026 ☐Other:	
Inverted Microscope	☐HPV_037 ☐Other:	
Eppendorf Centrifuge	☐HPV_007 ☐Other:	☐N/A
Sorvall Legend XTR Centrifuge	☐HPV_008 ☐Other:	
Cellometer Auto 2000	☐HPV_005 ☐Other:	☐N/A
2-8°C Refrigerator	☐HSL_084 ☐HSL_087 ☐Other:	☐N/A
☐N/A epMotion 96	☐HPV_102 ☐Other:	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	

Reagents

Reagent	Lot Number	Expiration Date
DPBS		
0.05% Trypsin-EDTA		
Vita Stain AOPI Staining Solution		☐N/A
PBNA_M		
HPV Sero Negative Control		☐N/A
HPV Sero Positive Control		☐N/A

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Form Title: HPV Neutralization Assay, Sample Preparation Form

Document ID: 30000-01		Version:	5.0
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Cell Culture Lot Number: _____ Working Passage #: _____ Confluency %: _____

Cell Count

Count 1 (x10^6 Cells/mL)	Viability 1 (%) (≥80%)	Count 2 (x10^6 Cells/mL)	Viability 2 (%) (≥80%)	Average Count (x10^6 Cells/mL)	Percent Difference (%) (≤ 25%)
	☐Pass ☐Fail		☐Pass ☐Fail		
☐ N/A Row Count 3 (x10^6 Cells/mL)	Viability 3 (%) (≥80%)	Count 4 (x10^6 Cells/mL)	Viability 4 (%) (≥80%)	Average Count (x10^6 Cells/mL) (**Counts 1-4**)	
	☐Pass ☐Fail		☐Pass ☐Fail		

Cell Dilution to 0.3 x 10^6 cells/mL

Average Cell Count (x10^6 Cells/mL)	Total Volume Required (mL) (~12 mL per plate)	Volume of Cells Used (mL)	Volume of PBNA_M (mL)

F96 Plate Incubation at 37°C, 5% CO_2(120-360 minutes) *Based on last plate

Start Time	End Time	Total Time (hrs/min)

Sample Pre-Dilution ☐ See Attached

Assay Control Pre-Dilution ☐ See Attached

Plate Map Used: ☐ See Attached

PsV Dilution Preparation: ☐ See Attached

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PsV Reagents

HPV Type	Pseudovirus Lot Number	HPV Type	Pseudovirus Lot Number	HPV Type	Pseudovirus Lot Number

PsV + Sample Plate Incubation at 2-8°C (40-80 minutes) *Based on last plate

Start Time	End Time	Total Time (mins)

Sample and PsV Incubation at 37°C, 5% CO_2 (70-74 hours) *Based on last plate

Start Date / Time:	

Comment:

☐N/A

Performed by/date:	
Reviewed by/date:	

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Day 2: Harvest

Equipment

Equipment Description	Equipment ID	Calibration Due Date
BSC	☐ HSL_117 ☐ HSL_074 ☐ HSL_075 ☐ HSL_076 ☐ Other:	
Inverted Microscope	☐HPV_037 ☐Other:	
Sorvall Legend XTR Centrifuge	☐HPV_008 ☐Other:	
☐N/A Eppendorf Centrifuge	☐HPV_007 ☐Other:	☐N/A
☐N/A epMotion 96	☐HPV_102 ☐Other:	
-20°C Freezer	☐ HSL_085 ☐ Bldg. 469, Rm 135 ☐Other:	☐N/A
☐N/A Pipette: µL	PIP_	
☐N/A Pipette: µL	PIP_	

Sample and PsV Incubation at 37°C, 5% CO_2 (70-74 hours) *Based on last plate

End Date / Time	Total Time (hrs)

Cell Confluency >75%? (If no – Comment)

Plate Number	Confluency	Plate Number	Confluency	Plate Number	Confluency
☐N/A 1	☐Yes ☐No	☐N/A 8	☐Yes ☐No	☐N/A 15	☐Yes ☐No
☐N/A 2	☐Yes ☐No	☐N/A 9	☐Yes ☐No	☐N/A 16	☐Yes ☐No
☐N/A 3	☐Yes ☐No	☐N/A 10	☐Yes ☐No	☐N/A 17	☐Yes ☐No
☐N/A 4	☐Yes ☐No	☐N/A 11	☐Yes ☐No	☐N/A 18	☐Yes ☐No
☐N/A 5	☐Yes ☐No	☐N/A 12	☐Yes ☐No	☐N/A 19	☐Yes ☐No
☐N/A 6	☐Yes ☐No	☐N/A 13	☐Yes ☐No	☐N/A 20	☐Yes ☐No
☐N/A 7	☐Yes ☐No	☐N/A 14	☐Yes ☐No	☐N/A 21	☐Yes ☐No

Sample and PsV Incubation at -20°C *Based on last plate

Start Date / Time:	

Comment:

☐N/A

Performed by/date:	
Reviewed by/date:	

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Attachment 7: 30000-02: HPV Neutralization Assay, Substrate Developmen

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Vaccine, Immunity and Cancer Directorate
Standard Operating Procedure Form

Form Title: HPV Neutralization Assay, Substrate Development Form			
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Day 3: Substrate Development

Equipment

Equipment Description	Equipment ID	Calibration Due Date
Convection Oven	□ HPV_041 □ Other:	
BSC	□ HSL_117 □ HSL_074 □ HSL_075 □ HSL_076 □ Other:	
2-8°C Refrigerator	□ HSL_084 □ HSL_087 □ Other:	□N/A
□N/A Eppendorf Centrifuge	□ HPV_007 □ Other:	□N/A
□N/A epMotion 96	□HPV_102 □Other:	
Sorvall Legend XTR Centrifuge	□ HPV_008 □ Other:	
Microplate Shaker	□ HSL_030 □ HSL_031 □ HSL_032 □ HSL_054 □ HSL_055 □ Other:	
M5 Microplate Reader	□ HSL_098 □ Other:	□N/A
-20°C Freezer	□ HSL_085 □ Bldg 469, Rm 135 □Other:	□N/A
□N/A Pipette: µL	PIP_	
□N/A Pipette: µL	PIP_	
□N/A Pipette: µL	PIP_	

Reagents

Reagent	Lot Number	Expiration Date
SEAP Substrate		□N/A
5X Buffer		□N/A
Distilled Water		

1X Buffer Preparation

Total Volume Required (mL)	Volume of 5X Buffer (mL)	Volume of Distilled Water (mL)

Plates Removed from -20°C Freezer (One Freeze/Thaw Cycle) *Based on Last Plate

Date and Time	>12 hrs
	□ Yes □ No:_____

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Plate Identification
Assay Plate Data Reference: _____

Plate #	HPV Type	Plate #	HPV Type	Plate #	HPV Type
□N/A 1		□N/A 8		□N/A 15	
□N/A 2		□N/A 9		□N/A 16	
□N/A 3		□N/A 10		□N/A 17	
□N/A 4		□N/A 11		□N/A 18	
□N/A 5		□N/A 12		□N/A 19	
□N/A 6		□N/A 13		□N/A 20	
□N/A 7		□N/A 14		□N/A 21	

Incubations

Condition	Start Time	End Time	Total Time (mins)
Plates at 65-70°C (45 ± 2 min)			
Plates at 4°C (5-10 min)			
Substrate (**1st plate only**)(20-25 min)		Read Time:	

System Suitability

Plate #	Mean (NB)	Mean (NS/NV)	%CV (NB)	PsV+NB S:N Ratio	Disposition
	≥ 80,000 RLU	≤ 2000 RLU (Pass); 2000-5000 RLU(Check); > 5000 RLU Repeat	≤ 50%	≥ 50	Pass / Fail
□N/A 1					□ Pass □ Fail
□N/A 2					□ Pass □ Fail
□N/A 3					□ Pass □ Fail
□N/A 4					□ Pass □ Fail
□N/A 5					□ Pass □ Fail
□N/A 6					□ Pass □ Fail
□N/A 7					□ Pass □ Fail
□N/A 8					□ Pass □ Fail
□N/A 9					□ Pass □ Fail
□N/A 10					□ Pass □ Fail
□N/A 11					□ Pass □ Fail
□N/A 12					□ Pass □ Fail
□N/A 13					□ Pass □ Fail
□N/A 14					□ Pass □ Fail
□N/A 15					□ Pass □ Fail
□N/A 16					□ Pass □ Fail
□N/A 17					□ Pass □ Fail
□N/A 18					□ Pass □ Fail
□N/A 19					□ Pass □ Fail
□N/A 20					□ Pass □ Fail
□N/A 21					□ Pass □ Fail

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Raw Data File Reference: _____

Raw Data File Reference: _____

Sample Results □ See Attached

Comments:

_N/A

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Reviewed by/date:	

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