

Laboratory Procedure Manual

Analyte: **Human Papillomavirus**

Matrix: **Serum**

Method: 9-plex competitive Luminex Immuno Assay (9-plex cLIA)

Revised:

as performed by: PPD Inc. for Center for Disease Control & Prevention
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Important Information for Users

NCEZID periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2005–2006 data.

A tabular list of the released analytes follows.

Dataset name	Variable name	Description
HPVSRM_D	PV06	HPV 6 Qualitative
	PV6N	HPV6 Quantitative
	PV11	HPV 11 Qualitative
	P11N	HPV 11 Quantitative
	PV16	HPV 16 Qualitative
	P16N	HPV 16 Quantitative
	PV18	HPV 18 Qualitative
	P18N	HPV 18 Quantitative
	PV31	HPV 31 Qualitative
	P31N	HPV 31 Quantitative
	PV33	HPV 33 Qualitative
	P33N	HPV 33 Quantitative
	PV45	HPV 45 Qualitative
	P45N	HPV 45 Quantitative
	PV52	HPV 52 Qualitative
	P52N	HPV 52 Quantitative
	PV58	HPV 58 Qualitative
	P58N	HPV 58 Quantitative

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human papillomaviruses (HPVs) are double-stranded DNA viruses that infect epithelial cells and are associated with low-grade cervical intraepithelial neoplasia, genital condyloma, cervical and other anogenital cancers and some oropharyngeal cancer. Cervical cancer is the second most common cause of cancer deaths in women worldwide, resulting in approximately 400,000 deaths per year. HPVs are the primary cause of cervical cancer and are the most common sexually transmitted viral pathogens in the United States. To date, at least 100 different HPV types have been described. “Low-risk” HPVs such as HPV-6 and -11 are associated with the production of benign genital warts, whereas “high-risk” types such as HPV-16 and -18 are associated with the development of cervical cancer. Strong epidemiological evidence that HPVs cause cervical carcinoma is suggested by the fact that HPV DNA is detected in more than 99.7% of cervical cancers. HPV-16 is the most prevalent oncogenic HPV, being present in more than 50% of all cervical tumor specimens worldwide. HPV-16 and -18, plus the less prevalent oncogenic types such as HPV-31, -33, -35, -45, -52, and -58, contribute to more than 90% of cervical carcinomas. Two vaccines are currently FDA approved and recommended by ACIP, Cervarix (manufactured by GlaxoSmith Kline; targeting HPV 16 and 18) and Gardasil (manufactured by Merck; targeting HPV 6, 11, 16 and 18). Merck is currently sponsoring a clinical trial of a 9-valent vaccine, targeting HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58).

The competitive Luminex Assay is based on the ability of antibodies in the serum to compete with labeled type-specific neutralizing monoclonal antibody for binding to beads coated with L1-Viral-like particles (VLPs). The assay is proprietary to Merck and licensed to Pharmaceutical Product Development (PPD), Inc. The 9-plex format includes beads with L1-VLPs and monoclonal antibodies specific for HPV 6, 11, 16, 18, 31,33,45,52 and 58. Any class of Ig is detected, but detection is restricted to the specific neutralizing epitope recognized by the monoclonal antibody. Quantitative results are reported in terms of arbitrary units (milliMerck units/ml). Threshold for positive results established by Merck are used to report qualitative results (positive - at or above threshold; negative – below threshold).

2. SPECIAL SAFETY PRECAUTIONS

Observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labeled as hazardous waste and stating the composition of waste being contained.

Protect all work surfaces by absorbent benchtop paper. Discard the benchtop paper into the biohazard waste container daily or whenever blood contamination occurs. Wipe down all work surfaces with 10% (v/v) sodium hypochlorite after work is complete.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- a. Each shipment of specimens received from the NHANES mobile unit contains a corresponding shipping manifest listing the samples in the box. An electronic data file (Excel worksheet), containing the specimen ID's, specimen locations in the box, collection dates and other relevant information concerning individual samples, is independently sent from Westat to the

laboratory via email. From the data file, a worksheet for each assay run is generated. Each specimen is checked against the worksheet for correct sample ID on the label and acceptable condition of the specimen prior to the assay.

- b. After the test results have been obtained and the final values approved by the reviewing supervisor for release, the result codes were transcribed into the data file originally sent from Westat. Data entry is proofed by the supervisor and clerk. The completed data file is then uploaded to the NHANES Westat laboratory data management website. A copy is archived in the local computer, with weekly backup, to maintain an independent record. The new data are also appended to a local database, which includes all the specimens with results obtained in the project to-date; hardcopies of data are generated periodically and filed.

- c. Documentation for data system maintenance is contained in hard copies of data records.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

- a. No special instructions such as fasting or special diets are necessary. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.
- b. Specimens for HPV analysis should be fresh or frozen serum.
- c. A 0.5 mL sample of serum is preferable. The minimum sample volume required for analysis is 0.25 mL. Specimens are rejected if insufficient quantity is available for analysis.
- d. Specimens collected in the field should be frozen, and then shipped on dry ice by overnight mail. Once received, specimens are stored at $\leq -70^{\circ}\text{C}$ until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at $\leq -70^{\circ}\text{C}$. Samples thawed and refrozen several times are not compromised, but extensively repeated freeze/thaw cycles should be avoided.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable to this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

- a. Instrumentation

Luminex platform using LabMAP technology

The type-specific HPV-VLP antibody responses are associated with specific Luminex microspheres that are identified by their distinct red and orange fluorescent dye spectral properties on the Luminex. Antibody titers are determined in a competitive format, where known, type-specific, phycoerythrin (PE)-labeled, neutralizing antibodies compete with patient serum antibodies for binding to conformationally sensitive, neutralizing epitopes on the VLPs.

b. Materials

VLPs.

VLPs for HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 formed by the expression of the L1 gene in yeast *Saccharomyces cerevisiae* were purified from lysates of *S.cerevisiae*.

Antibodies.

The antibodies —HPV-6 (H6.B10.5), HPV-11 (MAb 8740 or H11.B2; Chemicon, Temecula, Calif.), HPV-16 (H16.V5)), and HPV-18 (H18.J4)—were all previously shown to be HPV type-specific and to bind to neutralizing epitopes. The H6.B10.5, H11.B2, H16.V5, and H18.J4 antibodies were tagged with PE. Proprietary antibodies specific for HPV 31, 33, 45, 52 and 58 were developed by Merck, and tagged with PE in similar fashion. For use in the assay, the nine PE-tagged MAbs were combined.

c. Reagent Preparation

Covalent coupling of HPV VLPs to Luminex microspheres.

The HPV-VLPs were coupled to the Luminex microspheres by using an *N*-hydroxysulfosuccinimide enhanced carbodiimide-mediated coupling reaction. Luminex microspheres are fluorescent polystyrene beads approximately 5,600 nm in diameter with functional carboxyl groups for covalently attaching proteins. Microspheres were stored at 4°C, in the dark, at an optimized concentration. The microspheres were brought to room temperature, sonicated to obtain an equal distribution of microspheres. Microspheres were pelleted and resuspended. The carboxylated sites on the surface of the microspheres were activated by adding *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. Coupling of the HPV-VLPs to their respective microspheres was performed after the carboxyl sites on the microspheres were activated. HPV-VLPs for types 6, 11, 16, 18, 31, 33, 45, 52, and 58 were diluted in buffer. VLPs were added to the activated microspheres and vortexed to resuspend the microspheres. The VLPs were coupled to the microspheres chosen because of the good spectral resolution between the different microsphere sets. After addition of the VLPs, vials were wrapped in foil and placed on a rotator to allow VLPs to covalently bind to the microspheres by forming amide bonds with the open carboxylate sites on the microspheres. VLPs coupled to microspheres were washed and resuspended in histidine buffer (20 mM histidine, 0.5 M NaCl; pH 6.2), Column A buffer (50 mM morpholinepropanesulfonic acid, 0.5 M NaCl mM; pH 7.0), or PBS with or without 1% bovine serum albumin (BSA) to block any remaining open carboxyl sites on the microspheres. VLP-microspheres were stored at 4°C in light-resistant vials.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration Curve

A 12-point standard curve is generated by the user as part of these assay methods. Stock, reference standard into HPV-negative normal human serum (NHS) was added in duplicate to generate a 12-point standard curve on each plate.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

- (1) Ensure that all plates are subjected to the same process and incubation times.
- (2) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

b. Sample Preparation

- (1) Thaw serum specimens on ice.
- (2) Mix serum samples gently before testing to eliminate stratification, which may occur when serum is frozen or stored at 4°C for extended periods.
- (3) Identify the reaction tray wells for each specimen or control.

d. Operation of Assay Procedure

To perform the competitive immunoassay, VLP-microspheres of each of the nine HPV VLP types were pooled in equal volumes and diluted to a final concentration. An HPV standard reference serum was generated by pooling sera from individuals immunized with the 9-valent vaccine. To the microspheres, serial dilutions of the stock, reference standard in HPV-negative normal human serum (NHS) was added to generate a standard curve. A negative control and high and low controls were added in duplicate to each plate. Sera were also tested in duplicate at 1:4 and/or 1:40 dilution. Sera and VLP-microspheres were incubated in a foil-covered plate. The combined, PE-tagged, type-specific MAbs were added to each well of the plate and the sera, VLP-microspheres, and MAb-PE combinations were mixed. Plates were resealed with foil covers and allowed to incubate overnight at room temperature. After incubation, all samples were transferred to a filter plate (Millipore, Bedford, Mass.) prewet with PBS. The serum samples were washed and the VLP-microspheres were resuspended in buffer for analysis. Samples were analyzed on a Luminex instrument with the XY plate handler platform in multiplexed acquisition mode with gates set to exclude microsphere multimers.

e. Recording of Data

Relative inhibition of MAb-PE binding was compared to a standard curve by using a four-parameter logistic curve fit. The standard reference sera used for the standard curve were assigned arbitrary values expressed in mMU/mL. The cutoff values were determined relative to the standard curve.

Merck established the serostatus cut-off values for a positive result for each HPV type in the assay as listed below. A final titer below this value is negative and a value equal or above is considered positive.

Sero-Status Cutoff (mMU/mL): HPV6=30, HPV11=16, HPV16=20, HPV18=24, HPV31=10, HPV33=8, HPV45=8, HPV52=8, HPV58=8.

9. REPORTABLE RANGE OF RESULTS

Final qualitative reports express results as positive or negative for the presence of HPV-6 or HPV-11, or HPV-16, or HPV-18 or HPV-31, or HPV-33, or HPV-45, or HPV 52 or HPV 58 in the sample.

Final quantitative reports express results as mMU/mL for HPV-6, or HPV-11, or HPV-16, or HPV-18 or HPV-31, or HPV-33, or HPV-45, or HPV 52 or HPV 58 in the sample.

10. QUALITY CONTROL (QC) PROCEDURES

A negative control, high and low controls were added in duplicate to each plate.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

- a. Repeat the test if the controls don't agree.
- b. Do not report results from runs in which the controls did not meet expected reactivities.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Aggregation of the bead and particles in sera can sometimes lead to clogging of the needle in the Luminex instrument. Positive results indicate seroconversion but correlates of protection are not known. Only about 70% of individuals seroconvert after natural exposure to HPV.

13. REFERENCE RANGES (NORMAL VALUES)

A normal sample in unvaccinated person is negative for the presence of HPV-6 or HPV-11, or HPV-16, or HPV-18, or HPV-31, or HPV-33, or HPV-45, or HPV-52 or HPV-58.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this assay method.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at $\leq -70^{\circ}\text{C}$ until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at $\leq -70^{\circ}\text{C}$.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Other available methods have different performance characteristics (for example, lower sensitivity or specificity). If the analytical system fails, it is preferable to store specimens at $\leq -70^{\circ}\text{C}$ until the system is returned to functionality.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this assay method.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping involves using the computerized database and the hard copy results themselves to track specimens. Records are maintained indefinitely. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

For the NHANES study, residual serum $>100\ \mu\text{l}$ is retained at $\leq -70^{\circ}\text{C}$ for 1 year and then returned to NCHS serum bank.

19. SUMMARY STATISTICS AND QC GRAPHS

Qualitative assays are assays with a positive, negative or borderline/indeterminate result.

REFERENCES

Dias D, Van Doren J, Schlottmann S, Kelly S, Puchalski D, Ruiz W, Boerckel P, Kessler J, Antonello J, Green T, Brown M, Smith J, Chirmule N, Barr E, Jansen KU, Esser MT. Optimization and Validation of a multiplexed Luminex assay to quantify antibodies to neutralizing epitopes on human papillomaviruses 6, 11, 16, and 18. Clin Diag Lab Immunol 12:959-969, 2005.

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