

Laboratory Procedure Manual

Analyte: **Human Papillomavirus**

Matrix: **Serum**

Method: **Multiplexed Competitive Luminex
Immunoassay (4-plex CLIA)**

Revised:

as performed by: **Pharmaceutical Product Development (PPD) for Centers for
Disease Control & Prevention**

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Important Information for Users

PPD 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	SAS Label
HPVSER_D	LBXS06MK	HPV 06 (Merck competitive Luminex assay)
	LBXS11MK	HPV 11 (Merck competitive Luminex assay)
	LBXS16MK	HPV 16 (Merck competitive Luminex assay)
	LBXS18MK	HPV 18 (Merck competitive Luminex assay)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human papillomaviruses (HPVs) are double-stranded DNA viruses that infect epithelial cells. HPVs are among the most common sexually transmitted viral pathogens and are significantly associated with cervical intraepithelial neoplasia, genital condylomas, cervical and other anogenital cancers. Cervical cancer is the second most common cause of cancer deaths in women worldwide, resulting in approximately 400,000 deaths per year. 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. 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, -45, -52, and -58, contribute to more than 90% of cervical carcinomas. There are two vaccines currently marketed worldwide, Gardasil[®] (Merck) targets HPV6,11,16 and 18, whereas Cervarix[®] (Glaxo Smith-Kline) targets HPV16 and 18 only.

Antibody response to HPV infection is not clinically relevant. However, it can be used as a surrogate for lifetime exposure in the population or to measure vaccine immunogenicity and uptake. The competitive Luminex assay used here can simultaneously detect neutralizing antibodies to HPV 6, 11, 16 and 18. Type-specific antibodies of any Ig class are detected in patient sera by displacement of fluorescently tagged neutralizing monoclonal antibody from VLP-coated microspheres. This assay was developed by Merck & Co, and has been used to determine titers in the Gardasil[®] vaccine trials. The testing for the NHANES 2005-2006 sample set was performed by Pharmaceutical Product Development (PPD) using a similar protocol to that described in the Dias et.al 2005 with possible modifications not listed here.

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 weekly.

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 $\leq -20^{\circ}\text{C}$ until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at $\leq -20^{\circ}\text{C}$. 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, CALBRATORS (STANDARDS), AND CONTROLS

- a. Instrumentation
Bio-Plex Suspension Array System (Biorad, CA)

- b. Materials

Virus-Like Particles (VLPs).

VLPs for HPV types 6, 11, 16, and 18 formed by the expression of the type-specific L1 gene in yeast were purified from lysates of *Saccharomyces Cerevisiae*.

Antibodies.

The antibodies chosen for the assay—HPV-6 (H6.M48), HPV-11 (K11.B2), HPV-16 (H16.V5), and HPV-18 (H18.J4)—were all previously shown to be HPV type-specific and to bind to neutralizing epitopes.

c. Reagent Preparation

Covalent coupling of HPV VLPs to Luminex microspheres.

The HPV-VLPs were coupled to the xMAP Multi-Analyte COOH Microspheres (Luminex Corporation, TX) by using an *N*-hydroxysulfosuccinimide enhanced carbodiimide-mediated coupling reaction. Microspheres were stored at 4°C, in the dark, at a concentration of 1.25×10^7 microspheres/ml. The microspheres were brought to room temperature, sonicated followed by vortexing for 1 min to obtain a homogenous distribution of microspheres. The microspheres were divided into aliquots in 1.5-ml copolymer microcentrifuge vials (USA Scientific, FL) and pelleted. Microspheres were washed 500 μ l of 0.1 M sodium phosphate buffer (pH 6.2), resuspended with sonication and repelleted. The carboxylated sites on the surface of the microspheres were activated by adding 50 μ l of a 50-mg/ml solution of *N*-hydroxysulfosuccinimide and 50 μ l of a 50-mg/ml solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce, IL). The tubes were sonicated for 1 min, wrapped in foil and incubated for 20 min at room temperature (RT). After the activation step, the microspheres were washed once in 500 μ l of either 50mM 2-(*N*-morpholino)ethane sulfonic acid buffer, pH 6.0 (MES, Sigma. MO) or phosphate-buffered saline (PBS; pH 7.4) to remove residual activation agents.

HPV-VLPs for types 6, 11, 16, and 18 were diluted in MES to an optimized concentration. of HPV16. Upon dilution, 500 μ l of each VLP solution was added to the corresponding microsphere. The VLPs were coupled to the microspheres as follows: VLP-6 to microsphere 6, VLP-11 to microsphere 11, VLP-16 to microsphere 16, and VLP-18 to microsphere 18. After addition of the VLPs, vials were vortexed, sonicated for 1 min and wrapped in foil, and placed on a rotator overnight at room temperature. VLPs coupled to microspheres were washed twice with 1 ml of wash buffer, i.e. PBS pH 7.4 containing 1% Triton-X100 or containing 0.05% Tween 20. The microspheres were then resuspended in 1 ml of histidine buffer (20 mM histidine, 0.5 M NaCl; pH 6.2) containing 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.

VLP-microspheres of each of the four HPV VLP types were pooled in PBS with 1%BSA and 0.05% sodium azide (PBS-BN) or PBS-BN plus 1% TritonX 100 to a final concentration of 2.0×10^5 microspheres/ml per VLP type for use in the assay.

Antibodies.

Each of the 4 monoclonal antibodies were tagged with R-Phycoerythrin (PE) via succinyl esters containing either thiol or maleimide reactive groups by Chromaprobe Inc (Maryland Heights, MO.). For use in the assay, the PE-tagged MAbs were pooled and diluted in PBS with 1%BSA and 0.05% sodium azide (PBS-BN) containing varying concentrations of 0-20% of Triton-x 100.

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. The reference sera was heat inactivated prior to use for 30 ±2min at 56°C ±1°C. Two-fold dilutions in Antibody-depleted Human Serum (ADHS) (Valley Biomedical, VA) were prepared for the assay...

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) Bring serum specimens to room temperature (20–25°C).
- (2) Heat inactivate all samples for 30 ±2min at 56°C ±1°C prior to use.
- (3) Mix serum samples gently before testing to eliminate stratification, which may occur when serum is frozen or stored at 4°C for extended periods.
- (4) Identify the reaction tray wells for each specimen or control.

d. Operation of Assay Procedure

All standards, controls and samples were tested in duplicate. 25 µl of control sera was added to each assay well along with 25 µl of ADHS. This was followed by the addition of 25 µl of MAb-PE (at the optimized concentration) and 25 µl VLP microspheres (5000 microspheres per well per type) to the assay plate. For the calibration curve, 50 µl of twofold serial dilutions of the reference standard was added to wells containing 25 µl of MAb-PE and 25 µl VLP microspheres. The plate was then sealed and incubated at RT for 15-25h.. After incubation, all samples were transferred to a 1.2 µm low protein binding filter plate (Millipore, Bedford, Mass.) prewet with wash buffer. The plates were washed three times with 200 µl of wash buffer and resuspended in 125 µl of wash buffer for analysis. Samples were analyzed on the Bio-plex Suspension Array System.

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 test sample concentration was calibrated from the fitted standard curve. The reference sera used for the standard curve were assigned arbitrary values expressed in milli-Merck Units/milliliter (mMU/ml). The cutoff values were established by determining the lowest titer within a valid assay's quantifiable range that allowed for an acceptable level of discrimination between a set of "negative" sera and sera likely to contain a varying percentage of true positives.

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=20, HPV11=16, HPV16=20, HPV18=24

9. REPORTABLE RANGE OF RESULTS

Final reports express results as positive or negative for the presence of HPV-6 or HPV-11, or HPV-16 or HPV-18 in the sample. The reports also contain antibody titers for each HPV type expressed in mMU/ml.

10. QUALITY CONTROL (QC) PROCEDURES

A negative control, high and low controls were tested in duplicate on 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

As with any HPV serology assay, a major limitation is the lack of established clinical sensitivity and specificity measures to measure past or current infection. This is due to the fact that not all people seroconvert after infection and in others the antibody response can occur several months after initial infection.

Another limitation, is that the antibodies in patient sera that differ in neutralizing epitopes to that of the MAbs used in the assay will go undetected, thereby reducing the sensitivity of detection.

13. REFERENCE RANGES (NORMAL VALUES)

Not Applicable to this assay method.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this assay method.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at $\leq -20^{\circ}\text{C}$ until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at $\leq -20^{\circ}\text{C}$.

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), and also do not allow for multiplexed high-throughput format utilized here. If the analytical system fails, it is preferable to store specimens at $\leq -20^{\circ}\text{C}$ 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 $>200\ \mu\text{l}$ is retained at $\leq -20^{\circ}\text{C}$ 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. Quantitative values for the controls are monitored, but details are not available at this time.

REFERENCES

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