Laboratory Procedure Manual

Analyte: **Human Papillomavirus**

Matrix: **Serum**

Method: Multiplexed Luminex Assay

Revised:

as performed by: *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 2009–2010 data.

A tabular list of the released analytes follows.

Dataset name	Variable name	Description
HPVSER_F	LBXH6	HPV 6
	LBX11	HPV 11
	LBX16	HPV 16
	LBX18	HPV 18

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human papillomaviruses (HPVs) are double-stranded DNA viruses that infect epithelial cells and are significantly associated with low-grade cervical intraepithelial neoplasia, genital condyloma, and cervical 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, -45, -52, and -58, contribute to more than 90% of cervical carcinomas. Vaccines for both the low-risk and high-risk HPV genotypes are currently being tested in clinical trials.

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 HPVanalysis should be fresh or frozen serum.

   c. A 0.5 mL sample of serum is preferable. The minimum sample volume required for analysis is .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 ≤ -70°C until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at ≤ -70°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 100 platform using LabMAP3 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^{100}. 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, and 18 formed by the expression of the L1 gene in yeast *Saccharomyces cerevisiae* were purified from lysates of *S.*

      Antibodies.

      The antibodies chosen for the assay—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 (Chromaprobe, Aptos, Calif.). For use in the assay, the four PE-tagged MAbs were combined so that the final concentrations of each MAb were 2.5 µg/ml for H6.B10.5, 1.0 µg/ml for H11.B2, 1.0 µg/ml for H16.V5, and 1.25 µg/ml for H18.J4.

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 a concentration of 1.25×10^7 microspheres/ml. The microspheres were brought to room temperature, sonicated for 2 min to obtain an equal distribution of microspheres, and divided into aliquots in 1.5-ml vials (VWR, West Chester, Pa.) at 2.5×10^6 microspheres per vial. Microspheres were pelleted and resuspended in 400 µl of 0.1 M sodium phosphate buffer (pH 6.2). The carboxylated sites on the surface of the microspheres were activated by adding 50 µl of a 50-mg/ml solution of *N*-hydroxysulfosuccinimide and 50 µl of a 50-mg/ml solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The tubes were sonicated for 2 min, wrapped in foil and incubated for 20 min at room temperature. After the activation step, the microspheres were washed once in 500 µl of phosphate-buffered saline (PBS; pH 7.4) before the addition of the VLPs.

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, and 18 were diluted in PBS to a concentration of 12 µg/ml. A total 500 µl of the VLPs (12 µg/ml) was added to the activated microspheres and vortexed on a low setting for 10 to 20 s to resuspend the microspheres. The VLPs were coupled to the microspheres as follows: VLP-6 to microsphere 32, VLP-11 to microsphere 53, VLP-16 to microsphere 38, and VLP-18 to microsphere 18. The different microspheres were 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 for 2 h at room temperature 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 once with 1 ml of PBS containing 0.05% Tween 20 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 (1.0×10^6 microspheres/ml) were stored in 1-ml aliquots 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.

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) 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 four HPV VLP types were pooled in equal volumes and diluted with histidine buffer to a final concentration of 8.0×10^5 microspheres/ml. VLP-microspheres were added to each well of a 96-well, black opaque, microtiter plate (Costar, Corning, N.Y.) in a volume of 25 µl (20,000 VLP-microspheres total, 5,000 VLP-microspheres of each HPV type) per well. An HPV standard reference serum was generated by pooling sera from individual African green monkeys that had been immunized with either HPV-6, -11, or -18 VLPs and serum from a chimpanzee that had been immunized with HPV-16 VLPs. The titers for the four different sera had previously been determined in a pseudoneutralization assay The stock concentrations of the pooled, standard, reference sera for the different HPV types were 250 milli-Merck units (mMU)/ml for HPV-6 and 1,000 mMU/ml for HPV-11, HPV-16, and HPV-18. An antibody titer of >200 mMU/ml for HPV-11 has been shown to neutralize ~10^8 virions in the athymic mouse xenograft assay (7). To the 25 µl of microspheres, 50 µl of twofold serial dilutions of the stock, reference standard into HPV-negative normal human serum (NHS) was added in duplicate to generate a 12-point standard curve. A negative control was added in quadruplicate, and high and low controls were added in duplicate to each plate. Sera and VLP-microspheres were incubated at room temperature for 15 min in a foil-covered plate. The combined, PE-tagged, type-specific MAbs were added to each well of the plate in a volume of 25 µl and the sera, VLP-microspheres, and MAb-PE combinations were mixed three times by using a multichannel pipette. 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 three times with 200 µl of

PBS buffer and the VLP-microspheres were resuspended in 200 µl of PBS plus 1% BSA for analysis. Samples were analyzed on a Luminex^{100} instrument with the XY plate handler platform in multiplexed acquisition mode with gates set to exclude microsphere multimers. Instrument analysis time was approximately 30 s per sample.

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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/milliliter. 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=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.

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 ifthe 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

The effect of several different assay diluents on the immunoassay and the effect of adding a filtration-wash step to the procedure. Performing the assay in either a serum or a PBS-1% BSA matrix gave similar results suggesting that patient serum could be diluted into a PBS-1% BSA sample diluent for high-titer samples. The addition of a filtration-wash step did not significantly affect the standard curves but did significantly improve read times from 80 to 100 min per plate to

30 to 40 min per plate. The optimized assay was performed in a serum NHS matrix, and the samples were transferred to a filter plate that was washed three times in PBS-1% BSA before being placed on the Luminex^{100}.

To determine whether the individual HPV immunoassays could be multiplexed, we examined the four HPV standard sera singly or pooled together in the immunoassay in single and multiplex format. A 12-point standard curve was run in duplicate in both the single (simplex) and the multiplex formats. As mentioned, the four MAbs used for detecting HPV antibody responses bind to HPV type-specific epitopes and do not cross-react with the other genotypes. Pooling the four standards together had a minimal effect on the HPV-11, HPV-16, and HPV-18 standard curves. However, multiplexing the assay slightly affected the HPV-6 standard curve. The analytical limits of quantitation for the different standard curves in the multiplex assay were determined for HPV-6 (1.2 to 54.8 mMU/ml), HPV-11 (9.8 to 365.6 mMU/ml), HPV-16 (4.5 to 476.5 mMU/ml), and HPV-18 (11.3 to 203.0 mMU/ml), which were comparable to the limits of quantitation determined for the cRIA). The precision of the HPV-Luminex immunoassay was determined with eight replicate low-, medium-, and high-titer serum samples (data not shown). For within-run precision, the %CV values ranged from 4.3 to 4.5% for HPV-6, 3.6 to 7.4% for HPV-11, 7.4 to 22.0% for HPV-16, and 2.1 to 23.9% for HPV-18. These studies indicated that the Luminex assay could serve as a sensitive and precise multiplex assay to quantitate antibodies to neutralization epitopes on HPV-6, -11, -16, and -18.

To determine the accuracy of the Luminex assay to the currently employed individual cRIAs, we tested a panel of 45 human sera twice in both assay formats. The sera were from HPV-negative individuals enrolled in a quadrivalent HPV-VLP (types 6, 11, 16, and 18) vaccine clinical trial pre- and postvaccination. A total of 15 negative and 15 low-, 10 medium-, and 5 high-titer samples to all four genotypes were run in duplicate in both assay formats, the titers were measured, and the relative concordance between the two assays was determined. A representative set of two samples that were negative, low positive, or positive for antibodies to HPV-6, -11, -16, and -18 shows the inverse relationship between the MFI and the calculated serum titer in mMU/milliliter in the HPV-Luminex assay. Comparing the titers determined in both assay formats revealed that the two assays showed good concordance. Specifically, the Pearson correlation coefficients (R^2) between the two assay formats were greater than 0.75 (HPV-6, 0.837; HPV-11, 0.751; HPV-16, 0.768; and HPV-18, 0.775), suggesting good agreement between the two assays. In addition, the HPV-Luminex assay had a 0% false-positive rate and a 0% false-negative rate compared to the cRIAs. In summary, there was good agreement between the Luminex and cRIA assay formats, suggesting that the multiplex Luminex assays could be used in addition to or as a replacement for cRIAs.

13. REFERENCE RANGES (NORMAL VALUES)

A normal sample is negative for for the presence of HPV-6 or HPV-11, or HPV-16 or HPV-19 in the sample.
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14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this assay method.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at ≤ –70°C until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at ≤ –70°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 ≤ –70°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:l is retained at ≤ –70°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. Since the controls do not generate quantitative values, plots are not generated for quality control purposes.

REFERENCES

Dias D,Van Doren J, Schlottmann S, KellyS, 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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