

Laboratory Procedure Manual

Analyte: Human papillomavirus (HPV) 6,11,16,18,31,33,45,52 and 58 Antibodies

Matrix: Serum

Method: M9ELISA – Multiplexed VLP-based IgG ELISA on the Meso Scale Discovery Platform

Method No.:

Revised:

As performed by: Centers for Disease Control and 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 testing the items listed in the following table:

Data File Names	Variable Name	SAS Label
HPVN_D_R	LBX9M6N	HPV 6 (Quantitative)
	LBX9M11N	HPV 11 (Quantitative)
	LBX9M16N	HPV 16 (Quantitative)
	LBX9M18N	HPV 18 (Quantitative)
	LBX9M31N	HPV 31 (Quantitative)
	LBX9M33N	HPV 33 (Quantitative)
	LBX9M45N	HPV 45 (Quantitative)
	LBX9M52N	HPV 52 (Quantitative)
	LBX9M58N	HPV 58 (Quantitative)
	LBX9M6	HPV6 9-plex coded result
	LBX9M11	HPV11 9-plex coded result
	LBX9M16	HPV16 9-plex coded result
	LBX9M18	HPV18 9-plex coded result
	LBX9M31	HPV31 9-plex coded result
	LBX9M33	HPV33 9-plex coded result
	LBX9M45	HPV45 9-plex coded result
	LBX9M52	HPV52 9-plex coded result
	LBX9M58	HPV58 9-plex coded result

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

M9ELISA allows for simultaneous detection of antibodies to HPV 6,11,16,18, 31,33,45,52,and 58 in one microwell (Panicker et.al. 2021). Virus-like particles (VLP), self-assembled HPV L1+L2 protein capsids that resemble intact virions, are used as antigen in the assay. HPV-type specific antibodies present in test samples will bind to type-specific conformational epitopes on the VLPs adsorbed onto the surface of the microwell. Bound human IgG is detected with a biotinylated Anti-Human IgG followed by Streptavidin SulfoTAG™ using electrochemiluminescent detection on the MesoScale Discovery (MSD) plate reader that generates relative light units (RLU). The amount of antibodies in the sample is estimated relative to a reference sample using the parallel line method. Samples are scored as positive or negative using a pre-determined “cut-off” level. The assay is used for research purposes and does not have a clinical relevance.

2. SAFETY PRECAUTIONS

Observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all items like plastic tips, sample dilution plates, reservoirs etc. 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. Handle neat serum in a biosafety cabinet if available. 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 allowing appropriate contact time 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 Human Papillomavirus laboratory via email.
- b. From the data file, a worksheet for each assay run is generated. Each specimen is scanned and confirmed against the worksheet for correct sample ID on the label and acceptable condition of the specimen prior to the assay.
- c. After the test results have been obtained and the final values approved by the reviewing supervisor for release, the result codes are 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.
- d. 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.
- e. 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.
- b. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.

- c. Specimens for HPV analysis should be fresh or frozen serum.
- d. A 0.5 mL sample of serum is preferable. The minimum sample volume required for analysis is 0.1 mL.
- e. Specimens collected in the field should be processed for serum and frozen, and then shipped on dry ice by overnight mail. Once received, specimens are stored at $\leq -70^{\circ}\text{C}$ until analyzed.
- f. 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.
- g. Specimens are rejected if insufficient quantity is available for analysis.

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

Not applicable

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

- Plate Reader (Sector Imager 6000 OR Quickplex SQ120, Meso Scale Discovery)
- Automated Plate washer (e.g. 405Select, Biotek)
- Orbital Plate Shaker (e.g. MTS2/4, IKA)
- Microplate incubator/shaker (e.g. iEMS incubator/shaker, Thermo Scientific)
- Single and Multi-channel Pipettors, 20 μl , 100 μl , 200 μl , 1000 μl
- Automated Multichannel Pipettors, 1250 μl , 250 μl
- Vortexer
- Benchtop Centrifuge
- Weigh Balance
- Pipet-Aid
- Computer with Microsoft Excel[®] and program for PLL analysis
- Automated liquid handling system (e.g. JANUS[®], Perkin Elmer) (Optional: Required only for automated serial dilutions)

Reagents (Room Temperature, RT):

- 10X PBS (0.01 M; pH 7.4) – (e.g. Hyclone, Cat. #4550)
- 10X PBST (pH 7.4)- (e.g. Hyclone, Cat. #0125)
- 4X Read Buffer T (MSD, Cat. #R92TC-1)
- ddH₂O

Reagents (4°C):

- ECL blocking agent (GE Healthcare Life Sciences, Cat. #RPN2125)
- Sulfo-tag[™] Streptavidin (MSD, Cat. #R32AD-1)
- Biotinylated Anti-Human IgG (Biotrend, Cat. # I-125-250)

Preparation of Reagents

Note: Make all reagents on the day of testing. Do not store unless otherwise specified. Volumes shown are for one plate.

Wash Buffer (PBS-0.1% Tween 20) (1L)

10X PBST= 100 ml

ddH₂O= 900 ml

Store at room temperature for 1 month. Buffer can be made fresh on day of assay as needed.

5% Blocking Buffer (30 ml)

ECL blocking agent = 1.5 g

1X PBST = 30 ml (QS)

1% Assay Diluent (50ml)

5% Blocking Buffer = 10 ml

1X PBST = 40 ml

Biotinylated Anti-Human IgG (3 ml)

Refer to antibody vial for stock concentration and dilute as needed to 1 µg/ml in Assay Diluent.

Sulfo-tagTM Streptavidin (3 ml)

Refer to vial for stock concentration and dilute as needed to 1 µg/ml in Assay Diluent.

1X Read Buffer T (20 ml)

4X Read Buffer T = 5 ml

ddH₂O =15 ml

Supplies, Other Materials

- MSD pre-printed multiplex plates (10 spot plates)
- Costar 96-Well Untreated Round (e.g. Fisher, Cat. # 07-200-103)
- Pipettor tips
- Serological pipettes
- Adhesive plate seals (e.g. Thermo-Fisher, Cat. #AB-0580)
- Conical Tubes (e.g. BD Falcon, Cat. #352098)
- Solution Troughs (e.g. CDC Glassware, Cat. #98339)
- Paper towels

For automated serial dilutions

- Robopack tips 20 µl (Perkin Elmer, Cat# 6000679)
- Robopack tips 200 µl (Perkin Elmer, Cat# 6000683)
- MDT tips 235 µl (Perkin Elmer, Cat# 69000044)
- Janus associated troughs (e.g. Seahorse Bioscience, Cat.# S30014)

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

All equipment and instrumentation used were maintained and verified as per manufacturer instructions.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. General notes:

- i. A minimum of 3 dilutions were tested for each sample and control
 - ii. Reference sera along with positive and negative controls must be run on every plate.
 - iii. The MSD imager requires 30 min for the camera to warm up before the plate can be read.
 - iv. Avoid air bubbles when adding 1X Read Buffer. This is especially important at this step, since bubbles will cause an incorrect reading. Ensure that the bottom of each plate is devoid of moisture before reading the plate. Do not let the plate sit after adding the read buffer as it will affect the final reading.
- b. Sample Preparation**
- i. Test specimens and assay controls were thawed on ice
 - ii. Serum samples vortexed to mix before performing serial dilutions
- c. Assay procedure:**
- i. A plate diagram was created to identify location and dilution series of test sample and control sample wells on the microplate.
 - ii. Take out pre-printed plates from refrigerator and let them acclimate to Room Temperature (RT) ($24^{\circ}\text{C} \pm 2$) on the day of assay.
 - iii. Add 150 μl /well of 5% blocking buffer. Seal plate and incubate for 1 h at RT on an orbital shaker at 650 rpm.
 - iv. Wash plate 4 times with 150 μl /well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.
 - v. Prepare at least 3 serial dilutions of each sample in assay diluent in a new Costar 96 well dilution plate as described below. Serial 3.16-fold dilutions of human serum samples starting at 1:100 were prepared in assay diluent. Serial dilution can be performed by automated liquid handling workstation or manually.
Example: Add 11 μl sample to 99 μl assay diluent (1:10); from this dilution take 20 μl to 180 μl assay diluent (1:100), and from this dilution take 70 μl to 150 μl assay diluent (1:316).
 - vi. 25 μl of appropriate sample dilution was added to pre-printed multiplex MSD microplate according to the plate diagram. After adding all samples to the plate, plate was sealed and incubated at 37°C in a plate incubator-shaker at 650 rpm for 1 h.
 - vii. Plate was washed 4 times with 150 μl /well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.
 - viii. 25 μl per well of diluted Biotin labeled secondary antibody was added to the plate. Plate was sealed and incubated at 37°C in a plate incubator-shaker at 650 rpm for 1 h.
 - ix. Plate was washed 4 times with 150 μl /well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.
 - x. 25 μl per well of diluted Sulfo-TagTM Streptavidin was added to the plate. Plate was sealed and incubated at 37°C in a plate incubator-shaker at 650 rpm for 1 h.
 - xi. Plate was washed 4 times with 150 μl /well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.
 - xii. 150 μl /well of 1X read buffer without any bubbles was added to the plate and plate read immediately.
 - xiii. Read plate using the MSD plate reader.

- xiv. Save the .txt files in appropriate study folder for further calculations.
- d. **Calculations:**
 The parallel line method (PLL) as described in the WHO HPV Labnet Manual (Grabowska et al., 2002; Labnet, 2009) was used to calculate antibody titers relative to the reference.
- e. **Interpretation of results:**
 Serostatus cut-off is established using children’s sera (n= 200). Antibody titer in a test sample below established sero-status cut-off value is negative and a value equal or above is considered positive.

HPV type	SeroStatus Cut-off value
HPV6	0.7 AU/ml
HPV11	0.8 AU/ml
HPV16	1.3 IU/ml
HPV18	2.9 IU/ml
HPV31	3.9 AU/ml
HPV33	5.7 AU/ml
HPV45	8.3 AU/ml
HPV52	2.3 AU/ml
HPV58	7.1 AU/ml

9. REPORTABLE RANGE OF RESULTS

- a. Final qualitative reports express results as positive or negative for the presence of HPV6, or HPV11, or HPV16, or HPV18 or HPV31, or HPV33, or HPV45, or HPV52 or HPV58 in the sample.
- b. Final quantitative reports express results as AU/mL (arbitrary units/mL) for HPV6, or HPV11, or HPV31, or HPV33, or HPV45, or HPV52 or HPV58 in the sample and in IU/mL (International units/mL) for HPV16, or HPV18.
- c. Below table shows the lower limits of quantitation for each HPV type. Antibody titers above this limit have total error (imprecision + bias) ≤ 40%

Limits of quantitation

HPV Type	Lower Limits of Quantitation (Units)
HPV6	0.2 AU/ml
HPV11	0.4 AU/ml
HPV16	1.0 IU/ml
HPV18	0.3 IU/ml
HPV31	1.6 AU/ml
HPV33	3.0 AU/ml
HPV45	2.3 AU/ml
HPV52	1.6 AU/ml
HPV58	2.2 AU/ml

Note: NHANES reports the lower limit of quantitation (LLOQ) values for this assay as LLOD

10. QUALITY CONTROL (QC) PROCEDURE

A reference sample, negative control, high and low controls were tested 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 reactivity.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

- a. Only about 60-70% of individuals seroconvert after natural exposure to HPV.
- b. Positive results indicate type-specific sero-conversion, but correlates of protection are not known.

13. REFERENCE RANGES (NORMAL VALUES)

An unvaccinated and unexposed person is negative for the presence of HPV6 or HPV11, or HPV16, or HPV18, or HPV31, or HPV33, or HPV45, or HPV52 or HPV58.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable

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

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 $\leq -70^{\circ}\text{C}$ for 1 year and then returned to NCHS serum bank.

19. SUMMARY STATISTICS AND QC GRAPHS

Not available

20. REFERENCES

Panicker G, Rajbhandari I, Pathak HN, Brady AM, Unger ER. Multiplex immunoassay to measure antibody response to nine HPV vaccine types. *Journal of Immunological Methods* 2021. <https://doi.org/10.1016/j.jim.2021.113136>

Grabowska K, Wang X, Jacobsson A, Dillner J. Evaluation of cost-precision ratios of different strategies for ELISA measurement of serum antibody levels. *J Immunol Methods*. 2002 Dec 20;271(1-2):1-15.

Labnet, W.H. 2009 Human Papillomavirus Laboratory Manual (WHO/IVB/10.12). In, http://whqlibdoc.who.int/hq/2010/WHO_IVB_10.12_eng.pdf?ua=1