Laboratory Procedure Manual

Analyte:	**Human Papillomavirus (HPV)**
Matrix:	**Vaginal Swab**
Method:	**Digene Hybrid Capture II**
Method No.:	
Revised:	
as performed by:	*Viral Exanthems and Herpes Virus Branch* *Division of Viral and Rickettsial Diseases* *National Center for Infectious Diseases*
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Important Information for Users

The National Center for Infectious Diseases/CDC 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 2003–2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
I37_c	LBXH2RL	Hybrid Capture high risk (RLU) result
	LBXH3RL	Hybrid Capture low risk (RLU) result

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Digene HPV Test using Hybrid Capture II technology is a nucleic acid hybridization microplate assay with signal amplification that utilizes chemiluminescent detection. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase-conjugated antibodies specific for the RNA:DNA hybrids and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid, resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted that is measured as relative light units (RLUs) on a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen.

The presence of certain HPV types in the female genital tract is associated with a number of diseases, including condyloma, Bowenoid papulosis, cervical, vaginal, and vulgar intraepithelial neoplasia and carcinoma. It is generally accepted that these viruses are predominantly sexually transmitted and that high-risk HPV types are a major recognized risk factor for development of cervical cancer.

Human papillomaviruses are composed of an icosahedral viral particle (virion) containing an 8000 base pair double-stranded circular DNA molecule surrounded by a protein capsid. Following infection of epithelial cells, the viral DNA becomes established throughout the entire thickness of the epithelium, but intact virions are found only in the upper layers of the tissue. Thus, viral DNA can be found either in virions or as episomal or integrated HPV sequences, depending upon the type and grade of lesion.

To date, HPV cannot be cultured *in vitro*, and immunological tests are inadequate to determine the presence of HPV cervical infection. Indirect evidence of anogenital HPV infection can be obtained through physical examination and by the presence of characteristic cellular changes associated with viral replication in Pap smear or biopsy specimens. Alternately, biopsies can be analyzed by nucleic acid hybridization to directly detect the presence of HPV DNA.

Historically, HPV-16 and HPV-18 have been regarded as high-risk cancer-associated HPVs, and HPV types -6 and -11 have been regarded as low-risk HPVs. HPV types 31, 33, and 35 subsequently have been demonstrated to have an intermediate association with cancer. Despite this useful conceptual framework, these seven HPV types have been found in only about 70% of cervical neoplasms. Additional HPVs, including types 42, 43, 44, 45, 51, 52, 56, 58, 59 and 68, have been identified as the principal HPVs detectable in the remaining lesions. These HPV types can also be categorized into low-, intermediate-, and high-risk groups based on their relative distribution in various histopathological diagnosis categories.

HPV DNA has been shown to be present in approximately 10% of women with normal cervical epithelium, but the actual prevalence in specific groups of women is strongly influenced by age and other demographic variables. Prospective studies have shown that 15–28% of HPV DNA-positive women developed squamous intraepithelial neoplasia (SIL) within 2 years, compared to only 1–3% of HPV DNA-negative women. In particular, the risk of progression for HPV types 16 and 18 was greater (approximately 40%) than for other HPV types.

2. SAFETY PRECAUTIONS

A. HANDLE ALL ASSAY SPECIMENS AND DISPOSED MATERIALS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

(1) Patient specimens should be handled at the BSL 2 safety level as recommended for any potentially infectious human serum or blood specimen in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 1984, pp. 12–16.

(2) Do not pipette by mouth.

(3) Wear disposable powder-free gloves while handling reagents or specimens. Wash hands thoroughly after performing the test.

(4) All materials used in this assay, including reagents and specimens, should be disposed of in a manner that will inactivate infectious agents.

(5) Solid Wastes: Autoclave.

(6) Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0% (1:5 dilution of household bleach). Allow 30 minutes for decontamination before disposal.

(7) SPILLS: Non-base-containing spills should be wiped thoroughly with a 5% sodium hypochlorite solution (full-strength household bleach). Base-containing spills should be neutralized, wiped dry, and then the spill areas should be wiped with a 5% sodium hypochlorite solution. The wiped area should be covered with absorbent material, saturated with a 5% sodium hypochlorite solution and allowed to stand for at least 10 minutes. A glass or plastic cover or tray can be used to reduce exposure to fumes. All wiping materials should be treated as hazardous waste.

B. Handling Precautions

(1) Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not failing within the established time and temperature ranges must be repeated.

(2) Do not use the reagents beyond the expiration date on the outer box label.

(3) The Digene HPV Test Procedure, Quality Control, and the Interpretation of Specimen Results must be followed closely to obtain reliable test results.

(4) It is important to pipette the exact reagent volume indicated and to mix well after each reagent addition. Failure to do so could result in erroneous test results. Ensuring that the noted color changes occur will help confirm that these conditions have been met.

(5) These components have been tested as a unit. Do not interchange components from other sources or from different lots.

(6) Nucleic acids are very sensitive to environmental nuclease degradation. Nucleases are present on human skin and on surfaces or materials handled by humans. Clean and cover work surfaces with disposable pads and wear powder-free gloves when performing all assay steps.

(7) Care should be taken to prevent contamination of the Capture Microplate and Detection Reagent 2 with exogenous alkaline phosphatase during performance of the assay. Substances which may contain alkaline phosphatase include Detection Reagent 1, bacteria, saliva, hair and oils from the skin. Covering the Capture Microplate after the wash step and during Detection Reagent 2 incubation is especially important, since exogenous alkaline phosphatase may react with Detection Reagent 2 producing false positive results.

(8) Protect Detection Reagent 2 from prolonged exposure to direct light. Use reagent immediately after aliquoting and avoid direct sunlight.

(9) Care should be taken to deliver the correct volumes of reagents to the reaction tubes and microplates at all steps and to mix well after each reagent addition. The repeating pipettor should be primed in advance of reagent delivery and checked for large air bubbles periodically. Excessive amounts of large air bubbles in the repeating pipettor tip may cause inaccurate delivery and can be avoided by filling the pipettor, dispensing all the liquid, and refilling. See pipettor instruction manuals for specific directions for use.

(10) Multichannel pipetting should be performed using the reverse pipetting technique for dispensing Detection Reagents I and 2. Check each pipette tip on the multichannel pipettor for proper fit and filling.

(11) Care should be taken during washing to ensure that each microwell is washed thoroughly. Inadequate washing will result in increased background and may cause false positive results. Residual wash buffer in wells may result in reduced signal or poor reproducibility.

C. Reagent Preparation and Storage:

(1) Upon receipt, store the kit at 2–8°C. The Wash Buffer Concentrate may be stored at 2–25°C, if desired.

(2) The kit may be used through the expiration date on the outer box label as long as prepared reagents are within their stated shelf life (see below).

(3) All reagents are ready-to-use except Denaturation Reagent, HPV Probes A and B, and Wash Buffer.

To test specimens for the presence of any of the 18 HPV types, a Combined Probe Cocktail Method (CPC) has been provided. To test using this option, a Combined Probe Cocktail must be prepared by mixing diluted Probe A Cocktail and diluted Probe B Cocktail together in advance of performing the Digene HPV Test. The Two-Probe Method uses separate Probe Cocktails A and B. See directions below.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

RLU values are captured by assay-specific software on a dedicated computer attached to the luminometer. These values are downloaded to an Excel file for data storage and subsequent file transport to WESTAT. In addition, printed copies of each assay, together with quality control calculations, are stored in notebooks.

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

A. Specimens may contain infectious agents and should be handled accordingly.

B. Self-collected vaginal swabs are shipped dry at room temperature. Usually two batches with identifying container numbers are received weekly. Samples are refrigerated at 4ºC upon receipt. The Hybrid Capture II Test is performed and results reported back to NHANES within three weeks.

C. For pre-test sample preparation, see separate protocol:

Protocol for DNA Extraction from NHANES IV Cervical/vaginal Swabs Using QIAmp Midi Kit (Catalog 51183)

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

Not applicable for this procedure.

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

A. Reagents and Materials

Provided in kit 5101-1096; Catalog Number 96 Tests (42 specimens):

(1) 1x 160 µl HPV Probe A: HPV 6/11/42/43/44 RNA probe cocktail in buffered solution (green cap).
(2) 1x 160 µl HPV Probe B: HPV 16/18/31/33/35/39/45/51/52/66/58/59/68 RNA probe cocktail in buffered solution (red cap).
(3) 1x 2.0 ml Negative Control: Carrier DNA (Herring Sperm) in Specimen Transport Medium (STM).
(4) 1x 1.0 ml Calibrator A: 1.0 pg/ml cloned HPV 11 DNA and carrier DNA in STM.
(5) 1x 1.0 ml Calibrator B: 1.0 pg/ml cloned HPV 16 DNA and carrier DNA in STM.
(6) 1 each Capture Microplate: Coated with goat polygonal anti-RNA:DNA hybrid antibodies.
(7) 1x 12 ml Detection Reagent 1: alkaline phosphatase-conjugated murine monoclonal antibodies to RNA:DNA hybrids in buffered solution.
(8) 1x 12 ml Detection Reagent 2: CDP-Star with Emerald II (chemiluminescent substrate).
(9) 1x 100 ml Wash Buffer Concentrate.
(10) 1x 0.35 ml Indicator Dye: Contains sodium azide.

(11) 1x 50 ml Denaturation Reagent: Dilute sodium hydroxide (NaOH) solution.
(12) 1x 4 ml Probe Diluent.

NOTE: Positive assay controls are not supplied with this kit, but control material is available from Digene.

B. Instrumentation

(1) DML 2000 Luminometer and PC System* or equivalent
(2) Rotary Shaker I with adjustable speed setting*
(3) Wash Apparatus*
(4) Specimen Collection Tube Rack (to fit specimen collection tubes)*
(5) EXPAND-4 Pipettor and stand (optional)*
(6) 65 ± 2°C water bath of sufficient size to hold 2 specimen racks (each rack 28 cm X (7) 12.8 cm X 7.6 cm)
(7) Vortex mixer with cup attachment
(8) Microplate Heater I
(9) Single channel micropipettor; variable settings for 20–200 µl volumes
(10) Repeating positive displacement pipettor such as Eppendorf or equivalent
(11) 8-Channel pipettor
(12) Timer

C. Other Materials

(1) Positive assay controls* (Digene HPV DNA Panel,, see “Quality Control" section for additional details)
(2) 1.6 ml Microcentrifuge tubes
(3) Microcentrifuge tube racks
(4) Polystyrene 96-well plates*
(5) Extra long pipette tips for removal of specimen*
(6) Screw caps*
(7) Digene Sample Conversion Kit * (for PreservCyt specimens)
(8) Disposable reagent reservoirs*
(9) Disposable bench cover, paper towels, powder-free gloves, lint-free tissues
(10) Sodium hypochlorite solution, 5% (or household bleach)
(11) Parafilm or equivalent
(12) Disposable aerosol-barrier pipette tips for single channel pipettor (20 to 200 µl)
(13) Disposable tips for Eppendorf repeating pipettor (25 and 500 µl)
(14) Disposable tips for 8-channel pipettor (25 to 200 µl)
(15) KayDry wipers (Kimberly Clark Corp., Roswell, GA)
(16) 5 ml and/or 15 ml snap cap round bottom polypropylene tubes

* These items are available from Digene. A complete list of equipment and specifications is available upon request.

D. Reagent Preparation

All reagents are ready-to-use except Denaturation Reagent, HPV Probes A and B, and Wash Buffer.

(1) Denaturation Reagent

Add 5 drops indicator dye to the bottle of denaturation reagent and mix thoroughly. The color should be dark purple.

Once prepared, the denaturation reagent is stable for 3 months when stored at 2–8°C. Label it with the new expiration date. If the color fades, add 1 or 2 drops of indicator dye and mix thoroughly before using.

(2) HPV Probe A Cocktail (Prepared from HPV Probe A and Probe Diluent reagents)

Probe Mix: Prepare during specimen denaturation.

IMPORTANT: SOMETIMES PROBE GETS TRAPPED IN THE VIAL LID.

NOTE: Extreme care should be taken at this step to prevent RNase contamination of probe and probe mix. Use aerosol-barrier pipette tips for pipetting probe. Probe diluent is viscous. Care should be taken to ensure thorough mixing when preparing HPV probes. A visible vortex must form in the liquid during the mixing step. Incomplete mixing may result in reduced signal.

(a) Centrifuge both probe vials briefly to bring liquid to bottom of vial. Tap gently to mix.

(b) Determine the amount of probe mix required. It is recommended that extra probe mix be made to account for the volume which may be lost in the pipette tips or on the side of the vial. Refer to suggested volumes listed below. The smallest number of wells recommended for each use is 24. If fewer than 24 wells per run are desired, the total number of tests per kit may be reduced due to limited probe and probe diluent volumes.

(c) Transfer the required amount of probe diluent to a new disposable container. Depending on the number of tests, either a 5-ml or 15-ml snap-cap, round bottom, polypropylene tube is recommended. Make a 1:25 dilution of each HPV Probe in probe diluent to prepare probe mix.

Table 1.

No. of Tests/Strips	Volume Probe Diluent	Volume Probe
48/6	2.0 ml	80.0 µl
24/3	1.0 ml	40.0 µl
Per well	0.045ml	1.8 µl

These values include the recommended extra volume.

(d) Pipette HPV Probe A into probe diluent by placing the pipette tip against the inner wall of the tube just above the meniscus and expelling the contents. Do not immerse the tip into probe diluent.

(e) Vortex for at least 5 seconds at maximum speed to mix thoroughly. A visible vortex must be produced. Label as “HPV Probe A Cocktail”.

(f) Unused Probe Mix should be discarded.

(g) HPV Probe B Cocktail

Prepare as HPV Probe A above. Label as “HPV Probe B Cocktail”.

(h) Combined Probe Cocktail (if using)

Prepare probe A and Probe B Cocktails as described above. Add the entire contents of diluted Probe A Cocktail to the tube of diluted Probe B Cocktail. Mix thoroughly by vortexing for at least 5 seconds at maximum speed. A visible vortex must be produced. Label as “Combined Probe Cocktail”.

(i) Wash Buffer: Prepare during capture step.

(j) Dilute 100 ml of wash buffer concentrate with 2.9 L of distilled or deionized water and mix well (final volume should be 3 L).

(k) Seal container to prevent contamination or evaporation.

Once prepared, the wash buffer is stable for three months at 2–25°C. Label it with the new expiration date. If wash buffer has been refrigerated, equilibrate to 20–25°C before using.

It is recommended that the wash apparatus and tubing be cleaned with bleach and rinsed thoroughly with deionized water once every three months to prevent possible contamination from alkaline phosphatase present in bacteria and molds.

(3) Detection Reagent 1 & Detection Reagent 2

Mix reagent thoroughly, then carefully measure the appropriate volume of detection reagent 1 (or reagent 2) into a clean reagent reservoir following the guidelines shown below. To avoid contamination, these reagents MUST NOT be returned to the original bottles: discard unused material after use. If an 8-channel pipettor is not being used, a repeating pipettor may be substituted. In this case, aliquots of the reagent should be made into an appropriately sized polypropylene tube.

Table 2.

No. of Tests/Strips	Volume Detection Reagent 1 and 2
96/12	contents of bottle
72/9	7.0 ml
48/6	5.0 ml
24/3	3.0 ml
1	0.125 ml

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration curve assay calibration verification is performed to ensure that the reagents and furnished calibrator material are functioning properly, permitting accurate determination of the assay cut-off value. The Digene HCII HPV Test requires calibration with each run; therefore, it is necessary to verify each run using the following criteria. This verification procedure is not intended as a substitute for internal quality control testing.

B. Verification

(1) Negative Control

The negative control must be run in triplicate with each test run. The negative control mean must be < 250 RLUs in order to proceed. The negative control results should show a coefficient of variation (%CV) of <25%. If the %CV is > 25%, discard the control value with a RLU value furthest from the mean as an outlier, and recalculate the mean using the remaining two control values. If the difference between the mean and each of the two values is < 25%, proceed to step 2; otherwise, the assay calibration verification is invalid and the test run must be repeated for all patient specimens. Accordingly, patient specimen results should not be reported.

(2) Calibrator

The calibrator(s) must be run in triplicate with each test run. The calibrator results should show a coefficient of variation (%CV) of < 15%. If the %CV is > 15%, discard the calibrator value with a RLU value furthest from the mean as an outlier, and recalculate the mean using the remaining two calibrator values. If the difference between the mean and each of the two values is < 15%, proceed to step 3; otherwise, the assay calibration verification is invalid and the test run must be repeated for all patient specimens. Accordingly, patient specimen results should not be reported.

The assay calibration verification described above for the negative control is performed automatically by the DML 2000 software and printed on the data analysis report. For the calibrator, however, the DML 2000 software will NOT invalidate the assay unless the %CV is > 25%. Therefore the user must manually verify that the %CV calculated by the Luminometer software is less than or equal to 15% and proceed as indicated for Situation 1 in the table below. If the %CV of the calibrator replicates falls between 15 and 25%, refer to the instructions in Situation 2 or 3 in the table below and proceed with the indicated "User Action".

Table 3.

Situation	Reported %CV for the Calibrator Replicates	Action Taken by Software	User Action
1.	< 15%	Assay reported as “Valid”	Results may be reported; no further action required
2.	Between 15% and 25%	No outliers removed and assay reported as “Valid”	Remove the calibrator RLU value farthest from the mean. Recalculate the %CV of the calibrator with the two remaining values. If the %CV of the two remaining RLU values is >15%, the assay is invalid. The results must not be reported. If the %CV of the two remaining RLU values is <15%, recalculate the assay cutoff, then recalculate the RLU/cutoff ratio for each specimen using this cutoff. These recalculated values may be reported.
3.	Between 15% and 25%	One outlier removed and assay reported as “Valid”	Assay is invalid, results must not be reported. Assay must be repeated
4.	> 25%	One outlier removed and assay reported as “Invalid”	Assay is invalid, results must not be reported. Assay must be repeated

To manually calculate the %CV as required in Situation 2 above, the user should divide the standard deviation of the two remaining replicate RLU values by the mean of the two remaining replicate RLU values (CA or CB) and multiply that result by 100.

To calculate the %CV using Microsoft Excel (supplied with the DML 2000 Luminometer), the user can calculate the standard deviation of the calibrator replicates using the formula “STDEV” and determine the mean RLU of the Calibrator using the formula “AVERAGE”. Once these two values are obtained, divide the STDEV by the AVERAGE and multiply the result by 100 to obtain the %CV.

(3) STDEV/AVERAGE

If there are any questions related to calculating %CV's, recalculating the assay cutoff, or recalculating the RLU/cutoff of the specimens, please call Digene Technical Services.

The calibrator mean (CAII or CBII) and negative control mean (NCII) results are used to calculate the CA /NCII or CBII /NCII ratio for each probe. These ratios must meet the following criteria to verify the assay calibration before the specimen results can be interpreted:

Table 4.

CPC METHOD	TWO-PROBE METHOD
Assay Calibration Verification Acceptable Ranges	Assay Calibration Verification Acceptable Ranges
CAII / NCII > 2.0 CB / NCII > 2.0	CAII /NCAOO > 2.0 CBII / NCBII > 2.0

Calculate the appropriate CAII / NCII or CBII / NCII ratios for each of the probe sets. If the ratios are > 2.0 for CPC or > 2.0 for the Two Probe Method, proceed to the next step. If any of the ratios are <2.0, the assay calibration is invalid for that specific probe and must be repeated. All patient specimens should be repeated within the run.

NOTE: Acceptable ranges for the negative control and calibrators have been established only for the DML 2000 Luminometer. To be acceptable for use with the Digene HPV Test, other luminometers must meet the assay calibration verification criteria described above. Absolute RLU values on these luminometers may be different from the DML 2000 Luminometer and must be established by the laboratory. The Digene HPV DNA Panel described in the “Quality Control” section below may be used to confirm that the luminometer will measure the Digene HPV Test results correctly.

C. Cutoff Calculation

Once an assay has been validated according to the criteria stated above, the cutoff values for determining positive specimens are as follows:

(1) Combined Probe Cocktail method:(CAII + CBII) 2

(2) Two-Probe method:

Probe A Cutoff = CAII
Probe B Cutoff = CBII

Table 5. Example Cutoff Calculation:

	NC RLU Values	CA RLU Values
	97 101 91	312 335 307
Mean Value	96	318
%CV	4.9	4.7
/NCII	NA	3.31

Therefore, Positive Cutoff Value for Probe A (CAII) = 318

All specimen RLU values should be converted into a ratio to the appropriate cutoff value. For example, all assays tested with HPV Probe A should be expressed as specimen RLU/cutoff Value A. The same can be done with specimens tested with HPV Probe B or the CPC Probe. These ratios should be recorded on the data sheet provided. An example of these calculations is provided in the data sheet. NOTE: RLU/CO values and positive/negative results for all specimens tested for each probe are reported in the DML 2000 data analysis report.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Set Up

(1) Remove the specimens and all required reagents from the refrigerator prior to beginning the assay. Allow them to reach 20–25°C for at least 15 to 30 minutes.

(2) Place controls, calibrators and specimens to be tested in a test tube rack, in the same order in which they will be tested. The negative controls and calibrators must be tested FIRST. Negative control (NC), calibrator A (CA), calibrator B (CB) and specimens should be run in an eight microwell column configuration. See example layout below for each probe type. The control/calibrator/specimen plate layout file can be created using the Digene Qualitative Software. See the DML 2000 user manual for more detail.

Table 6. Example layout for a run of 24 microwells:

Row	**Column**		
	1	2	3
A	NC	Spec. 3	Spec. 11
B	NC	Spec. 4	Spec. 12
C	NC	Spec. 5	Spec. 13
D	CA or CB	Spec. 6	Spec. 14
E	CA or CB	Spec. 7	Spec. 15
F	CA or CB	Spec. 8	Spec. 16
G	Spec. 1	Spec. 9	Spec. 17
H	Spec. 2	Spec. 10	Spec. 18

(3) NC and CA are tested in triplicate with HPV Probe A Cocktail on the left side of the microplate, and NC and CB are tested in triplicate with HPV Probe B Cocktail starting in column 7 on the right side of the microplate. The calibrator and control positions in the microplate are determined by the Digene software. See Digene DML 2000 User Manual for further details.

(4) If using the Combined Probe Cocktail method (CPC), NC, CA and CB are tested in triplicate with the combined probe cocktail in the same microplate. Use wells A1, B1 and C1 for the NC and wells D1, E1, F1, G1, H1 and A2 for CA and CB, respectively.

(5) If using the Two-Probe Method, NC and CA are tested in triplicate with HPV Probe A Cocktail on the left side of the microplate, and NC and CB are tested in triplicate with HPV Probe B Cocktail starting in column 7 on the right side of the microplate. The calibrator and control positions in the microplate are determined by the Digene software. See Digene DML 2000 User Manual for further details.

(6) Alternately two separate microplates can be used for controls, calibrators, and specimens tested with probe A and B. NC and CA are tested in triplicate with HPV Probe A Cocktail in one microplate, and NC and CB are tested in triplicate with HPV Probe B Cocktail in a second

microplate. Use wells A1, B1 and C1 for the NC and wells D1, E1 and F1 for CA or CB, respectively.

(7) Specimens may be tested once with the combined probe cocktail if using the CPC method or once with HPV Probe A Cocktail and once with HPV Probe B Cocktail if using the two-probe method.

(8) Sample preparation

For pre-test sample preparation, see separate protocol:

B. Instrument setup

Read the microplate on the DML 2000 luminometer immediately after 15 minutes of incubation (and no later than 30 minutes of incubation). (See DML 2000 Operator's Manual for details on entering specimen identification, measuring a plate and performing data reduction and analysis.)

C. Denaturation: Combined Probe Cocktail and Two-Probe Methods

NOTES:

Caution: Denaturation reagent is corrosive. Use care and wear powder-free gloves when removing tape seal from bottle and when handling.

Important: Some specimens may contain blood or other biological material which may mask the color changes upon addition of denaturation reagent and probe mix. Specimens who exhibit a dark color prior to the addition of denaturation reagent may not give the proper color changes at these steps. In these cases, failure to exhibit the proper color change will not affect the results of the assay.

Do not remove specimen collection device prior to denaturation.

During the denaturation and hybridization steps, be sure that the water level in the water bath is adequate to immerse the entire volume of specimen in the tube.

Calibrators, controls and specimens may be prepared up through the denaturation step and stored at 2–8°C overnight, or at –20°C for up to 3 months. A maximum of 4 freeze thaw cycles may be performed with a maximum of 2 hours at room temperature during each thaw cycle. Mix well before using.

To avoid false positive results, it is critical that all calibrator, control and specimen material come into contact with denaturation reagent. Mixing after denaturation Reagent addition is a critical step: make sure that each calibrator, control and specimen is mixed individually by vortexing each for at least 5 seconds at full speed such that the liquid vortex washes the entire inner surface of the tube.

Following denaturation and incubation, the specimens are no longer considered infectious.

(1) Remove and discard caps from calibrator, control and specimen tubes.

(2) Pipette denaturation reagent with indicator dye into each calibrator, control or specimen using a repeating or adjustable pipettor. Take care not to touch the sides of the tube or cross-contamination of specimens could occur. The volume of denaturation reagent needed is equivalent to half the sample volume. The exact volume for each type of calibrator, control and specimen is listed in the table below.

Table 7.

Calibrator, Control or Specimen	Vol. of Denaturation Reagent Required
Negative Control	1000 µl
Calibrator	500 µl*
Cervical Specimen	125 µl* (added the day before testing)-see extraction protocol.

*If using an Eppendorf repeating pipettor, use a 12.5-ml tip and a pipettor setting of 2.

(3) Recap the calibrator, control and specimen tubes with clean screw caps.

(4) Mix each tube thoroughly by vortexing individually, at high speed, for 5 seconds. There must be a visible vortex of liquid inside each tube during mixing such that the liquid washes the entire inner surface of the tube. The calibrators, controls and specimens should turn purple.

(5) Invert specimen tube one time to wash the inside of the tube, cap and rim.

(6) Return tube to rack.

Incubate in a 65 ± 2°C water bath for 4.5 ± 5 minutes (denatured calibrators, controls and specimens may be tested immediately, or stored as described in Notes above). Prepare CPC or HPV probe A and HPV Probe B Cocktails during this incubation. See **Reagent Preparation** section.

D. Hybridization: Combined Probe Cocktail and Two Probe Methods

NOTE: HPV probe mixes are viscous. Care should be taken to ensure thorough mixing and that the required amount is completely dispensed into each well. See **Reagent Preparation** section.

(1) Remove calibrators, controls and specimens from the water bath after incubation.

(2) Vortex each tube individually for at least 5 seconds.

(3) Pipette 75µl of each control, calibrator, or specimen into the bottom of a clear polystyrene 96-well plate following the template created under Setup. NOTE: False positive results can occur if sample aliquots are not carefully transferred. During sample transfer, DO NOT TOUCH PIPETTE TIP TO INSIDE OF TUBE WHEN REMOVING THE 75 µl.

(4) Cover the plate and allow standing at room temperature for 10 minutes to allow the samples to equilibrate to room temperature.

(5) Pipette 25 µl of appropriate probe mix from a disposable reagent reservoir into each well using an eight-channel pipettor. Use fresh tips for each row.

(6) Cover the plate with a plate lid. Shake the plate on the Hybrid Capture System Rotary Shaker I set at 1400 ± 100 rpm for 3 ± 2 minutes. The calibrators, controls and specimens should turn yellow after shaking. Wells that remain purple may not have received the proper amount of probe mix. Add an additional 25 µl of probe mix to samples that remain purple and shake again. If tubes remain purple after following this procedure, specimens should be retested.

NOTE: Some specimens may contain blood or other biological material, which may mask the color changes upon addition of denaturation reagent and probe mix. Specimens that exhibit a dark color prior to the addition of denaturation reagent may not give the proper color changes at these steps. In these cases, failure to exhibit the proper color change will not affect the results of the assay. Proper mixing can be verified by observing the color change of the controls and calibrators.

Incubate in a preheated Microplate Heater I at 65 + 2°C for 60 ± 5 minutes. Create a plate layout file using the Digene Qualitative Software v.2 during this incubation if this has not been completed earlier.

E. Hybrid Capture

(1) Remove all but the required number of Capture microplate wells for the run from the preassembled plate. Return the unused microwells to the original bag and reseal. With a marker, number each column 1, 2, 3... The samples will be added to the wells according to the example layout shown below and/or the template previously prepared under Preliminaries:

Table 8. Example layout for a run of 24 microwells.

Row	Column		
	1	2	3
A	NC	Spec. 3	Spec. 11
B	NC	Spec. 4	Spec. 12
C	NC	Spec. 5	Spec. 13
D	CA or CB	Spec. 6	Spec. 14
E	CA or CB	Spec. 7	Spec. 15
F	CA or CB	Spec. 8	Spec. 16
G	Spec. 1	Spec. 9	Spec. 17
H	Spec. 2	Spec. 10	Spec. 18

(2) Carefully remove plate containing calibrators, controls and specimens from the heater.

(3) Transfer the entire contents of the calibrators, control and specimen microtubes to the bottom of the corresponding Capture microwell using an 8-channel pipettor set at 100 µl. Use new pipette tips for each column transferred and allow each pipette tip to drain well to ensure complete sample transfer. If desired, the pipettor may be steadied by resting the middle of the pipette tips on the top edge of the microwells.

(4) Do not pipette vertically. Avoid backsplash.

(5) Do not allow tip to touch side of well.

(6) Cover microplate with a lid and shake on the Rotary Shaker at 1100 ± 100 rpm, at 20–25°C for 60 ± 5 minutes.

(7) Prepare Wash Buffer during this incubation. See Reagent Preparation.

(8) When the capture step is complete, remove the Capture microplate from the shaker and carefully remove the plate sealer. Remove the liquid from the wells by discarding into a sink: fully invert plate over sink and shake hard with a downward motion, being careful not to cause a back splash by decanting too closely to the bottom of the sink. Do not reinvert plate; blot by tapping firmly 2–3 times on clean Kay Dry absorbent paper. Ensure that all liquid is removed from the wells and the top of the plate is dry.

F. Hybrid Detection

NOTES:

1. Make additions across the plate in a left-to-right direction using an 8-channel pipettor.
2. It is recommended that the reverse pipetting technique be utilized to improve consistency of reagent delivery.
3. With this technique, the pipette tips are initially over-filled by using the second stop on the pipettors aspirate/dispense control (plunger). See procedure below. Wipe tips on reagent reservoir or on a clean lint-free pad to remove excess reagent before delivery to plate.
4. If desired, the pipettor may be steadied by resting the middle of the pipette tips on the top edge of the microwells. Take care not to touch the sides of the microwells or cross-contamination of specimens could occur. Refer to Diagram 1 shown earlier.

(1) Aliquot the appropriate volume of detection reagent 1 into a reagent reservoir (see Reagent Preparation Section for instructions). Carefully pipette 75 µl of detection reagent 1 into each well of the Capture microplate using an 8-channel pipettor and the reverse pipetting technique.

(a) Insert tips into 8-channel pipettor; ensure all tips are firmly seated.

(b) Push the plunger of the pipettor past the first stop to the second stop.

(c) Immerse tips into the DR1 solution.

(d) Release plunger and allow solution to fill the tips.

(e) Dispense solution into microwells (75 µl) by depressing plunger to the first stop. Do not release plunger until pipette tips have been reimmersed into the DR1 solution.

(f) Refill tips and repeat until all wells are filled. Fill well of microplate from left to right. Verify that all wells have been filled accurately by observing the intensity of the pink color. All wells should have similar intensity.

(2) Cover plates with clean Parafilm or equivalent and incubate at 20–25°C for 30 ± 3 minutes.

G. Washing

(1) Remove detection reagent 1 from the wells by placing clean KayDry absorbent paper on top of the plate and carefully inverting. Before inverting, ensure that the paper is in contact with the entire surface area of the plate. Allow the plate to drain for 1–2 minutes. Blot well on clean KayDrys. Carefully discard the used KayDrys to avoid alkaline phosphatase contamination of later steps.

(2) Using Digene's wash apparatus, hand wash the plate six times. Each well must be washed to overflowing to remove conjugate from the top of the wells. Washing begins at A1 and continues in a serpentine fashion to the right and downward. After all the wells have been filled, decant liquid into sink with a strong downward motion. The second wash is started at well H12, moving in a serpentine motion to the left and upward. This sequence of two washes is repeated two more times, for a total of six washes per well.

(3) After washing, blot the plate by inverting on clean KayDrys and tapping firmly 3 or 4 times. Replace the toweling and blot again. Leave plate inverted and allow to drain for 5 minutes. Blot the plate one more time.

H. Signal Amplification

NOTES

1. Use a clean pair of gloves for handling detection reagent 2.
2. Aliquot only the amount of reagent required to perform the assay into the reagent reservoir in order to avoid contamination of detection reagent 2. See Reagent Preparation Section. DO NOT return detection reagent 2 to the original bottle.-Discard unused material after use.
3. Detection reagent 2 additions should be made without interruption. The incubation time of all wells must be consistent.
4. Take care not to touch the sides of the microwell or splash reagent back onto tips because cross-contamination of specimens could occur.

(1) Carefully pipette 75 µl of detection reagent 2 into each well of the Capture microplate using an 8-channel pipettor and the reverse pipetting technique, as previously described. All microwells should turn a yellow color. Verify that all wells have been filled accurately by observing the intensity of the color. All wells should have similar intensity.

(2) Cover microplates with clean Parafilm or equivalent and incubate at 20–25°C for 15 minutes. Avoid direct sunlight.

(3) Read the microplate on the DML 2000 Luminometer or an equivalent plate luminometer immediately after 15 minutes of incubation (and no later than 30 minutes of incubation). (See

DML 2000 Operator's Manual for details on entering specimen identification, measuring a plate and performing data reduction and analysis.)

(4) If the DML 2000 Luminometer is used to read the results, assay specific software will allow the entry of pertinent run information directly into the spreadsheet. Alternatively, if assay verification and specimen results are calculated manually, record the lot number, date and operator name for each test run.

(5) If a full microplate was not used, remove used microwells from the microplate holder, rinse the holder thoroughly with deionized water, dry and reserve for next assay.

I. Recording of Data

The Digene software on the hc2-dedicated computer automatically calculates the RLU values and cutoff values for each run. The data is printed out and stored by run number and date in notebooks. The data are also transferred to another computer as Excel worksheets. The Excel files serve as the source of results sent to WESTAT for the hybrid capture 2 assay within 3 weeks of receipt of the sample.

J. Calculations

See verification section.

9. REPORTABLE RANGE OF RESULTS

A. Results: Interpretation of Specimen Results

(1) Specimens with RLU/Cutoff value ratios > 1.0 with the combined probe cocktail are considered positive for one or more of HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59 and 68.

(2) Specimens with RLU/Cutoff Value ratios > 1.0 with HPV probe A only are considered positive for one or more of HPV types 6, 11, 42, 43 or 44.

(3) Specimens with RLU/Cutoff Value ratios > 1.0 with HPV probe B only are considered positive for one or more of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.

(4) Specimens with RLU/Cutoff Value ratios > 1.0 for both HPV probe A and HPV probe B are considered positive for one or more HPV types from each group of probes.

(5) Specimens with RLU/Cutoff Value ratios < 1.0 for combined probe cocktail or both HPV Probe A and HPV Probe B are considered negative for the eighteen HPV types tested and should be reported as “No HPV DNA detected”. HPV DNA sequences are either absent or the HPV DNA levels are below the detection limit of the assay.

(6) If the RLU/C ratio of a specimen is close to but less than 1.0 and HPV infection is suspected, alternate testing methods and/or a repeat specimen should be considered.

Table 9. Interpretation of Result for HPV STD Screening:

HPV Result		**Interpretation**
Probe A	Probe B	
Negative	Negative	The person is not likely to be infected with HPV
Negative	Positive	The person is likely to be infected with HPV
Positive	Negative	
Positive	Positive	

10. QUALITY CONTROL (QC) PROCEDURES

A water blank is carried through the entire procedure, and tested with each run. Two and one-half percent of samples are retested and then compared with previous results. If any run has more than 10% of samples with RLU/cut off values between 1.0 and 5.0, the entire assay is repeated with a new kit.

Since quality control samples are not supplied with the Digene HPV Test kit, alternate control material is needed. To facilitate internal quality control testing, Digene offers an HPV DNA Panel (Catalog No. 5101-1024) consisting of six samples (one negative sample and five positive samples) which contain varying concentrations of HPV, ranging from low levels detectable around the assay cutoff to high levels of HPV. For the purpose of internal quality control, it is recommended that a negative sample (Target 1) and at least two positive samples be selected. The tested specimens should represent one low risk HPV type (Targets 2 or 5) and one high risk HPV type (Targets 3, 4, or 6). In order to monitor the ability of the Digene HPV Test to reproducibly detect HPV at levels around the assay cutoff, it is recommended that the low level controls (Targets 2 and 3) be tested. Testing of the intermediate and high level specimens (Targets 4- 6) will serve to monitor for substantial reagent failure only. It is recommended that the samples be tested during each user-defined run.

The expected results for each specimen included in the HPV DNA Panel (Targets 1–6) are summarized below.

Table 10.

Target No.	HPV Level	HPV Type	Expected Results (Target RLU Π /Cutoff Value)		
			Probe A	**Probe B**	**CPC**
1	Negative	None	<1	<1	<1
2	Low	Low risk (HPV 6)	>2	<1	>2
3	Low	High risk (HPV 16)	<1	>2	>2
4	Intermediate	High risk (HPV 18)	<1	>8	>8
5	High	Low risk (HPV 43)	>30	<1	>30
6	High	High risk (HPV 56)	<1	>80	>80

The mean of Negative Control results should be < 250 RLUs. If the mean of the Negative Control is > 250 RLUs, or if any of the Expected Results ratios listed above are not obtained, the assay is invalid and must be repeated.

The specimens contained in the HPV DNA Panel are cloned HPV DNA targets and not derived from wild-type HPV. This is the same material used for the calibrators supplied with the Digene HPV Test. Please confirm that different lots of the specimens are utilized for internal quality control testing, as recommended by NCCLS Document C24-A.

This control material will not act as an appropriate control for the specimen transport medium. Refer to the HPV DNA Panel package insert for additional information regarding these specimens.

The samples contained in the HPV DNA Panel may be used for internal quality control. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations. Refer to NCCLS C24-A for additional guidance on appropriate internal quality control testing practices.

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

If any quality control data are outside the acceptable range, the test must be repeated with a new kit.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. The Digene HPV Hybrid Capture II Test for human papillomavirus types is not recommended for evaluation of suspected sexual abuse.

B. Prevalence of HPV infection in a population may affect performance. Positive predictive values decrease when testing populations with low prevalence or individuals with no risk of infection.

C. A negative result does not exclude the possibility of HPV infection since very low levels of infection or sampling error may cause a false negative result.

D. The Digene HPV Test can only be used with vaginal specimens collected using the Digene vaginal sampler or Digene Specimen Transport Medium or vaginal specimens collected using a broom-type collection device and placed in Cytyc ThinPrep Pap Test PreservCyt Solution. Biopsy specimens may be assayed only if they are placed immediately in Digene Specimen Transport Medium and stored at –20°C until assayed.

E. The Digene HPV Test distinguishes between two groups of HPV types: HPVs 6/11142/43/44 and 16/18/31/33/35/39/45/51/52/56/58/59/68. It will not distinguish among the viral types within these groups

F. Cross-reactivity may be observed in the presence of HPV type 13, since both HCII HPV test probes cross-react with HPV 13. This cross reactivity is not considered to be clinically relevant for anogenital specimens, since HPV 13 is commonly detected in lip lesions of certain ethnic groups and rarely, if ever, detected in the anogenital tract.

G. A small amount of cross-hybridization between HPV types 6 and 42 (low risk HPV types) and the high risk Probe B group exists. Specimens with high levels (4 ng/ml or higher) of HPV 6 or HPV 42 DNA may be positive when tested with both probes. These patients may incorrectly be referred to colposcopy.

H. The Digene HPV Test is designed to detect high risk HPV types including 39, 58, 59, and 68. Analytical studies conducted by Digene, using cloned HPV plasmid DNA, demonstrate that the assay using Probe B detects these types at levels ranging from 0.82 pg/ml to 1.39 pg/ml. This is equivalent to the detection characteristics of the other HPV types targeted by the Digene HPV Test. Digene was able to validate the detection of these HPV types in only a limited number of clinical specimens. Due to the low prevalence of these types in the general population, the performance characteristics of the Digene HPV Test for the detection of HPV types 39, 58, 59, and 68 has not been statistically confirmed.

I. If high concentrations of anti-fungal cream, contraceptive jelly, or douche are present at the time a specimen is collected for HPV testing, there is a likelihood of obtaining a false-negative result should these specimens contain HPV DNA levels that yield RLU/CO values near the assay cutoff.

J. Cross reactivity between both HCII HPV test probes and the plasmid pBR322 is possible. The presence of pBR322 homologous sequences has been reported in human genital samples and false positive results could occur in the presence of high levels of bacterial plasmid.

13. REFERENCE RANGES (NORMAL VALUES) N/A

A negative result is considered normal for this type of testing.

14. CRITICAL CALL RESULTS (PANIC VALUES) N/A

There are no associated critical call results for this type of testing.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

All testing is done on a portion of the sample that has had DNA extracted from it. The DNA aliquot required for testing is removed from the tube, and the remainder is stored at –20°C.

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

None available.

Problematic assays are repeated with new test kits. If problem seems lot-specific, Digene is notified.

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

All results are reported electronically to WESTAT within three weeks of sample receipt.

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

All sample leftover from testing is stored at – 86°C. DNA aliquots are stored at –20°C. Sample logs with location data are kept. Samples will not be transferred to other individuals without approval from NHANES.

19. SUMMARY STATISTICS and QC GRAPHS

Qualitative assays are qualitative assays with a positive or negative result. The absorbance or reactivity values of specimens are compared with a RLU/cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

20. ACKNOWLEDGMENTS

Digene Corporation HPV DNA Test package insert. Catalog No. 5101-1096
Gaithersburg, MD.