

Laboratory Procedure Manual

Analyte: **High-risk human papillomavirus (HPV)**

Matrix: **Self-collected vaginal swabs**

Method: *digene* HC2 HPV DNA Test (Qiagen)

As performed by: Chronic Viral Diseases Branch
Division of High-Consequence Pathogens
and Pathology
National Center for Emerging and Zoonotic
Infectious Diseases
Centers for Disease Control and Prevention

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Important Information for Users

The HPV Laboratory of the Chronic Viral Diseases Branch/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 testing the items listed in the following table:

Data File Name	Variable Name	SAS Label
HPVSWR_F & HPVS_F_R	LBXH2RL	Digene HPV Swab High Risk

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This protocol describes procedures for DNA extraction and high-risk HPV detection from self-collected vaginal swabs.

DNA extraction is performed with a modified protocol and the commercial QIAamp kit (Qiagen, Valencia, CA). Because of the stability of DNA, extraction of cellular material dried on an inert matrix generally yields DNA of sufficient quality and quantity for PCR testing. The non-invasive subject-performed collection method is generally acceptable to participants. Room-temperature stability simplifies storage and transportation to the laboratory. Typical DNA extraction methods can be used, with modifications to accommodate re-hydration and removal of cellular material from the collection matrix. The protocol was developed for Catch-All™ Sample Collection Swabs (Epicentre, Madison WI) and includes a final concentration and wash of the extract using Ultracell spin columns. Modifications may be required for other types of swabs.

Presence of high-risk HPV in the extracted DNA is determined with the digene HC2 HPV DNA Test (Qiagen), a nucleic acid hybridization microplate assay with signal amplification that utilizes chemiluminescent detection. Specimens containing the target DNA hybridize with the high risk HPV RNA probe cocktail (whole genomic probes for 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). 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 measured as relative light units (RLUs) on a luminometer. Alkaline phosphatase is not inactivated by its substrate, allowing amplification of signal with increasing reaction time. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen. This qualitative in-vitro diagnostics test detects 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and also frequently detects HPV 66 because of cross-reaction. Results are reported as positive or negative. There is no internal control for sample adequacy. The test has been modified from the FDA approved format (sample type and extraction) and results cannot be used clinically (See also 12.0 Limitations of the Method).

2. SAFETY PRECAUTIONS:

Appropriate PPE must be worn throughout all lab procedures. General precautions must be followed as outlined in the CCID Safety Manual (CDC). All specimen handling (pre-lysis) is to be performed in a biosafety cabinet.

The *digene*HC2 wash buffer contains sodium azide which is toxic and the denaturation reagent contains sodium hydroxide which is corrosive. Follow procedure as outlined in the OIA Safety Manual and Chemical Hygiene Manual.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Final high-risk HPV results, dates and notes from DNA extraction process are recorded in a password protected database on a secure server. Original data from the digene HPV result files are archived. Additional observations and comments kit/reagent lot numbers, and name of operator is recorded on a worksheet accompanying the entire procedure. The results from the hybrid capture 2 assays are sent to the Westat ftp site (<ftp://ftp.westat.com/lab37/>) within three weeks of receipt of the sample.

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

NHANES MEC personnel explains the process for self-collection of vaginal samples to study participants and provide an individually packaged foam swab (Catch-All, Epicentre) in a transport sleeve. Vaginal and cervical exfoliated cells are collected by study participants by inserting the swab vaginally (similar to tampon insertion) and rotating as directed. The participants then place the swab in the sleeve and return it to NHANES personnel. The closed sleeve is kept at room temperature and sent to CDC at ambient temperature in weekly or bi-weekly shipment. At the testing laboratory the sample is kept at 4°C until extraction (within 3 weeks).

DNA extracts are stored temporarily at 4 °C or for long term storage, in -80°C freezer.

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

N/A

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

6.1 Supplies for Cell Lysis and DNA extraction

6.1.1 Equipment

- Eppendorf Microcentrifuge and rotor for 1.5ml tubes (or similar centrifuge reaching 20,000 x g)
- Biosafety cabinet level II
- Pipettes: 20µl, 200 µl, 1000 µl (Rainin, cat# L-20XLS, L-200XLS, and L-1000XLS)
- Vortex Tube Mixer
- Waterbath, temperature set at 56°C (Daigger, cat# EF8943E) or Bead bath (Lab Armor Beads from Fisher Scientific, cat# 50-550-333)
- Multi-dispense pipettor (Rainin, cat# AR-E1)

- Drummond Pipet-Aid or equivalent

6.1.2 Reagents and Media

Reagents at room temperature:

- Water, nuclease-free (Ambion, Cat. #AM9938)
- Alcohol molecular grade, 200 proof (Sigma, cat# E7023)
- Reagent Alcohol, 70%, in spray bottle (Fisher Scientific, cat# 2546-70-1)
- Physiological Saline (PS) (0.9% Sodium chloride, Fisher Scientific Cat. # LC23485-2 or similar)
- Reagents from QIAamp kit (below): AL, lysis buffer, AW1, wash buffer, AW2, wash buffer, AE, elution buffer
- Tris-HCl/EDTA (TE buffer), DNA suspension buffer, 10mM Tris-HCl, 0.1mM EDTA, (Teknova, Cat. #T0223)
- Sodium Hydroxide, 1.0N solution (Fisher Scientific, cat# AC12426-0010)
- Proteinase K (below)

Reagents at 4°C:

- Dissolved Proteinase K- refer to Reagent Preparation under Procedure

6.1.3 Supplies, Other Materials

- QIAamp Mini Kit (Qiagen, cat.# 51304 for 50 samples or #51306 for 250 samples)
- Amicon Ultra 0.5 Ultracel-100 membrane (Fisher Scientific Cat. No. UFC5100BK)
- Screw-cap polypropylene tubes, 5 ml (TTE Laboratories, Catalog # 580-GRDS)
- Tube racks for 5 ml tubes and 1.5 ml microcentrifuge tubes, heat resistant
- Sterile solution basins (VWR, Cat. No. 21007-972)
- Aerosol barrier pipette tips, 20µl, 200µl, and 1000µl (Rainin, cat. # RT-L10F, RT-L200F, and RT-L1000F)
- Autorep tips, sterile, 5.0ml and 12.5ml (Rainin, cat. #ENC-5MLS and ENC-12MLS)
- 50ml Falcon tubes (Fisher Scientific, cat. # 14-959-49A)
- Screw-Cap polypropylene storage tubes with O-ring, 1.5ml and 0.5ml (Daigger, cat. #EF4219G and EF4219C)
- 1.5ml tubes (Eppendorf, lock cap, Fisher Scientific, cat. #05-402-25)
- Kaydry towels (Kimberly-Clark, cat. #34721) or similar lab towels
- Biohazard wipers, 4"x 4" (Fisher Scientific, cat. #06-670-35)
- Absorbent bench pads (Fisher Scientific, cat. #15235101)
- Lab coat with ribbed knit cuffs (Daigger, cat. #EF1463)
- Gloves, latex or nitrile, powder-free (Fisher Scientific)
- Lab markers, waterproof

- Labels, Extreme temperature tolerant, (Tough-Tags and/or Tough-Spots, Diversified Biotech, cat. #TTLW-1000, T-Spots)
- Autoclavable discard pan

6.1.4 Controls

Nuclease-free water is used as a negative quality control for the DNA extraction procedure as well as PCR assays. The water is processed in the same manner as specimens used in the assay. At least one water controls is included in each DNA extraction. The control and specimens processed are tested by downstream assays.

6.2 Supplies for the *digene* HC2 HPV Test

6.2.1 Equipment

- Microcentrifuge Eppendorf 5415D
- DML 3000 Luminometer and PC System or equivalent with *digene* Hybrid Capture System Version 3 (DHCS v.3) software (Qiagen, cat. # 5000-00031)
- Automated Plate Washer I (Qiagen, cat. # 6000-00174)
- Water bath (65°C)
- Vortex mixer with cup attachment
- Microplate Heater I (Qiagen, cat. # 6000-1110U)
- Rotary Shaker I (Qiagen, cat# 6000-2110E) or equivalent Orbital Plate Shaker with variable speed setting and timer.
- Lumicheck Plate and Software User Package (Qiagen, cat# 6000-5013)
- Pipettes: 20µl, 200 µl, 1000 µl (Rainin, cat# L-20XLS, L-200XLS, and L-1000XLS) Multi-dispense pipettor (Rainin, cat# AR-E1)
- 8-Channel pipette, 200 µl (Rainin, cat# 17013805)

6.2.2 Reagents and Media

Reagents at room temperature:

- Physiologic saline

Reagents at 2-8°C:

- Digene HPV HR DNA Test Kit, cat. no. 5199-1220, Qiagen, Inc. Valencia, CA. includes:
 - Indicator Dye: Contains Sodium Azide
 - Denaturation Reagent: Dilute Sodium Hydroxide (NaOH) solution
 - Probe Diluent
 - High-Risk HPV Probe: HPV 16/18/31/33/35/39/45/51/52/66/58/59/68 RNA probe cocktail in buffered solution (red cap)
 - Negative Calibrator: Carrier DNA (Herring Sperm) in Specimen Transport Medium (STM)

High Risk Calibrator : 1.0 pg/ml cloned HPV 16 DNA and carrier DNA in STM
Low-risk HPV Quality Control: 5pg/ml cloned HPV 6 and carrier DNA in STM (serves as additional negative calibrator)
High-risk HPV Quality Control: 5pg/ml cloned HPV 16 and carrier DNA in STM
1 Capture Microplate: Coated with goat polyclonal anti-RNA:DNA hybrid antibodies
Detection Reagent 1: Alkaline phosphatase-conjugated murine monoclonal antibodies to RNA:DNA hybrids in buffered solution
Detection Reagent 2: CDP-Star with Emerald II (chemiluminescent substrate)
Wash Buffer Concentrate (contains 1.5% sodium azide)

6.2.3 Supplies, Other Materials

- Timer
- 1.6 ml Microcentrifuge tubes
- Microcentrifuge tube racks
- Microcentrifuge tube waterbath racks
- Wire Tube Rack for 15ml tubes
- Hybridization Microplates (Polystyrene 96-well plates) and lids (Fisher Scientific, cat. #07-200-694)
- Extra long pipette tips for removal of specimen
- Screw Caps (Qiagen, cat#5080-1000)
- Disposable reagent reservoirs
- Disposable bench cover, paper towels, powder-free gloves, lint-free tissues, Sodium hypochlorite solution, 5% (or household bleach)
- Disposable aerosol-barrier pipette tips for single channel pipette (20 to 200 µl)
- Disposable tips for Rainin pipette (12.5ml and 5ml)
- Disposable tips for 8-channel pipette (25 to 200 µl)
- Kaydry wipers (Kimberly Clark Corp., Roswell, GA)
- 5 ml or 15 ml snap/screw cap round bottom polypropylene tubes
- Labeling tape (Fisher Scientific, cat. # 15-950)
- Absorbent bench pads (Fisher Scientific, cat. #15235101)
- Lab coat with ribbed knit cuffs (Daigger, cat. #EF1463)
- Gloves, latex or nitrile, powder-free

6.2.4 Quality Control

Quality control specimens are supplied with the hc2 High-Risk HPV DNA Test. These controls must be included in each assay and the RLU/CO of each control must fall within the acceptable ranges for it to be considered valid.

Control	HPV Type	Expected Result (RLU/Cutoff Value) High-Risk HPV Probe		
		Minimum	Maximum	Average
QC1-LR	Low-Risk (HPV 6)	0.001	0.999	0.5
QC2-HR	High-Risk (HPV 16)	2	8	5.0

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Negative Calibrator:

The negative calibrator must be tested in triplicate with each assay. The negative calibrator mean must be ≥ 10 and ≤ 250 RLUs in order to proceed. The negative calibrator results should show a coefficient of variation (%CV) of $\leq 25\%$. If the %CV is $> 25\%$, discard the calibrator value with a RLU value farthest from the mean as an outlier, and recalculate the mean using the remaining two values. If the difference between the mean and each of the two values is $\leq 25\%$, proceed to next calibrator check. The assay is invalid if the %CV is $> 25\%$ after removing the outlier and the test will have to be repeated with new calibrators and controls.

High-Risk Calibrator:

The High-Risk calibrator (HRC) must be tested in triplicate with each assay. The calibrator result should show a coefficient of variation (%CV) of $\leq 15\%$. If the %CV is $\geq 15\%$, discard the calibrator value with a RLU value farthest 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 $\leq 15\%$, the assay is valid; otherwise, the assay calibration verification is invalid and the test must be repeated for all samples using new calibrators and controls.

The assay calibration verification described above for the calibrators is performed automatically by the Digene assay analysis software and printed in test result reports.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

8.1 DNA Extraction

Prepare NHANES – DNA Extraction worksheet with container ID and sample IDs from electronic shipping manifest. Record all reagent and kit lot numbers on sheet, along with the date of extraction. Any unusual observations of the specimens and issues is recorded on the worksheet.

Reagent Preparations:

Prepare Buffer AW1 and Buffer AW2, supplied as a concentrate in the QIAamp kit. Add 125ml 100% Ethanol to Buffer AW1 and 160 ml 100% Ethanol to Buffer AW2 buffers prior to use. Preheat water bath/bead bath to 56°C. For Ultracel preparations, create a 0.1N NaOH solution in 50 ml conical tube by making a 1:10 dilution of 1N NaOH stock solution.

Lysis Process:

1. Turn on biosafety cabinet blower, and clean working surface with 70% ethanol. Cover work surface with absorbent bench pads. Place the following items in the cabinet: a plastic bag-lined discard pan, tubes racks for 5ml tubes, biohazard wipers and self-collected, dry swab samples.
2. On the lab bench covered by an absorbing bench pad, remove from stock containers the total amount of each reagent (PS, buffer AL and Proteinase K) required to make the lysis buffer cocktail for the number of samples being processed and aliquot into a sterile solution basin prior to dispensing.
3. Label one 5 ml polypropylene tube per sample and dispense 460 µl physiological saline, 460 µl buffer AL, and 80 µl Proteinase K into each using multi-dispense pipettor and sterile 12.5ml tips. Alternatively, a master mix can be prepared from these three components prior to dispensation into the specimen tubes. After adding lysis mixture, place caps on the tubes to prevent possible cross-contamination. Transfer rack of prepared 5ml tubes to the biological safety cabinet.
Note: For every container set, process a blank as “negative control” that contains all reagents but no cellular material.
4. Take the cap off the first tube and place upside down on the absorbent pad. Use a biohazard wipe to grasp the handle of the swab from the collection sleeve.
Note: Newer versions of the Epicentre swab devices have a protective sleeve guard so a wipe may not be needed for grasping the swab handle. However, sleeve guards may not always grasp the swab handle and a wipe will be required for removal of swab from the protective sleeve.
5. Carefully, without allowing the sponge pad of the swab to touch gloves or any surfaces, insert the self-collected swab into the 5ml tube containing the lysis cocktail so that the sponge pad is completely submerged in the liquid.
6. Using a biohazard wiper to grasp the handle end of the swab, slightly lift the handle and carefully snap off the end of the handle by bending the plastic shaft over the edge of the tube, directed away from your body. The biohazard wiper will block the opening of the tube during this process. The swab should then be short enough to allow the screw cap to fit onto the tube. Discard the snapped-off portion of the swab handle and the biohazard wiper into the discard pan. Recap the tube, and move it to a separate rack for completed samples. Process samples in numerical order.

Note: In order to avoid cross-contamination, handle only one sample at a time. Change gloves if there is any suspicion that they have been contaminated. Check that all screw caps are tightly closed. Mix each tube by vortexing. Change gloves before removing rack of samples from the cabinet.

7. Place the rack of tubes in the 56°C waterbath or beadbath and incubate overnight (about 16 h).

Note: When using a water bath, place weights on tubes if tubes are slightly floating from rack. This will assure that the samples are thoroughly heated. The water level should be below cap level. Do not submerge the tubes underwater.

QIAamp Extraction Process:

8. For each sample, label a 1.5 ml screw-cap tube for storage and a 1.5 ml microcentrifuge tube for extraction. Using pipettor, add 500 µl 100% ethanol to each 1.5 ml “extraction” tube.
9. After incubation, remove the rack of tubes containing the swabs from the waterbath/beadbath, dry off excess water, and place on a clean absorbent bench pad on the lab bench. Allow the samples to equilibrate to room temperature for at least 15 minutes.
Note: After overnight lysis, the tubes containing swabs are okay to handle on lab benchtop.
10. Vortex each tube for at least 5 seconds. Remove the screw cap from the first sample and discard the cap. Using a 1000 µl pipette, transfer 500 µl of sample to the corresponding 1.5 ml “extraction” microcentrifuge tube. Transfer remaining sample to the 1.5 ml “storage” screw-cap tube. Close microcentrifuge and storage tubes. Discard the pipette tip and sample tube containing the swab into the autoclave pan. Repeat process for each sample. Change gloves if contamination is suspected. Place “storage” aliquots in -80°C freezer.
11. Label two QIAamp Mini spin columns placed in 2 ml collection tubes for each sample. Vortex each 1.5 ml “extraction” tube thoroughly. Transfer one-half of the DNA-ethanol mixture for each sample into each column using a 1000 µl pipette. Do not moisten the rim of the columns. Close the attached cap and discard pipette tip. Repeat for each sample. Centrifuge in Eppendorf 5415D at 8000 rpm (6000 xg) for 1 min.
Note: Always keep columns in an upright position in rack. All centrifugation steps should be carried out at room temperature (21°C-25°C.)
12. If solution has not entirely passed through the membrane, increase speed and repeat spin.
13. Discard the tubes containing filtrates. Place each spin column in a clean 2ml collection tube.

Note: Wipe off any spillage from the columns before inserting into fresh collection tubes.

14. Carefully, without moistening the rim, add 500 μ l buffer AW1 to each column. Replace caps and centrifuge at 8000 rpm (6000 rcf) for 1 min.
15. Discard the collection tubes containing filtrates. Place each spin column in a clean 2 ml collection tube. Carefully, without moistening the rim, add 500 μ l buffer AW2 to each column. Replace caps and centrifuge at 13,000 rpm (15,700 xg) for 3 min.
16. Discard the tubes containing filtrates. Place QIAamp spin columns in a new collection tube. Add 100 μ l buffer AE directly onto the membrane of each column, cap, and incubate at room temperature for 5 min. Centrifuge at 8000 rpm (6000 xg) for 1 min. Add another 100 μ l buffer AE and repeat incubation and centrifugation.

Note: Do not change collection tubes between centrifugations. Filtrates from both centrifugations will be in the same tube. Because sample was divided at beginning of extraction, each sample will have filtrate in two tubes.

Concentration and Purification of the Extracts:

17. Label one set of 0.5ml screw-cap tubes for storage of extracts and set aside. Label one 0.5 Ultracel-100 membrane for each sample.
18. Prepare each 0.5 Ultracel-100 membrane filter by adding 300 μ l of 0.1N NaOH into each column. Centrifuge at 14,000 x g for 30 minutes.
19. Remove filtrate from the collection tube and discard filtrate in autoclave pan. Replace the original 0.5 Ultracel-100 membrane filter in the collection tube.
20. Add 300 μ l of Nuclease-free water to the membrane filter. Centrifuge columns at 14,000 x g for 30 minutes.
21. Remove filtrate from the collection tube and discard water filtrate in autoclave pan. Replace the original 0.5 Ultracel-100 membrane filter in the collection tube.
22. Place both DNA elution filtrates from the QIAamp collection tubes of the corresponding sample into the Ultracel filter column. Centrifuge at 14,000 x g for 30 min.
23. Remove filtrate from the collection tube and discard filtrate in autoclave pan. Replace the original 0.5 Ultracel-100 membrane filter in the collection tube.
24. Add 300 μ l of nuclease free water to the filter. Centrifuge at 14,000 x g for 30 min.
25. Discard filtrate and repeat step 20. Discard collection tube containing the water wash filtrate into an autoclave discard pan.
26. Carefully invert the 0.5 Ultracel-100 membrane filter into a new collection tube. Centrifuge at 1000 x g for five min to collect the retentate (extracted DNA).
27. Bring extracts to 100 μ l with TE buffer and transfer the 100 μ l into a 0.5 ml O-ring screw-capped storage tubes. If not testing immediately, store extract tubes at -

20°C. An aliquot of the extract is used for HPV genotyping in Roche Linear Array (see laboratory methods – HPV Genotypes in Self-Collected Vaginal Swab).

8.2 High-risk HPV Detection (*digeneHC2* HR HPV)

Reagent Preparation:

Denaturation Reagent: Add 5 drops indicator dye to the bottle of Denaturation Reagent and mix thoroughly. The color should be dark purple.

Note: Once prepared, the Denaturation Reagent is stable for 3 months when stored at 2-8°C. Label it with a new expiration date. If the color fades, add 3 drops of Indicator Dye and mix thoroughly before using.

Probe Mix: Prepare during specimen denaturation step

- Centrifuge both probe vials briefly to bring liquid to bottom of vial. Tap gently to mix.
- Make a 1:25 dilution of HPV Probe in Probe Diluent to prepare Probe Mix. 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.

No. of Wells (Tests)/Strips	Volume Probe Diluent*	Volume Probe*
48/6	2.0 ml	80 ul
24/3	1.0 ml	40 ul
1 well	0.045 ml	1.8 ul

*These values include the recommended extra volume

- 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.

Wash Buffer: Prepare during capture step

- Dilute 100 ml Wash Buffer Concentrate with 2.9 L of distilled or deionized water and mix well (final volume should be 3 L).
- Seal container to prevent contamination or evaporation.

Note: Once prepared, the Wash Buffer is stable for three months at 2-25°C. If Wash Buffer has been refrigerated, equilibrate to 20-25°C before using.

Equipment Preparation

- DML 3000 Luminometer and computer – turn on before start of assay. Run lumicheck plate in luminometer following operating procedure and maintenance protocol for digene plate luminometer and DHCS v3.0 software. Lumicheck is designed to detect cross-talk between wells. Passing results from the lumicheck plate is critical prior to start of assay. Failing results requires additional maintenance on DML 3000 luminometer.
- Microplate Heater and Waterbath – turn on one hour prior to initial start of procedure

Procedure for Assay

1. Remove extracts from the refrigerator or freezer and allow them to reach 20-25°C for at least 15-30 minutes
2. In a clean 1.6 ml microcentrifuge tube, place 50 µl of extracted specimen with 200ul physiological saline solution and 125 µl of Denaturation Reagent.
3. Add 500 µl of Denaturation Reagent to the High-risk HPV Calibrator and Low-risk and High-risk HPV Quality Controls; add 1ml of Denaturation Reagent to the Negative Calibrator.
4. Vortex the Calibrators, controls, and specimens and place in waterbath at 65°C for 45 min. **Note:** *The procedure can be interrupted here. Specimens, calibrators and controls may be stored overnight at 4°C or at -20°C up to 3 months. If frozen, the calibrators and controls can withstand a maximum of 3 freeze/thaw cycles with a maximum of 2 hours at room temperature during each thaw cycle.*
5. Vortex the calibrators, controls, and specimens and add 75 µl of each control, calibrator, specimen to the bottom of the wells of a Hybridization Microplate using Extra-Long pipette tips (reference plate layout from the *digene* HC2 software for proper placement of each). Do not touch pipette tip to inside of tube when removing the 75 µl aliquot. Cover plate with lid and incubate for 10 minutes at 20-25°C.
6. Add probe-mix into a reagent reservoir and add 25 µl of probe mix to each well of the microplate using an 8-channel pipette and fresh tips for each row.
7. Cover plate with lid and place on the orbital shaker set at 1100 rpm for 2-3 min. Color will change from purple to yellow.
8. Add an additional 25 µl of Probe Mix to samples that remain purple and shake again. If wells remain purple results for that sample will be reported as invalid.
9. Place the hybridization microplate (covered) in the Microplate Heater I at 65°C for 1 hour.
10. After incubation, remove microplate from heater and transfer the contents from the Hybridization microplate to the Capture microplate (provided in kit), using an 8-channel pipette. Ensure all contents have been transferred. It is important to not let the pipette tip touch any interior surface of coated capture well during transfer.
11. Cover capture plate with plate lid securely with lab tape and shake capture plate on the orbital plate shaker at 1100 rpm for 60 min.

12. Decant the liquid from the microplate wells by fully inverting the plate over a sink. Shake hard in a downward motion being careful not to cause a backsplash by decanting too closely to the bottom of the sink. Keep plate inverted and blot firmly several times on clean Kaydry wipes until all liquid is removed from the wells. Do not blot over previously used areas of the wipes.
13. Pour Detection Reagent 1 (DR1) into a new reagent reservoir and pipette 75 μ l of DR1 into each well using an 8-channel pipette and the reverse pipetting technique.
14. Cover plate with lid and incubate at 20-25°C for 30-45 minutes.
15. Wash Capture microplate using the Automated Plate Washer I.
 - Verify that the Rinse Reservoir is filled with distilled or deionized water and that the Waste Reservoir is empty.
 - Make sure all caps are securely fastened. Remove the plate lid and place the microplate horizontally on the washer platform.
 - Select the number of rows to be washed by pressing the Rows key and then + or - to adjust the number of rows.
 - Press Start/Stop key to begin washing.
 - Change gloves immediately after washing the plate.
16. Pour the Detection Reagent 2 (DR2) into a new reagent reservoir. Pipette 75 μ l of the DR2 into each well using an 8-channel pipette and the reverse pipetting technique.
17. Cover the microplate with a plate lid and incubate at 20-25°C for 15 min in a dark area. Avoid direct sunlight.
18. During this incubation time turn on the computer and open the DHCS v 3.0 software. The main menu dialogue box opens, click on the "Plates" tab.
19. Click on "New" button to create a plate ID for each test run. "ID Entry" dialogue box opens and type in the plate identification under *Enter New Plate ID*. Click "OK" to close the window.
20. The "Create/Edit Layout" dialogue box opens automatically and a blank plate layout is displayed. Click on "Add New Assay" located on the bottom left screen. The "Select Protocols" dialogue box opens.
21. Select "High Risk HPV" by clicking once on the name, then click on "OK". The "Header Information" automatically opens.
22. On the "Header Information" dialogue box, select the kit lot using the drop-down list beside the label, *Kit Lot Number*. The expiration date for the selected kit lot number will automatically appear beside the *Expiration Date* label. To enter in new kit lot information, refer to procedure in manufacturer manual, HC2 HPV DNA Test User Guide.
23. In the box adjoining the label *Room Temperature*, type in the current room temperature of the lab. Click "OK" to close the header box. In comment box, record temperature of both Microplate Heater I and the waterbath used in procedure.

24. The “Create/Edit Layout” dialogue box opens and the calibrators and quality controls are automatically added on the plate layout. Click on “New Specimens”, located on the bottom center of the box, to enter the sample identifications.
25. The “New Specimens” dialogue box opens and there are three options to enter in sample ID’s. The first tab, “Single ID” allows the samples to be entered individually. Type in the identification in the *Specimen ID* box, and then click on “Add” to register each sample ID in the *Specimen ID List*. The second option for entering ID’s is through “Series of ID’s”. This option allows the samples be entered in form of a numerical series; best for doing replicate or triplicate testing of a sample. The last tab, “Import ID’s”, supplies the option to import the sample ID’s from a preexisting Excel spreadsheet.
26. After adding all specimen identifications in the *Specimen ID List*, select “Specimen List and Plate Layout” option located under the label “*When OK is pressed add new specimen to*”. This will automatically place the ID’s onto the blank plate layout. Click “OK” to close the dialogue box.
27. The “Create/Edit Layout” dialogue box is shown again with the specimens added to the plate layout. Click “OK” to save the plate layout. This will bring back the main menu.
28. Click on the “Plates” tab. The saved plate layout and the date it was created will now be listed under *Unmeasured Plates*. Click on “Measure” tab to run the plate on the luminometer.
29. The “Header Information” box opens again. Make sure all information is correct before clicking “OK” to proceed on to the “Measure” dialogue box. The plate id is shown in the “Measure” box located on the left hand side. Click on the “Measure” button.
30. Software will ask to run a mechanical test to test operating function of the luminometer. Click on “Yes”. When mechanical test is complete, a report will be displayed. This report can be printed or saved, then click on the “Close” button to proceed on.
31. A dialog box asking to insert plate into luminometer. Place microplate in the DML 3000 Instrument with notched corner of the plate in the back right of the plate carrier. In the same dialog box, The RCS serial number is requested. Do not input a number in this box unless running with the RCS automated instrument. Click “OK”. This begins the measurement of the plate.
32. After the measurement is completed, click “OK” to accept the results. The main menu returns with plates tab open. The measured plate will be shown in the center box named “measured plates”. If results are good, then click on “Accept Results”. If test is invalid repeat test and click on “Re-measure”. This will place the plate under the “unmeasured plate” box located on the left side of the plates tab. [NOTE: If a plate is invalid a second time, review instrument and assay troubleshoot guides before testing additional specimens. Specimens in failed plates will need to be re-tested, or reported as invalid if there is no residual material.] Accepted plates will be shown on the right side in the box named “accepted plates”. Export results and print

Qiagen results. Record name of operator, date of testing and kit/reagent lot numbers on *digene* hc2 Qualitative Column Report.

33. 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. For the NHANES study the Excel files serve as the source of results sent to the Westat ftp site (<ftp://ftp.westat.com/lab37/>).

9. REPORTABLE RANGE OF RESULTS

The RLU of the High Risk Calibrator is used to establish the threshold for a positive result – ratio of sample RLU to High Risk Calibrator RLU of 1.0 or greater are considered positive. Results are reported as positive or negative for the high risk probe mix.

10. QUALITY CONTROL (QC) PROCEDURES

The digene software must accept plate readings. If any run has more than 10% of samples with RLU/Cutoff values between 1.0 and 5.0, the entire assay is repeated with a new kit.

A water-blank is carried through the entire procedure, and tested with each run. If any negative control or quality controls do not fall within the acceptable range, the assay is invalid and must be repeated. Accordingly, no patient results should be reported for any invalid assay.

2.5% of samples are randomly selected for QC retesting, and extent of agreement is monitored (expected 100% agreement). HC2 results are also correlated with linear array results quarterly and extent of agreement is monitored.

Laboratory participates in CAP Proficiency testing for HR HPV HC2.

All observations and comments including date of testing, kit/reagent lot numbers, and name of operator should be recorded on the *digene* hc2 Qualitative Column Report. The technical supervisor reviews the results and signs the print copy of the *digene* hc2 Qualitative Column Report. Any incidences or problems are documented on the *digene* hc2 Qualitative Column Report and reported to the supervisor.

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

All samples on plates that were not accepted by software need to be repeated or reported as assay failure.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

- Detection of HPV is dependent on the number of viral genomes present in the sample and may be affected by sample collection methods, particularly by self-collected samples.

- The *digene* HC2 hrHPV Test detects DNA of the high-risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. HPV 66 frequently cross-reacts with this probe mix.
- A small amount of cross-hybridization between the LR-HPV types 6 and 42 with the High Risk HPV probe exists. Specimens with high levels (≥ 4 ng) of these types may be positive for the HR probe.
- A negative result does not exclude the possibility of HPV infection because very low levels of infection or sampling error may cause a false-negative result.
- The performance characteristics of the *digene* HC2 hrHPV Test have not been validated in connection with a self-collected vaginal swab and manual DNA extraction. Therefore the results of this test should not be used for the diagnosis, treatment, or assessment of patient health and management.

13. REFERENCE RANGES (NORMAL VALUES)

N/A

14. CRITICAL CALL RESULTS ("PANIC VALUES")

N/A

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

All specimens are stored in 4°C conditions up to two weeks during processing and testing unless specified differently by the procedure.

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

No alternative test method is available. In the event that the DNA extraction or the *digene* HC2 hrHPV Test fails the procedure can be repeated. Specimens are stable 2 weeks at 4°C but must be kept at -80°C for long-term storage.

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

HPV results are submitted to Westat electronically within 21 days of receipt of the specimen. Result files in the format of the NHANES shipping manifest are uploaded to the Westat ftp server. Unexpected delays are communicated to Westat.

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

Original biological specimens are collected at the NHANES Mobile Examination Clinics and shipped to the HPV laboratory at CDC via FedEx. At the HPV lab, all specimens and resulting DNA extracts are tracked via a LIMS system. DNA

extracts are also used for determination of HPV genotypes using Roche Linear Array assay (see Laboratory Methods – HPV Genotypes in Self-Collected Vaginal Swab).

19. Summary Statistics and QC graphs

N/A

20. REFERENCES

QIAamp DNA Mini Kit Handbook, Version Date February 2003. QIAGEN Corp.
HC2 HPV DNA Test User Guide, 2005 Digene Corp Gaithersburg MD.

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