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digene® HC2 HPV DNA Test Instructions for Use

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An in Vitro Nucleic Acid Hybridization Assay with Signal Amplification using Microplate Chemiluminescence for the Qualitative Detection of Human Papillomavirus (HPV) Types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59, and 68 and Analysis of HPV DNA Low- and High-Risk Groups in Cervical Specimens: HPV Types 6/11/42/43/44 and 16/18/31/33/35/39/45/51/52/56/58/59/68.

For use with:
digene HC2 DNA Collection Device
digene Specimen Transport Medium
Hologic® PreservCyt® Solution

For Professional Use Only, by trained and validated laboratory personnel.
Read these instructions carefully before using the test.

IVD

REF 5198-1220

QIAGEN, 19300 Germantown Road, Germantown, MD 20874, USA

R4 MAT 1059754

Sample to Insight

QIAGEN

Contents

Intended Use

The *digene*® Hybrid Capture® 2 (HC2) HPV DNA Test is an *in vitro* nucleic acid hybridization assay with signal amplification using microplate chemiluminescence for the qualitative detection of eighteen types of human papillomavirus (HPV) DNA in cervical specimens. The *digene* HC2 HPV DNA Test can differentiate between two HPV DNA groups: low-risk HPV types 6/11/42/43/44; and high/intermediate-risk HPV types 16/18/31/33/35/39/45/51/52/56/58/59/68, but cannot determine the specific HPV type present.

CAUTION 	Federal law restricts this device to sale by or on the order of a physician.

Specimens collected with the *digene* HC2 DNA Collection Device

Biopsies collected in *digene* Specimen Transport Medium (STM)

Specimens collected using a broom-type collection device and placed in HOLOGIC PreservCyt® Solution (refer to the *digene* HC2 Sample Conversion Kit instructions for use for complete details).

The use of this test is indicated:

- To aid in the diagnosis of sexually transmitted HPV infections with HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59, and 68.
- To screen patients with ASC-US (atypical squamous cells of undetermined significance) Pap smear results to determine the need for referral to colposcopy. The results of this test are not intended to prevent women from proceeding to colposcopy.

- In women with low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL) Pap smear results, prior to colposcopy, a *digene* HC2 HPV DNA Test result will aid the physician in patient management by assisting with risk assessment of women to determine absence of high-grade disease.

The *digene* HC2 HPV DNA Test is not intended for use as a screening device in the general population.

The *digene* HC2 HPV DNA Test is designed to augment existing methods for the detection of cervical disease and should be used in conjunction with clinical information derived from other diagnostic and screening tests, physical examinations and full medical history in accordance with appropriate patient management procedures.

digene HC2 HPV DNA Test results **should not** be used as the sole basis for clinical assessment and treatment of patients.

For high-volume sample-throughput testing, the *digene* HC2 HPV DNA Test may be performed using the Rapid Capture® System (RCS) Instrument Application. Only the High-Risk HPV Probe was approved for high-volume testing for HPV Types 16/18/31/33/35/39/45/51/52/56/58/59/68. Performance was not evaluated using the Low-Risk HPV Probe. Therefore, QIAGEN recommends using the *digene* HC2 High-Risk HPV DNA Test for testing with the Rapid Capture System.

Summary and Explanation

In women, human papillomaviruses (HPVs) can infect the cervix, vagina, vulva, urethra, or the area around the anus. More than 70 types of HPV have been identified, and are generally classified as high-risk or low-risk depending on their known association or lack of association with cancer and its precursor lesion, high-grade cervical intraepithelial neoplasia (CIN 2-3). 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 vulvar intraepithelial neoplasia and cancer.[1,2] 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.[2,3,4,5,6] Infection of the cervix with high-risk HPV types can be associated with cytological and histological changes that are detected by Pap screening, colposcopy, or biopsy. The natural history of how HPV infection progresses to cancer, however, is not completely understood. Low-risk HPV types 6 and 11 have been associated with the presence of genital warts, or condylomas, but have been linked infrequently with precancerous or cancerous cervical changes. There are many other low-risk HPV types that are not associated with genital warts or cervical cancer.[7,8]

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. Alternatively, 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 HPV types.[2,9,10] HPV types 31, 33, and 35 have been demonstrated to have an intermediate association with cancer.[2,11] This intermediate association is due to the fact that these types are more frequently detected in CIN 2-3 rather than in cancers. Therefore, cancers associated with the presence of these types are less common than cancers that are associated with high-risk HPV DNA types 16 and 18.[2,12] These five HPV types together account for about 80% of cervical cancers.[2,13,14] Additional high- and intermediate-risk HPV DNA types, including types 39, 45, 51, 52, 56, 58, 59 and 68, have been identified as the principal HPVs detectable in the remaining cancers.[2,14–20]

HPV infection is common in adults who have had more than one sexual partner (or a single partner who has had multiple partners) and can persist for years with no symptoms. Infection with some HPV types is an important risk factor for cervical cancer; however, most women with HPV infection do not develop cervical cancer or CIN 2-3 and infections regress. Most infections cause mild cytologic changes that resolve. 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.[2,13,21] Prospective studies (age 16–60 years) have shown that 15%–28% of HPV-DNA-positive women developed squamous intraepithelial lesions (SIL) suggestive of CIN 1-3 or cancer within 2 years compared to only 1%–3% of HPV-DNA-negative women.[4,22,23] In particular, the risk of progression for HPV types 16 and 18 was greater (approximately 40%) than for other HPV types.[4,6,10,23,24] Most SIL was low-grade.

Very few HPV-DNA-positive women develop cytologic HSIL indicating underlying CIN 2-3 or cancer.[25] The absolute risk of developing an incident cytologic abnormality following an HPV infection with types detected by the *digene* HC2 HPV DNA Test has not been adequately described and is known to vary in different populations.[6]

Although current scientific literature suggests that persistent infection with high-risk HPV is the main risk factor for development of high-grade cervical neoplasia and cancer[2,4,5,10,24,26–31], apparent persistence may represent continuous infection with a single HPV type, with multiple

HPV types, or reinfection. Nonetheless, women who are repeatedly Pap-negative and High-Risk-HPV-negative appear to be at low risk for having or developing cervical precancerous lesions.[5,24,32,33]

Principle of the Procedure

The *digene* HC2 HPV DNA Test using Hybrid Capture 2 technology is a nucleic acid hybridization assay with signal amplification that utilizes microplate 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.

An RLU measurement equal to or greater than the Cutoff Value (CO) indicates the presence of HPV DNA sequences in the specimen. An RLU measurement less than the Cutoff Value indicates the absence of the specific HPV DNA sequences tested or HPV DNA levels below the detection limit of the assay.

Reagents and Materials Provided

There are 96 tests in one *digene* HC2 HPV DNA Test kit (REF 5198-1220). The number of patient results will vary, depending on the number of uses per kit:

1 use = 40 patient results (Low-Risk) and 40 patient results (High-Risk) or 85 patient results (combined Low- and High-Risk using the combined-probe method)

2 uses = 32 patient results (Low-Risk) and 32 patient results (High-Risk) or 74 patient results (combined Low- and High-Risk using the combined-probe method)

1 x 0.35 ml	Indicator Dye Contains 0.05% w/v of sodium azide.
1 x 50 ml	Denaturation Reagent* Dilute sodium hydroxide (NaOH) solution.
1 x 5 ml	Probe Diluent* Buffered solution with 0.05% w/v sodium azide.
1 x 150 µl	Low-Risk HPV Probe HPV 6/11/42/43/44 RNA probe in buffered solution (green cap).
1 x 100 µl	High-Risk HPV Probe HPV 16/18/31/33/35/39/45/51/52/56/58/59/68 RNA probe in buffered solution (red cap).
1 x 1 ml	Low-Risk HPV Quality Control* 5 pg/ml (500,000 copies/ml) cloned HPV 6 DNA and carrier DNA in STM with 0.05% w/v sodium azide.
1 x 1 ml	High-Risk HPV Quality Control* 5 pg/ml (500,000 copies/ml) cloned HPV 16 DNA and carrier DNA in STM with 0.05% w/v sodium azide.
1 x 2.0 ml	Negative Calibrator* Carrier DNA in Specimen Transport Medium with 0.05% w/v of sodium azide.

1 x 1.0 ml Low-Risk HPV Calibrator*
1 pg/ml cloned HPV 11 DNA and carrier DNA in STM with 0.05% w/v of sodium azide.

1 x 1.0 ml High-Risk HPV Calibrator*
1 pg/ml cloned HPV 16 DNA and carrier DNA in STM with 0.05% w/v of sodium azide.

1 x 1 Capture Microplate
Coated with anti-RNA:DNA hybrid antibodies.

1 x 12 ml Detection Reagent 1
Alkaline phosphatase-conjugated antibodies to RNA:DNA hybrids in buffered solution with 0.05% w/v of sodium azide.

1 x 12 ml Detection Reagent 2
CDP-Star® with Emerald II (chemiluminescent substrate).

1 x 100 ml Wash Buffer Concentrate*
Contains 1.5% w/v sodium azide.

* See *Warnings and Precautions* section of these instructions for use for health and safety information.

Materials Required but not Supplied

Hybrid Capture System *In Vitro* Diagnostic Equipment and Accessories[A]

digene Hybrid Capture 2 System ("*digene* HC2 System"), consisting of a QIAGEN-approved luminometer ("luminometer"), QIAGEN-approved personal computer and computer peripherals (monitor, keyboard, mouse, printer, and printer cable), *digene* HC2 System Software ("*digene* assay analysis software"), *digene* HC2 System Assay Protocols for HPV, LumiCheck Plate Software, and *digene Hybrid Capture 2 System User Manual;* or the above-listed equipment with *digene* Qualitative Software version 1.3 or earlier ("*digene* assay analysis software") and *digene Qualitative Software User Manual*

Hybrid Capture System Rotary Shaker I	Empty Microplate Strips (available from Costar, Model #2581); optional for use with the Automated Plate Washer
Hybrid Capture System Microplate Heater I	Extra-Long Pipette Tips for removal of specimen
Hybrid Capture System Automated Plate Washer (optional)	Specimen Collection Tubes
Hybrid Capture System Multi-Specimen Tube (MST) Vortexer 2 (optional)	Specimen Collection Tube Rack
Conversion Rack and Rack lid (optional)	Specimen Tube Rack
digene Specimen Rack and Rack lid (optional)	Specimen Collection Tube Screw Caps
digene HC2 DNA Collection Device	Disposable Reagent Reservoirs
Tube Sealer Dispenser and cutting device (optional, used with the MST Vortexer 2)	DuraSeal® Tube Sealer Film
Rapid Capture System (optional for high-volume sample-throughput testing)[D]	Hybridization Microtubes[C]
Wash Apparatus	Microtube Racks[C]
Hybridization Microplate	Plate Sealers[C]
Microplate Lids	

General Laboratory Use Equipment and Accessories

65±2°C water bath of sufficient size to hold either one MST Vortexer 2 Rack (36 x 21 x 9 cm) or two specimen racks (each 31.7 x 15.2 x 6.4 cm)	Disposable aerosol-barrier Pipette Tips for single-channel pipettor (20 to 200 µl)
Microcentrifuge (optional for centrifuging probe vials to obtain maximum probe volume)	Disposable Tips for Eppendorf Repeater Pipette (25 and 500 µl)
Vortex mixer with cup attachment	Disposable Tips for eight-channel pipettor (25 to 200 µl)
Single-channel Micropipettor; variable settings for 20-200-µl volumes	Kimtowels® Wipers or equivalent low-lint paper towels
Repeating positive displacement Pipettor, such as Eppendorf® Repeater® Pipette or equivalent	Power Surge Protector
Eight-channel Pipettor: variable settings for 25-200-µl volumes	Disposable bench cover
Timer	Powder-free gloves
Sodium hypochlorite solution, 5% v/v (or household bleach)	5-ml and/or 15-ml snap-cap, round-bottom Polypropylene Tubes (for Probe dilution)
Parafilm® or equivalent[C]	Disposable 5-ml serological pipette or single-channel pipettor and tips capable of 1000-µl volume (for probe Diluent and PreservCyt Solution Specimen processing)

PreservCyt Solution Specimen Processing needed Equipment and Accessories

digene HC2 Sample Conversion Kit[A] (for PreservCyt Solution specimen processing)	Sarstedt® 10-ml Conical tubes with Caps (REF 62 9924-283) or VWR or Corning brand 15-ml conical-bottom polypropylene centrifuge tubes with caps ((for use with the Multi-Specimen Tube Vortexer 2 procedure)
Swinging Bucket Centrifuge capable of reaching 2900 ± 150 x *g* and holding 10-ml or 15-ml conical polypropylene centrifuge tubes	Disposable tips for Eppendorf Repeater Pipette (50 and 100 µl)

[A] Only equipment and accessories validated with *digene* HC2 HPV DNA Tests are available from QIAGEN.

[B] Custom item. Other custom expandable multi-channel pipettes can be used, provided tip spacing of 3.2 cm is achievable when expanded. Alternatively, a single-channel pipette capable of pipetting 75 µl may be used.

[C] These items are used for the water bath method only and are not required when the Microplate Heater I Hybridization method is used.

[D] Refer to Rapid Capture System User Manual for instructions specific to the use of that system for high-volume sample-throughput testing with this assay.

Warnings and Precautions

For *in vitro* diagnostic use.

Safety precautions

- HANDLE ALL ASSAY SPECIMENS AND DISPOSED MATERIALS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS. Patient specimens should be handled at the BSL 2 level as recommended for any potentially infectious human serum or blood specimen in the CDC-NIH manual, *Biosafety in Microbiological and Biomedical Laboratories*, 3rd Edition, 1993, pp. 10–13 and Clinical and Laboratory Standards Institute/NCCLS Approved Guideline M29-A, *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.*
- Do not pipette by mouth.
- Do not smoke, eat, or drink in areas where reagents or specimens are handled.
- Wear disposable powder-free gloves while handling reagents or specimens. Wash hands thoroughly after performing the test.

- All materials used in this assay, including reagents and specimens, should be disposed of in a manner that will inactivate infectious agents in accordance with national and local regulations.
  - Solid Wastes: Autoclave.
  - Liquid Wastes: Add sodium hypochlorite to a final concentration of 0.5% (1:10 dilution of household bleach). Allow 30 minutes for decontamination before disposal.[34,35]
- SPILLS: Clean and disinfect all spills of specimens using a tuberculocidal disinfectant such as 0.5% sodium hypochlorite solution (1:10 dilution of household bleach) or other suitable disinfectant. Base-containing spills should be neutralized, wiped dry, and then the spill areas should be wiped with a 0.5% sodium hypochlorite solution.
- The wiped area should be covered with absorbent material, saturated with a 0.5% 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.
- Treat all wiping materials as hazardous waste and dispose of in accordance with national and local regulations.

RCS Automated testing

Refer to the *Rapid Capture System User Manual* for additional Warnings and Precautions specific to the use of that system for high volume sample-throughput testing.

Safety and risk statements for components

The following risk and safety phrases apply to components of the *digene* HC2 HPV DNA Test kit:

Denaturation Reagent

Contains: sodium hydroxide. Danger! May be corrosive to metals. Causes severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

High-Risk HPV Calibrator

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

High-Risk HPV Quality Control

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Low-Risk HPV Calibrator

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Low-Risk HPV Quality Control

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Negative Calibrator

Warning! Causes mild skin irritation. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Probe Diluent

Contains: acetic acid; polyacrylic acid. Danger! Causes severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

Wash Buffer Concentrate

Contains: sodium azide. Warning! Harmful if swallowed. Harmful to aquatic life with long lasting effects. Wear protective gloves/ protective clothing/ eye protection/ face protection.

Further information

Safety Data Sheets: www.qiagen.com/safety

Handling precautions

- For *in vitro* diagnostic use.
- Performing the assay outside the established time and temperature ranges may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
- Do not use the reagents beyond the expiration date on the outer box label.
- The *digene* HC2 HPV DNA Test Procedure, Quality Controls, assay Calibration and Verification criteria, and the Interpretation of Specimen Results must be followed closely to obtain reliable test results.
- 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.

- These components have been tested as a unit. **Do not** interchange components from other sources or from different lots.
- 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.
- 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 that 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 because exogenous alkaline phosphatase may react with Detection Reagent 2 producing false-positive results.
- Protect Detection Reagent 2 from prolonged exposure to direct light. Use reagent immediately after aliquoting and avoid direct sunlight.
- 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.
- Multichannel pipetting should be performed using the reverse pipetting technique for dispensing Detection Reagents 1 and 2. Check each pipette tip on the multichannel pipettor for proper fit and filling. Refer to the manufacturer's multichannel pipettor User Manual.
- Care should be taken during washing to ensure that each microwell is washed thoroughly, as indicated in the Manual Washing instructions. 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.

- The cervical brush is for use with non-pregnant women only.
- Allow 60 minutes for the Hybrid Capture System Microplate Heater I to equilibrate to 65 ± 2°C from a cold start. Not allowing for this warm-up period could result in melting of the Hybridization Microplate. Consult *Microplate Heater I User Manual* for details.

Reagent Preparation and Storage

- Upon receipt, store the kit at 2°–8°C. The Wash Buffer Concentrate, Denaturation Reagent, and Indicator Dye may be stored at 15°–30°C, if desired.
- Do not use after the expiration date printed on the outer box label or the expiration date of the prepared reagents (see below).
- All reagents are provided ready-to-use except Denaturation Reagent, Low-Risk & High-Risk HPV Probes, and Wash Buffer.

To test specimens for the presence of any of the 18 HPV types, a Combined-Probe Cocktail (CPC) Method has been provided. To test using this option, a Combined-Probe Cocktail must be prepared by mixing diluted Low-Risk HPV Probe Cocktail and diluted High-Risk HPV Probe Cocktail together in advance of performing the *digene* HC2 HPV DNA Test. The Two-Probe Method uses separate Low-Risk and High-Risk HPV Probe Cocktails. See directions below.

Refer to *Rapid Capture System User Manual* for the preparation of the High-Risk HPV Probe Mix, the Wash Buffer, Detection Reagent 1, and Detection Reagent 2, as those instructions are specific to the use of that system for high-volume sample-throughput testing.

Reagent	Preparation Method
Denaturation Reagent	PREPARE FIRST: • Add five drops of Indicator Dye to the bottle of Denaturation Reagent and mix thoroughly. The Denaturation Reagent should be a uniform, dark-purple color. Once prepared, the Denaturation Reagent is stable for three months when stored at 2°–8°C. Label it with the new expiration date. If the color fades, add three drops of Indicator Dye and mix thoroughly before using. Warning: Denaturation Reagent is corrosive. Wear suitable protective clothing, gloves, eye/face protection. Use care when removing cap from bottle and when handling.
Low-Risk HPV Probe Cocktail (Prepared from Low-Risk HPV Probe and Probe Diluent reagents)	PREPARE DURING SPECIMEN DENATURATION INCUBATION: 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. • Centrifuge the vial of Low-Risk HPV Probe briefly to bring liquid to bottom of vial. Tap gently to mix. • Determine the amount of Probe Mix required (25 µl/test). It is recommended that extra Probe Mix be made to account for the volume that 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 assay are desired, the total number of tests per kit may be reduced due to limited Probe and Probe Diluent volumes.

<table>
<tr>
<td>Low-Risk HPV Probe Cocktail

(Prepared from Low-Risk HPV Probe and Probe Diluent reagents)</td>
<td>
• 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 Low-Risk HPV Probe in Probe Diluent to prepare Probe Mix.
<table>
<tr><th>No. of Tests/Strips</th><th>Volume Probe Diluent*</th><th>Volume Probe*</th></tr>
<tr><td>48/6</td><td>2.0 ml</td><td>80 µl</td></tr>
<tr><td>24/3</td><td>1.0 ml</td><td>40 µl</td></tr>
<tr><td>1 test</td><td>0.045 ml</td><td>1.8 µl</td></tr>
</table>
*These values include the recommended extra volume.

• Pipette Low-Risk HPV Probe 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.
• Vortex for at least 5 seconds at maximum speed to mix thoroughly. A visible vortex must be produced. Label as "LR HPV Probe Cocktail."

Unused Probe Mix should be discarded.
</td>
</tr>
<tr>
<td>High-Risk HPV Probe Cocktail

(Prepared from High-Risk HPV Probe and Probe Diluent reagents)</td>
<td>Substituting the High-Risk HPV Probe for the Low-Risk HPV Probe, preparation is the same as above.

Label as “HR HPV Probe Cocktail.”</td>
</tr>
<tr>
<td>Combined-Probe Cocktail

Note: For combined-probe cocktail method only. Do not prepare if two-probe method is to be used.</td>
<td>Prepare Low-Risk HPV Probe and High-Risk HPV Probe Cocktails as described above. Add the entire contents of diluted Low-Risk HPV Probe Cocktail to the tube of diluted High-Risk HPV Probe Cocktail. Mix thoroughly by vortexing for at least 5 seconds at maximum speed. A visible vortex must be produced. Label as “Combined-Probe Cocktail.”</td>
</tr>
</table>

Wash Buffer

PREPARE DURING CAPTURE STEP:

For the Hybrid Capture System Automated Plate Washer, the Wash Buffer can be prepared as described below and stored in a covered container, or prepare 1 L at a time and place in the Automated Plate Washer Reservoirs. See the table below for mixing volumes:

Warning: Wash Buffer Concentrate is toxic by ingestion. Wear suitable protective clothing, gloves, eye/face protection. To minimize exposure, add water to the Wash Buffer Concentrate when preparing.

Amount of Wash Buffer Concentrate	Amount of Distilled or Deionized Water	Final Volume of Wash Buffer
33.3 ml	966.7 ml	1 L
66.7 ml	1,933.3 ml	2 L
100.0 ml	2,900.0 ml	3 L

It is very important to always leave the power to the unit on at all times. This allows the maintenance rinse to be performed after eight hours of nonuse.

Prior to each assay, make sure the waste reservoir is empty and the rinse reservoir is filled with deionized water.

See *Automated Plate Washer User Manual* for additional care and maintenance instructions.

Alternative manual plate washing method:

- Mix Wash Buffer Concentrate well.
- Dilute 100 ml Wash Buffer Concentrate with 2.9 L of distilled or deionized water in Wash Apparatus and mix well (final volume should be 3 L).
- Seal the 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 distilled or deionized water once every three months to prevent possible contamination from alkaline phosphatase present in bacteria and molds.

Detection Reagent 1

&

Detection Reagent 2

IMMEDIATELY PRIOR TO USE:

Mix reagent thoroughly, then carefully measure the appropriate volume of Detection Reagent 1 (or Detection 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 eight-channel pipettor is not being used, an appropriate repeating pipettor may be substituted. In this case, aliquots of the reagent should be made into a polypropylene tube of sufficient size to hold the required volume as indicated below.

No. of Tests/Strips Volume Detection Reagent 1 and 2	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 test	0.125 ml

Specimen Collection and Handling

The types of cervical specimens recommended for use in the *digene* HC2 HPV DNA Test are listed below. Specimens taken with other sampling devices or transported in other transport media have not been qualified for use with this assay. The *digene* HC2 HPV DNA Test's performance characteristics with other specimen types and collection devices are unknown. Cervical specimens must be collected prior to the application of acetic acid or iodine if colposcopic examination is being performed. See the *digene* HC2 DNA Collection Device instructions for use for additional specimen collection and handling procedures.

Cervical brushes*

The *digene* HC2 HPV DNA Test is designed for use with specimens collected and transported using the *digene* HC2 DNA Collection Device (cervical brush and STM). Specimens may be held for up to two weeks at room temperature and shipped to the testing laboratory, after which specimens can be stored an additional week at 2°–8°C. If the assay will be performed more than three weeks from collection, specimens can be placed at -20°C for up to three months prior to testing. A preservative has been added to the STM to retard bacterial growth and to retain the integrity of DNA. It is **not intended** to preserve viability of organisms or cells. The *digene* HC2 DNA Collection Device should not be used for collection of specimens from pregnant women.

* **Note:** To prevent caps from popping off specimens that are shipped or stored frozen (for STM specimens or converted PreservCyt Solution specimens):

1. Cover caps with Parafilm or equivalent prior to shipping specimens previously frozen. Specimens may be shipped frozen or at 20°–25°C.
2. When removing specimens from the freezer for testing, replace caps immediately with specimen collection tube screw caps.

Time prior to testing	Storage duration	Storage temperature
3 weeks	Up to 2 weeks	Room Temperature
	Up to an additional week	2°–8°C
Greater than 3 weeks	Up to 3 months	-20°C

Specimens may be shipped without refrigeration to a testing laboratory; however, specimens should be shipped in an insulated container using either an overnight or 2-day delivery vendor.

Cervical biopsies*

Freshly collected cervical biopsies, 2–5 mm in cross-section, may also be analyzed with the *digene* HC2 HPV DNA Test. The biopsy specimen must be placed immediately into 1.0 ml of STM and stored frozen at -20°C. Biopsy specimens may be shipped at 2°–30°C for overnight delivery to the testing laboratory and stored at -20°C until processed. Biopsies less than 2 mm in diameter should not be used.

Specimens in Hologic PreservCyt solution

Specimens collected with a broom-type collection device and placed in PreservCyt Solution for use in making ThinPrep® Pap Test slides can be used in the *digene* HC2 HPV DNA Test. Specimens should be collected in the routine manner, and the ThinPrep Pap Test slides should be prepared according to HOLOGIC instructions.

* **Note:** To prevent caps from popping off specimens that are shipped or stored frozen (for STM specimens or converted PreservCyt Solution specimens):

1. Cover caps with Parafilm or equivalent prior to shipping specimens previously frozen. Specimens may be shipped frozen or at 20°–25°C.
2. When removing specimens from the freezer for testing, replace caps immediately with specimen collection tube screw caps.

There must be at least 4 ml of PreservCyt Solution remaining for the *digene* HC2 HPV DNA Test. Specimens with less than 4 ml after the ThinPrep Pap Test has been prepared may contain insufficient material and could be falsely negative with the *digene* HC2 HPV DNA Test.

PreservCyt Solution specimens may be held for up to three months at 2°–30°C, following collection and prior to processing for the *digene* HC2 HPV DNA Test. PreservCyt Solution specimens cannot be frozen. To process these specimens, refer to the *digene* HC2 Sample Conversion Kit instructions for use. For convenience, the specimen processing steps have also been included in the *Test Procedure* section below.

Test Procedure

Specimens may contain infectious agents and should be handled accordingly.

High-volume sample-throughput testing using the RCS

The Rapid Capture System is a general-use automated pipetting and dilution system that can be used with the *digene* HC2 HPV DNA Test (High-Risk HPV Probe only) for high-volume sample-throughput testing. This system handles up to 352 specimens in eight hours, including a 3.5-hour period during which user intervention is not required; up to 704 specimen results can be generated in 13 hours. Denaturation of the specimens in preparation for testing is performed independently of the RCS, in the primary collection tube, as performed for the manual method of the *digene* HC2 HPV DNA Test described below, prior to placing on the RCS platform. In addition, chemiluminescent signal detection and result reporting are performed using the offline luminometer system common to both the manual and RCS methods. Each of the *digene* HC2 HPV DNA Test's procedural steps is performed in the exact sequence as the manual test procedure. The RCS Application allows for the staggered processing of up to 4 microplates, each plate containing specimens and the required assay Calibrators and Quality Controls. Because the required accessories for the *digene* HC2 HPV DNA Test and procedural steps are the same, the assay can also be performed manually as described in the next section.

When using the Rapid Capture System, refer to *Rapid Capture System User Manual* provided with the instrument, in addition to these instructions for use, for necessary procedural and descriptive information. The RCS application was only approved using the High-Risk HPV Probe for HPV types 16/18/31/33/35/39/45/51/52/ 56/58/59 and 68.

Setting up

1. Allow 60 minutes for the HCS Microplate Heater I to equilibrate to 65 ± 2°C from a cold start. Consult *Microplate Heater I User Manual* for details. Confirm a water bath is at 65°C and the water level is high enough to immerse the entire specimen volume in the specimen tubes.
2. 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 15–30 minutes.

   Note: Prepare PreservCyt Solution specimens prior to equilibrating any previously denatured specimens and kit reagents to room temperature.
3. Create a plate layout file using the *digene* assay analysis software with *digene* assay protocols for HPV. See the applicable software User Manual for details.
4. Place Calibrators, Quality Controls, and specimens to be tested in a test tube rack, in the same order in which they will be tested. **The Negative, Low-Risk and High-Risk HPV Calibrators must be tested FIRST**. Negative Calibrator (NC), Low-Risk HPV Calibrator (LRC), High-Risk HPV Calibrator (HRC), Low-Risk HPV Quality Control (QC1-LR), High-Risk HPV Quality Control (QC2-HR), and specimens should be tested in an eight-microwell column configuration. See example layout below for each probe type.

Example layout for a test using 24 Microwells: Two-Probe method

Row	Column		
	1	2	3
A	NC	Spec. 1	Spec. 9
B	NC	Spec. 2	Spec. 10
C	NC	Spec. 3	Spec. 11
D	LRC or HRC	Spec. 4	Spec. 12
E	LRC or HRC	Spec. 5	Spec. 13
F	LRC or HRC	Spec. 6	Spec. 14
G	QC1-LR	Spec. 7	Spec. 15
H	QC2-HR	Spec. 8	Spec. 16

5. If using the Combined-Probe Cocktail Method (CPC), NC, LRC and HRC 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 LRC and HRC, respectively. Use wells B2 for QC1-LR and C2 for QC2-HR. The first specimen will be in well D2.
6. If using the Two-Probe Method, NC and LRC are tested in triplicate with Low-Risk HPV Probe Cocktail on the left side of the microplate, and NC and HRC are tested in triplicate with High-Risk HPV Probe Cocktail starting in column 7 on the right side of the microplate. *digene* assay analysis software determines the Calibrator and Quality Control positions in the microplate. See the applicable *digene* assay analysis software User Manual for proper Calibrator/Quality Control/specimen setup in the software. Alternately two separate microplates can be used for Calibrators, Controls, and specimens tested with Low-Risk and High-Risk HPV Probe. NC and LRC are tested in triplicate with Low-Risk HPV Probe Cocktail in one microplate, and NC and HRC are tested in triplicate with High-Risk HPV Probe Cocktail in a second microplate. Use wells A1, B1, and C1 for the NC and wells D1, E1, and F1 for LRC or HRC, respectively. Use wells G1 and H1 for the QC1-LR and QC2-HR, respectively, for each plate.
7. Specimens may be tested once using the Combined-Probe Cocktail method or twice, once each with Low-Risk HPV Probe Cocktail and High-Risk HPV Probe Cocktail using the Two-Probe method.

Denaturation: Combined-probe cocktail and two-probe methods

Notes:

- Caution: Denaturation Reagent is corrosive. Use care and wear powder-free gloves when handling.
- Important: Some cervical specimens may contain blood or other biological material, which may mask the color changes upon addition of Denaturation Reagent. Specimens that exhibit a dark color prior to the addition of Denaturation Reagent may not give the proper color changes at this step. In this case, failure to exhibit the proper color change will not affect the results of the assay.
- Do not remove *digene* HC2 specimen collection device prior to denaturation.
- During the denaturation step, be sure that the water level in the water bath is adequate to immerse the entire volume of specimen in the tube.
- Calibrators, Quality 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 three months. A maximum of three freeze/thaw cycles may be performed with a maximum of two hours at room temperature during each thaw cycle. Mix well before using.
- To avoid false-positive results, it is critical that all Calibrator, Quality Control, and specimen material come into contact with Denaturation Reagent. Mixing after Denaturation Reagent addition is a critical step. If using the Hybrid Capture System Multi-Specimen Tube (MST) Vortexer 2, make sure it is set to 100 (maximum speed) and a visible vortex of liquid is observed during mixing such that the liquid washes the entire inner surface of the tube. If performing manual vortexing, make sure that each Calibrator, Quality 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, followed by inverting the tube once.
- Following denaturation and incubation, the specimens are no longer considered infectious[26]. However, laboratory personnel should still adhere to practical universal precautions.

Calibrators, Quality Controls, and STM specimens (including biopsy or *digene* HC2 DNA Collection Device specimens)

Note: This procedure is not for preparation and denaturation of PreservCyt Solution specimens.

Procedure

1. Remove and discard caps from Calibrator, Quality Control, and specimen tubes.
2. Pipette Denaturation Reagent with Indicator Dye into each Calibrator, Quality Control or specimen using a repeating or adjustable pipettor. Take care not to touch the sides of the tube, as cross-contamination of specimens could occur. The volume of Denaturation Reagent needed is equivalent to half the specimen volume. The exact volume for each type of Calibrator, Quality Control and specimen is listed in the table below.

Calibrator, Quality Control or Specimen	Vol. of Denaturation Reagent Required
Negative Calibrator	1000 µl
Low-Risk or High-Risk HPV Calibrator	500 µl*
Low-Risk or High-Risk HPV Quality Controls	500 µl*
Cervical Specimen	500 µl*

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

Note: Dilute the remaining Denaturation Reagent in the bottle prior to disposing. Dispose of in accordance with local, state, and federal regulations.

3. Mix the specimens using one of the two methods described below:

Manual/Individual Tube Vortexing Method

3a. Recap the Calibrator, Quality Control, and specimen tubes with clean specimen collection tube screw caps.

3b. Mix each tube thoroughly by vortexing individually, at high speed, for 5 seconds.
3c. Invert the specimen tube one time to wash the inside of the tube, cap, and rim.
3d. Return the tube to the rack.
3e. Incubate in a 65 ± 2°C water bath for 45 ± 5 minutes (denatured Calibrators, Quality Controls and specimens may be tested immediately, or stored as described in **Notes** above). Prepare CPC or Low-Risk and High-Risk HPV Probe Cocktails during this incubation. See *Reagent Preparation and Storage* section.
3f. Remove rack from the water bath.
3g. Proceed to the *Hybridization* step below or see *Optional Stop Point* for storage and treatment of denatured specimens.

Multi-Specimen Tube (MST) Vortexer 2 Method

Note: QIAGEN specimens mixed using the MST Vortexer 2 **must** be hybridized using the hybridization microplate and Microplate Heater I method. See *MST Vortexer 2 User Manual* for further instructions, as needed.

3a. Cover the Calibrator/Quality Control/specimen tubes with DuraSeal Tube Sealer film by pulling the film over the tubes in the rack.
3b. Place the rack lid over the film-covered tubes and lock the lid into place with the two side clamps. Cut the film with the cutter device after the lid is securely fastened.
3c. Move the red-handled lever up so that it is in a horizontal position.
3d. Place the Conversion Rack and Lid on the MST Vortexer 2 so that the largest notched corner of the Conversion Rack is located in the right front corner. Position the rack and lid on the MST Vortexer 2 platform so that it fits securely within the guides. Secure the rack in place by moving the red-handled lever down to the vertical position. This will lock the rack in place.
3e. Verify that the speed setting is at 100 (maximum speed) and the Pulser button is in the OFF position.
3f. Turn the Vortexer power switch to the ON position. **Vortex the tubes for 10 seconds.**
3g. Turn the Vortexer power switch to the OFF position.

3h. Remove the Conversion Rack from the Vortexer by lifting up on the red-handled lever.

3i. Incubate in a 65 ± 2°C water bath for 45 ± 5 minutes (denatured Calibrators, Quality Controls and specimens may be tested immediately, or stored as described in **Notes** above). Prepare CPC or Low-Risk and High-Risk HPV Probe Cocktails during this incubation. See *Reagent Preparation and Storage* section.

3j. Remove the rack from the water bath, dry the rack, and secure on the vortexer.

3k. Turn the vortexer power switch to the ON position. Vortex the tubes for 5 seconds.

3l. Turn the vortexer power switch to the OFF position. Remove the rack.

3m. Immediately remove the rack lid and DuraSeal Tube Sealer Film from the specimens.

3n. Proceed to the *Hybridization* step below or see *Optional Stop Point* for storage and treatment of denatured specimens.

Independent of the vortexing method utilized, 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, Quality Controls, and specimens should turn purple.

PreservCyt solution specimen preparation and denaturation procedure

Notes:

- Consult the *digene* HC2 Sample Conversion Kit instructions for use for complete details.
- Processing a 4-ml aliquot of PreservCyt Solution produces enough for two tests, when tested manually. The minimum volume that can be processed is 4 ml.
- Prepare PreservCyt Solution specimens in batches of 36 or fewer; otherwise, pellets may become dislodged when decanting the supernatant. This is important for maintaining the integrity of the cell pellet during the decanting step. If preparing additional PreservCyt Solution vials, do not start to prepare them until after completing the preparation of the first batch (see the table below).

Reagent preparation

If not performed previously, prepare the Denaturation Reagent (DNR) from the *digene* HC2 Sample Conversion Kit by adding 3 drops of Indicator Dye to the bottle of DNR and mix well. The solution should be a uniform, dark-purple color.

Volume requirements are determined based on the number of replicates to be tested per specimen. When using the Two-Probe method, the low-risk results require one replicate and the high-risk result requires another replicate (i.e., two replicates per specimen).

Volume requirements: Reagent preparation

Number of Tests	PreservCyt Solution Volume	Conversion Buffer volume
1–2	4 ml	0.4 ml
3	6 ml	0.6 ml
4	8 ml	0.8 ml
5	10 ml	1.0 ml
6	12 ml	1.2 ml

1. Label the 10-ml conical Sarstedt tube or a 15-ml VWR or Corning brand conical tube with the appropriate specimen identification number.
2. Handling one specimen at a time:
   2a. Shake the PreservCyt vial vigorously by hand or by vortexing each vial individually using a vortex mixer at maximum speed setting for approximately 5 – 10 seconds to resuspend cells and ensure homogeneity.
   2b. Immediately, as cells settle very quickly, pipette the appropriate volume of the PreservCyt specimen into the labeled tube. Deliver the PreservCyt Solution to the bottom of the conical tube to minimize cellular material adhering to the inside of the tube.
3. Add the appropriate volume of Sample Conversion Buffer to each tube (see the table *Volume Requirements: Reagent Preparation* above).
4. Recap and mix the contents of each tube thoroughly by using a vortex mixer with cup attachment.
5. Centrifuge the tubes in a swinging bucket rotor at 2,900 ± 150 x *g* for 15 ± 2 minutes.
6. During centrifugation, prepare the *digene* Specimen Transport Medium (STM) + Denaturation Reagent (DNR) mixture in a 2:1 ratio, according to the table *Volume Requirements, STM/DNR* table below.

   Note: Solution must be prepared fresh each day the test is being performed.

6a. To determine the total volume of STM/DNR mixture required, use the starting volume of the PreservCyt Solution specimen as a guide and then multiply the STM and DNR "per tube" volumes by the number of specimens to be processed (see the table *Volume Requirements, STM/DNR* below).

Volume requirements: STM/DNR

No. of Tests	PreservCyt Solution Volume	STM Volume per tube for final STM+DNR Mixture*	DNR Volume per tube for final STM+DNR Mixture*	STM+DNR Mixture added to tube
1–2	4 ml	120 µl	60 µl	150 µl
3	6 ml	170 µl	85 µl	225 µl
4	8 ml	220 µl	110 µl	300 µl
5	10 ml	270 µl	135 µl	375 µl
6	12 ml	320 µl	160 µl	450 µl

* The volumes listed in these columns should not be added directly to the specimen tube.

6b. Mix the solution thoroughly by vortexing.

7. Remove tubes from the centrifuge one tube at a time and place into a Rack or MST Vortexer 2 Conversion Rack. A pink/orange pellet should be present in the bottom of each tube.

8. Handling each tube individually:

8a. Remove the cap and set aside on a clean low-lint paper towel.

8b. Carefully decant supernatant.

8c. Maintain the inverted tube position and gently blot (approximately six times) on absorbent low-lint paper towels to remove the excess liquid. Use a clean area of the towel each time. **Do not** allow the cell pellet to slide down the tube during blotting.

8d. Place the tube in a Rack or the MST Vortexer 2 Conversion Rack.

Notes:

- Do not blot in the same area of the absorbent low-lint paper towel.
- It is important to remove the maximum amount of PreservCyt Solution by blotting. However, it is normal to see residual PreservCyt Solution after blotting.

Manual/Individual Tube Vortexing Method

1. Add the appropriate volume of *digene* Specimen Transport Medium + Denaturation Reagent to each pellet. Resuspend each pellet by vortexing each tube individually for at least 30 seconds at the highest speed setting. If a pellet is difficult to resuspend, vortex for an additional 10–30 seconds or until the pellet floats loose from the bottom of the tube. If a pellet remains undissolved after additional vortexing (a total of 2 minutes maximum), note the specimen identification and proceed to the next step.
2. Place tubes in 65 ± 2°C water bath for 15 ± 2 minutes. Ensure that the water level is sufficient to cover all liquid in the tubes.
3. Remove the rack with specimens from the water bath and vortex specimens individually for about 15–30 seconds.

   Note: Make sure that all pellets are completely resuspended at this point. Specimens that still have visible pellets are not acceptable for testing.
4. Return the rack to the 65 ± 2°C water bath and continue denaturation for another 30 ± 3 minutes.
5. Proceed to the *Hybridization* step below or see *Optional Stop Point* for storage and treatment of denatured specimens.

Multi-Specimen Tube (MST) Vortexer 2 Method

Notes:

- The MST Vortexer 2 Method is validated for the processing of PreservCyt Solution specimens following centrifugation and decanting of the supernatant.
- The MST Vortexer 2 procedure has not been validated for vortexing PreservCyt Solution specimens with Sample Conversion Buffer prior to centrifugation.
- Only the MST Vortexer 2 is designed for PreservCyt Solution specimen processing.
- The Conversion Rack and Lid are specifically designed to accommodate VWR or Corning brand 15-ml conical tubes. The user should use only one tube type on the Conversion Rack at a time. Other brands are not validated for use.
- Strict adherence to the specified vortexing times of the Conversion Rack and Lid is required.
- The Conversion Rack and Lid cannot be used to vortex the *digene* HC2 DNA Test kit Calibrators or Quality Controls. The height of the STM tubes prevents adequate vortexing using the Conversion Rack.

Procedure

1. After blotting each labeled 15-ml conical tube, place each in its proper position in the Conversion Rack.
2. Add 150 µl *digene* Specimen Transport Medium + Denaturation Reagent mixture to each pellet.
3. Cover the 15-ml conical tubes with DuraSeal Tube Sealer Film by pulling the film over the tubes in the rack.
   3a. Place the rack lid over the film-covered tubes and lock the lid into place with the two side clamps. Cut the film with the cutter device after the lid is securely fastened.
4. Move the red-handled lever up so that it is in a horizontal position.

5. Place the Conversion Rack and Lid on the MST Vortexer 2 so that the largest notched corner of the Conversion Rack is located in the right front corner. Position the rack and lid on the MST Vortexer 2 platform so that it fits securely within the guides. Secure the rack in place by moving the red-handled lever down to the vertical position. This will lock the rack in place.
6. Verify that the speed setting is at 100 (maximum speed) and the Pulser button is in the OFF position.
7. Turn the Vortexer power switch to the ON position. Vortex the tubes for 30 seconds.
8. Turn the Vortexer power switch to the OFF position.
9. Remove the Conversion Rack from the Vortexer by lifting up on the red-handled lever.
10. Place the rack in the 65 ± 2°C water bath for 15 ± 2 minutes. Be sure the water level completely covers all liquid in all of the tubes.
11. After the 15-minute incubation, remove the rack with specimens from the water bath.
12. To prevent splashing, dry the rack of excess water prior to placing it on the MST Vortexer 2.
13. Secure the Conversion Rack on the MST Vortexer 2 as described in *Step 6*.
14. Verify speed setting is at 100, and turn the vortexer power switch to the ON position. Vortex the tubes for 1 minute.
15. Turn the power switch to the OFF position.
16. Return the rack to the 65 ± 2°C water bath, and continue denaturation for 30 ± 3 minutes.
17. Remove the rack from the water bath, dry the rack, and secure on the vortexer.
18. Turn the Vortexer power switch to the ON position. Vortex for 10 seconds at the maximum setting.
19. Turn the Vortexer power switch to the OFF position. Remove the rack.
20. Immediately remove the rack lid and DuraSeal Tube Sealer Film from the specimens.
21. Proceed to the *Hybridization* step below or see *Optional Stop Point* for storage and treatment of denatured specimens.

Optional stop point

After denaturation, specimens may be stored at 2°–8°C overnight or at -20°C for up to three months. For overnight refrigeration, specimens may be left in the Conversion Rack with new DuraSeal film and Rack Lid replaced. Prior to storage at -20°C, the Rack Lid and DuraSeal film must be removed, and caps placed on the tubes. If the manual vortex procedure was used, place the rack of capped tubes in the desired storage temperature. In either case, the specimens must be equilibrated to room temperature (20°–25°C) and thoroughly vortexed before proceeding to the Hybridization step.

Note: Do not store or ship denatured specimens on dry ice.

A maximum of three freeze/thaw cycles may be performed with a maximum of 2 hours at room temperature during each thaw cycle. For specimens processed using the MST Vortexer 2, remove the Conversion Rack Lid and DuraSeal Tube Sealer Film from the 15-ml conical tubes, and cap each tube before storing specimens at -20°C.

Hybridization

Hybridization: Combined-Probe Cocktail (CPC) and Dual-Probe Methods

Notes: HPV Probe Mixes are viscous. Care should be taken to ensure thorough mixing and that the required amount is completely dispensed into each microplate well. See *Reagent Preparation and Storage* section.

Important: Some cervical specimens may contain blood or other biological material that may mask the color changes upon addition of Probe Mix. Specimens that exhibit a dark color prior to the addition of Denaturation Reagent may not give the proper color change at this step. 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 Calibrators and Quality Controls.

Hybridization Method using Hybridization Plate and Microplate Heater I

Notes:

- Specimens collected in STM with the digene HC2 DNA Collection Device and processed using the MST Vortexer 2 method can be hybridized utilizing the Microplate Heater I method only.
- If the denatured specimen has been frozen or refrigerated, equilibrate to 20°–25°C and vortex on the maximum speed setting for 5–10 seconds if using the MST Vortexer 2 with PreservCyt Solution specimens.
- Preheat the Microplate Heater I to 65 ± 2°C for 60 minutes prior to use. See Microplate Heater I User Manual for further instructions, as needed.

1. Obtain and label a Hybridization Microplate.
2. Pipette 75 µl of each vortexed Calibrator, Quality Control, or specimen into the bottom of empty hybridization microplate wells following the plate layout created under *Setup*. Avoid touching the sides of the wells and limit formation of air bubbles. Use a clean

extra-long pipette tip for each transfer to avoid cross-contamination of Calibrators, Quality Controls, or specimens. It is not necessary to remove the specimen collection device from the specimen transport tube. Denatured specimens may be capped with specimen collection tube screw caps and stored at -20°C with specimen collection devices remaining in the tubes.

Note: False-positive results can occur if specimen aliquots are not carefully transferred. During transfer of specimen, do not touch pipette tip to inside of tube when removing the 75-µl aliquot.

3. After transferring the last specimen, cover with Parafilm or a plastic lid and incubate the hybridization microplate for 10 minutes at 20°–25°C.
4. Aliquot the prepared and thoroughly vortexed Probe Mix into a disposable reagent reservoir. Carefully pipette 25 µl of the Probe Mix into each well of the Hybridization Microplate using an eight-channel pipettor and fresh tips for each row. Dispense the volume of probe into each hybridization well, preventing back splashing. Avoid touching the sides of the wells.

   Note: For the above step, use an eight-channel pipettor that is equipped with 25–200 µl tips and that is capable of delivering 25–75 µl. For a small number of wells, use a single-channel pipettor (equipped with 25–200 µl tips) in place of an eight-channel pipettor.
5. Cover the Hybridization Microplate with a plate lid and shake on Hybrid Capture System Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. The Calibrators, Quality 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 specimens that remain purple and shake again. If wells remain purple after following this procedure, specimens should be retested.

   Notes:

   - After shaking, PreservCyt Solution specimens should turn pink instead of yellow.
   - When placing the Hybridization Microplate in the Microplate Heater I, care should be taken not to cause splashing.

6. Incubate in a preheated and equilibrated to 65 ± 2°C Microplate Heater I for 60 ± 5 minutes.

   Note: Create a plate layout file using the *digene* assay analysis software with *digene* assay protocols for HPV if this has not been completed earlier.

Hybridization Method using Microtubes and Water Bath

Notes:

- The processing of specimens collected with the *digene* HC2 DNA Collection Device in STM using the MST Vortexer 2 method for mixing and the water bath method for hybridization **has not been validated**. Specimens collected with the *digene* HC2 DNA Collection Device in STM and processed using the MST Vortexer 2 method can be hybridized using the Microplate Heater I method only.
- If the denatured specimen has been stored at -20°C, allow the specimen to thaw to 20°–25°C, and thoroughly vortex the specimen before proceeding with hybridization.

Procedure

1. Label and place the required number of clean hybridization microtubes into the microtube rack.
2. Remove Calibrators, Quality Controls, and specimens from the water bath after incubation. Vortex each tube individually for at least 5 seconds just prior to removing aliquots.
3. Pipette 75 µl of each Calibrator, Quality Control, or specimen into the **bottom** of empty hybridization microtubes following the plate layout created under *Setup*. Use a clean extra-long pipette tip for each transfer to avoid cross-contamination of Calibrators, Quality Controls, or specimens. It is not necessary to remove the specimen collection device from the specimen transport tube. Denatured specimens may be capped with

specimen collection tube screw caps and stored at -20°C with the specimen collection devices remaining in the tubes.

Note: False-positive results can occur if specimen aliquots are not carefully transferred. During transfer of specimen, do not touch the pipette tip to the inside of the tube when removing the 75-µl aliquot.

4. After transferring the last specimen, incubate the hybridization microtubes for 10 minutes at 20°–25°C.
5. Aliquot the prepared and thoroughly vortexed Probe Mix into a Disposable Reagent Reservoir. Carefully pipette 25 µl of the Probe Mix into each microtube containing Calibrators, Quality Controls, and specimens using an eight-channel pipettor and fresh tips for each row. Dispense the volume of probe into each hybridization microtube, preventing back splashing.
6. Cover the microtubes with a plate sealer. Place the rack cover on top of the rack. Shake the microtube rack on Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. The Calibrators, Quality Controls, and specimens should turn yellow after shaking. Tubes that remain purple may not have received the proper amount of Probe Mix. Add an additional 25 µl of Probe Mix to specimens that remain purple and shake again. If tubes remain purple after following this procedure, specimens should be retested.

   Note: After shaking, PreservCyt Solution specimens should turn pink instead of yellow.
7. Incubate in a 65 ± 2°C water bath for 60 ± 5 minutes. Ensure that the water level in the water bath is sufficient to cover the entire volume of hybridization mixture. The microtube rack may be allowed to float in the water bath.

   Note: Create a plate layout file using the *digene* assay analysis software with *digene* assay protocols for HPV if this has not been completed earlier.

Hybrid capture

1. Remove all but the required number of Capture Microplate wells from the plate frame. Return the unused microwells to the original bag and reseal. With a marker, number each column 1, 2, 3. . . . , and label the microplate with an appropriate identifier. The specimens will be added to the wells according to the example layout prepared under *Setup*.
2. Carefully remove the Hybridization Microplate containing Calibrators, Quality Controls, and specimens from the Microplate Heater I. Immediately remove the Plate Lid and place it on a clean surface. Alternatively, remove the microtube rack from the water bath. Immediately remove the rack lid, and slowly pull the plate sealer up and across the rack.
3. Transfer the entire contents (approximately 100 µl) of each Calibrator, Quality Control, and specimen from the Hybridization Microplate wells or microtubes to the bottom of the corresponding capture microwell using an eight-channel pipettor. Use **new pipette tips** on the eight-channel pipettor for each column transferred and allow each pipette tip to drain well to ensure complete specimen transfer. If desired, the pipettor may be steadied by resting the **middle** of the pipette tips on the top edge of the capture microwells (see *Diagram 1: Correct Pipetting* below).

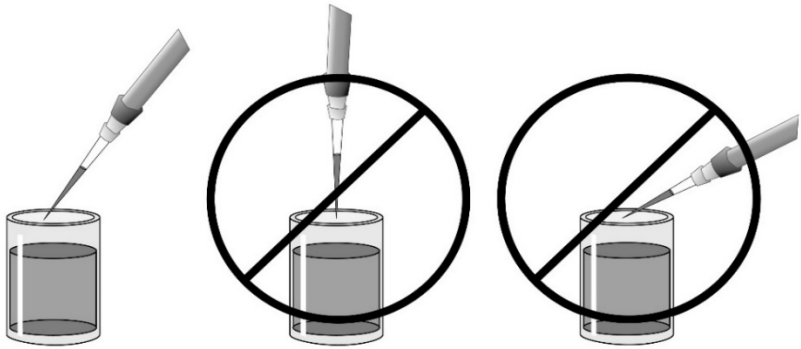

Diagram 1. Correct pipetting.

4. Cover the microplate with the plate lid or new plate sealer, and shake on the Rotary Shaker I at 1100 ± 100 rpm, at 20°–25°C for 60 ± 5 minutes.

5. Prepare Wash Buffer. If using the Automated Plate Washer, check rinse and waste reservoirs during this incubation. See *Reagent Preparation and Storage* section.
6. When the capture step is complete, remove the Capture Microplate from the Rotary Shaker I, and carefully remove the plate lid or 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 backsplash by decanting too closely to the bottom of the sink. **Do not reinvert the plate**; blot by tapping firmly 2–3 times on clean Kimtowels Wipers or equivalent low-lint paper towels. Ensure that all liquid is removed from the wells and the top of the plate is dry.

Hybrid detection

Notes:

- Make additions across the plate in a left-to-right direction using an eight-channel pipettor.
- It is recommended that the reverse-pipetting technique be utilized to improve consistency of reagent delivery. With this technique, the pipette tips are initially over-filled by using the second stop on the pipettor's aspirate/dispense control (plunger). See the procedure below. Wipe tips on a disposable reagent reservoir or clean low-lint pad to remove excess reagent before delivery to the plate.
- 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, as cross-contamination of specimens could occur. See *Diagram 1: Correct Pipetting* above.

Procedure

1. Aliquot the appropriate volume of Detection Reagent 1 into a reagent reservoir (see *Reagent Preparation and Storage* section for instructions). Carefully pipette 75 µl of Detection Reagent 1 into each well of the Capture Microplate using an eight-channel pipettor and the reverse-pipetting technique. Verify that all wells have been filled

accurately by observing the intensity of the pink color. All wells should have similar intensity.

Reverse-Pipetting Procedure

1a. Insert tips into eight-channel pipettor; ensure all tips are firmly seated.
1b. Push the plunger of the pipettor past the first stop to the second stop.
1c. Immerse tips into the Detection Reagent 1 solution.
1d. Release the plunger slowly and allow solution to fill the tips.
1e. Dispense 75 µl of solution into microwells by pressing the plunger to the first stop. Do not release the plunger until pipette tips have been reimmersed into the Detection Reagent 1 solution.
1f. Refill tips and repeat until all wells are filled. Fill wells 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 a plate lid or clean Parafilm or equivalent, and incubate at 20°–25°C for 30–45 minutes.

Washing

Wash the Capture Microplate using one of the two methods below.

Automated Plate Washer Method

Note: Always keep the Automated Plate Washer on. Ensure that the Rinse Reservoir is filled and the Waste Reservoir is empty. The Automated Plate Washer will routinely rinse the system for cleaning. See *Automated Plate Washer User Manual* for further instruction, as needed.

Before Each Use:

- Verify that the Wash Reservoir is filled at least to the 1 L mark. If not, prepare the Wash Buffer solution. See *Reagent Preparation and Storage* section.
- Verify that the Rinse Reservoir is filled with distilled or deionized water.
- Verify that the Waste Reservoir is empty and the cap is securely fastened.
- The Automated Plate Washer will automatically prime itself before each wash and rinse after each wash.

Procedure

1. Remove the plate lid and place the plate on the Automated Plate Washer platform.
2. Verify that the power is on, and that the display reads "Digene Wash Ready" or "P1."

   Note: If only a partial strip of capture wells is being used, empty microplate wells will need to be placed in the Capture Microplate to complete the column prior to washing. See *Accessories* for ordering information.
3. Select the number of strips to be washed by pressing the ROWS key and then + or - to adjust. Press the ROWS key to return to "Digene Wash Ready" or "P1."
4. Press START/STOP to begin.

   The Automated Plate Washer will perform six fill and aspirate cycles, taking approximately 10 minutes. There will be a brief pause during the program, so be sure

not to remove the plate prematurely. When the Automated Plate Washer is finished washing, it will read "Digene Wash Ready" or "P1."

5. Remove the microplate from the Automated Plate Washer when the program is finished. Plate should appear white, and no residual pink liquid should remain in the microwells.

Manual Washing Method

1. Remove Detection Reagent 1 from the wells by placing clean Kimtowels Wipers or equivalent low-lint paper towels 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 Kimtowels Wipers or equivalent low-lint paper towels. Carefully discard the used Kimtowels Wipers or equivalent low-lint paper towels to avoid alkaline phosphatase contamination of later steps.
2. Using the Wash Apparatus, hand wash the plate six times. Each well must be washed to overflowing to remove Detection Reagent 1 from the top of the wells. Washing begins at well A1 and continues in a serpentine fashion to the right and downward. After all the wells have been filled, decant liquid into the 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 twice more for a total of six washes per well.
3. After washing, blot the plate by inverting it onto clean Kimtowels Wipers or equivalent low-lint paper towels and tapping firmly 3–4 times. Replace the toweling and blot again. Leave the plate inverted and allow it to drain for 5 minutes. Blot the plate one more time.
4. The plate should appear white, and no pink residual liquid should remain in the microwells.

Signal Amplification

Notes:

- Use a new, clean pair of gloves for handling Detection Reagent 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 and Storage* section. Do not return Detection Reagent 2 to the original bottle. Discard unused material after use.
- Detection Reagent 2 addition should be made without interruption. The incubation time of all wells must be as close as possible.
- Take care not to touch the sides of the microwell or splash reagent back onto tips, as cross-contamination of specimens could occur (see *Diagram 1: Correct Pipetting*).

Procedure

1. Carefully pipette 75 µl of Detection Reagent 2 into each well of the capture microplate using an eight-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 a plate lid or clean Parafilm or equivalent, and incubate at 20°–25°C for 15–30 minutes. Avoid direct sunlight.
3. Read the microplate on the luminometer after 15 minutes of incubation (and no later than 30 minutes of incubation).
4. If a full microplate was not used, remove used microwells from the microplate holder, rinse the holder thoroughly with distilled or deionized water, dry, and reserve for the next assay.

Assay Calibration and Verification Criteria

Assay Calibration Verification is performed to ensure that the reagents and furnished Calibrator material are functioning properly, permitting accurate determination of the assay cutoff value. The *digene* HC2 HPV DNA Test requires calibration with each assay. Therefore, it is necessary to verify each assay using the following criteria. This verification procedure is not intended as a substitute for internal quality control testing.

- 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 ≤ 25%. If the %CV is > 25%, discard the control 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 ≤ 25%, proceed to step 2; otherwise, the assay calibration verification is invalid and the test must be repeated for all patient specimens. Accordingly, patient specimen results should not be reported.

- Low-Risk and High Risk Calibrators

  The Calibrator(s) must be tested in triplicate with each assay. The Low-Risk and High-Risk HPV Calibrator results should each show a coefficient of variation (%CV) of ≤ 15%. If the %CV is > 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 remaining values is ≤ 15%, proceed to step 3; otherwise, the assay calibration verification for that specific Probe is invalid and the test must be repeated for all patient specimens. Accordingly, patient specimen results should not be reported.

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

digene assay analysis software with version 4.01 or higher *digene* assay protocols for HPV automatically verifies that the Low-Risk and High-Risk HPV Calibrator %CV is ≤ 15%. However, previous versions (1.0.2 and 1.0.3) of the *digene* Qualitative Software will NOT invalidate the assay unless the %CV is > 25% for the Calibrators. Therefore, users of version 1.0.2 or 1.0.3 *digene* Qualitative Software assay protocols for HPV must manually verify that the %CV calculated by the *digene* Qualitative Software is ≤ 15% for both the Low-Risk and the High-Risk HPV Calibrators and proceed as indicated for Situation 1 in the table below. If the %CV of the Calibrator replicates falls between 15% and 25% for either or both Calibrators, refer to the instructions in Situation 2 or 3 in the table below and proceed with the indicated *User Action*.

Note: For CPC, the % CV of LRC, HRC, and LRC-HRC combined must show a % CV of ≤ 15%. Only 1 LRC and 1 HRC replicate may be deleted.

Situation	Reported %CV for the Calibrator Replicates	Action Taken by *digene* Qualitative 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.

In order to manually calculate the %CV as required in Situation 2 above, the user should divide the standard deviation of the remaining replicate RLU values by the mean of the remaining replicate RLU values (LRC or HRC) and multiply that result by 100.

To calculate the %CV using Microsoft® Excel® (supplied with the *digene* Qualitative Software), 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.

(STDEV/AVERAGE) * 100 = %CV

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

To determine Calibrator reproducibility and estimate the frequency in which manual recalculations may be necessary, the results from three clinical evaluations involving 152 assays performed with the *digene* HC2 HPV DNA Test were compiled. The results showed that the average %CV for these 152 assays was 8.1%. Considering all three replicates of the Calibrator per assay, Calibrator reproducibility of greater than 15%CV was observed for only 17 out of 152 runs (11.2%), with 10 out of these 17 assays resulting in %CV between 15%–25% (Situation 2). For the 17 assays that yielded a %CV greater than 15%, a single outlier was removed and the %CV recalculated. Following the User Action for Situation 2, only one of the %CVs remained greater than 15%, invalidating the assay. The %CVs of the remaining 151 assays were calculated for an average %CV of 6%.

- The Calibrator mean (LRC or HRC$\bar{X}$) and Negative Calibrator mean (NC$\bar{X}$) results are used to calculate the LRC$\bar{X}$/NC$\bar{X}$ or HRC$\bar{X}$/NC$\bar{X}$ ratio for each Probe. *digene* assay protocols for HPV version 4.01 and higher automatically verify the HRC$\bar{X}$/NC$\bar{X}$ or LRC$\bar{X}$ /NC$\bar{X}$ ratio acceptable range within 2.0–15.0. However, previous versions (v1.0.2 and v1.0.3) of the *digene* Qualitative Software assay protocols do not verify the upper limit of this range. This ratio must meet the following criteria to verify the assay calibration before the specimen results can be interpreted.

CPC METHOD	DUAL-PROBE METHOD
Assay Calibration Verification Acceptable Ranges	Assay Calibration Verification Acceptable Ranges
$2.0 \leq LRC\overline{\chi} / NC\overline{\chi} \leq 15$	$2.0 \leq LRC\overline{\chi} / NCLR\overline{\chi} \leq 15.0$ (LR side)
$2.0 \leq HRC\overline{\chi} / NC\overline{\chi} \leq 15$	$2.0 \leq HRC\overline{\chi} / NCHR\overline{\chi} \leq 15.0$ (HR side)
$2.0 \leq$ (LRC and HRC) $\overline{\chi} / NC\overline{\chi} \leq 15$	

- Calculate the appropriate $LRC\overline{\chi}/NC\overline{\chi}$ or $HRC\overline{\chi}/NC\overline{\chi}$ ratios for each of the Probe sets. If the ratios are ≥ 2.0 and ≤ 15.0, proceed to the next step. If any of the ratios are < 2.0 or > 15.0, **the assay is invalid for that specific probe and must be repeated.** Repeat all patient specimens within the assay.

  Note: Acceptable ranges for the Negative Calibrator, Low-Risk, and High-Risk HPV Calibrators have been established only for QIAGEN-approved luminometers.

Cutoff Calculation

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

- Combined-Probe Cocktail Method:

$$\frac{\text{(LRC replicates + HRC replicates)}}{\text{\# of replicates}}$$

- Dual-Probe Method:

$$\text{Low-Risk HPV Probe Cutoff} = \text{LRC}\overline{\chi}$$

$$\text{High-Risk HPV Probe Cutoff} = \text{HRC}\overline{\chi}$$

Example Cutoff Calculations

for:		Low-Risk or High-Risk HPV Probe Two-Probe Method	Low-Risk HPV Probe CPC Method	High-Risk HPV Probe CPC Method	Combined HPV Probe CPC Method
	NC RLU Values	LRC or HRC RLU Values	LRC RLU Values	HRC RLU Values	LRC and HRC RLU Values
	97	312	330	235*	330
	101	335	305	295	305
	91	307	385	279	385
					295
					235*
					279
Mean RLU Value	96	318	340	287*	318.8*
%CV	4.9	4.7	12.0	3.9*	13.0
$LRC\overline{X}/NC\overline{X}$	N/A	3.31	3.54	3.00	3.32

The mean RLU value for the Positive Calibrator determines the assay cutoff RLU. In the Two-Probe Method example above, the Cutoff RLU Value is 318.

* Mean %CV of all 6 replicates was 16.8. Replicate with a value of 235 was deleted as an outlier. % CV of replicates remaining was 13.0 with a mean of 318.8. The initial %CV of HRC was 11.5.

All specimen RLU values should be converted into a ratio to the appropriate Cutoff (CO) RLU Value. For example, all assays tested with Low-Risk HPV Probe should be expressed as Specimen RLU/Low-Risk Cutoff Value. The same can be performed with specimens tested with High-Risk HPV Probe or the CPC.

Note: RLU/CO values and positive/negative results for all specimens tested for each probe are calculated and reported in the *digene* assay analysis software test result reports.

Quality Control

Quality Controls are supplied with the *digene* HC2 HPV DNA Test. Consult the applicable *digene* assay analysis software User Manual for instructions on how to input the Lot Numbers and Expiration Dates of the Quality Controls. These controls must be included in each assay, and the RLU/CO of each control must fall within the following acceptable ranges for it to be considered valid. Version 4.01 and higher *digene* assay protocols for HPV automatically invalidate an assay if the controls are not within the specified limits. Earlier protocol versions will not automatically invalidate an assay if the Quality Controls are not within the specified limits. If the Quality Controls do not fall within these ranges, the assay is invalid and must be repeated. Accordingly, no patient results should be reported for any invalid assay.

The expected results for each specimen included in the *digene* HC2 HPV DNA Test are summarized below.

Control	HPV Type	Expected Result (RLU/Cutoff Value) Low-Risk HPV Probe		
		Minimum	Maximum	Expected Mean
QC1-LR	Low-Risk (HPV6)	≥ 2	≤ 8	5.0
QC2-LR	High-Risk (HPV16)	0	< 1	0.5

Control	HPV Type	Expected Result (RLU/Cutoff Value) High-Risk HPV Probe		
		Minimum	Maximum	Expected Mean
QC1-LR	Low-Risk (HPV6)	0	< 1	0.5
QC2-LR	High-Risk (HPV16)	≥ 2	≤ 8	5.0

Control	HPV Type	Expected Result (RLU/Cutoff Value) HighRrisk HPV Probe		
		Minimum	Maximum	Expected Mean
QC1-LR	Low-Risk (HPV6)	≥ 2	≤ 8	5.0
QC2-LR	High-Risk (HPV16)	≥ 2	≤ 8	5.0

digene Hybrid Capture 2 System users: if predefined controls (QC1, QC2, QC3, and QC4) are in the Controls list, delete them within the software and define the controls (QC1-LR and QC2-HR) listed in the table above. If the laboratory chooses to run replicates of QC1 and QC2 on a plate, the *digene* Hybrid Capture 2 System software will not report patient results if the CV of QC1 or QC2 exceeds 25%.

- The High-Risk HPV Quality Control material provided in the kit is cloned HPV DNA target and is not derived from wild-type HPV. The QC2-HR is 5 pg/ml HPV 16 DNA while the Calibrator contains 1 pg/ml of the same material.
- This Control material will not act as an appropriate specimen processing control for the PreservCyt Solution specimen.
- The controls provided with this test kit must be used for internal quality control. Alternatively, external controls may be tested according to guidelines or requirements of local, state, and/or country regulations or accrediting organizations.

Users may develop these Quality Control materials, as defined by NCCLS (currently known as CLSI) C24-A.[36] Please refer to NCCLS C24-A for additional guidance on appropriate internal quality control testing practices.

Interpretation of Specimen Results

- Specimens with RLU/CO ratios ≥ 1.0 with the CPC 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.
- Specimens with RLU/CO ratios ≥ 1.0 with Low-Risk HPV Probe only are considered positive for one or more of HPV types 6, 11, 42, 43, or 44.
  - Specimens with RLU/CO ratios ≥ 1.0 with High-Risk HPV Probe 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.
  - Specimens with RLU/CO Value ratios ≥ 1.0 for both Low-Risk HPV and High-Risk HPV Probes are considered positive for one or more HPV types from each group of probes.
- Specimens with RLU/CO Value ratios < 1.0 for CPC or both Low-Risk and High-Risk HPV Probes 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.
- When testing PreservCyt Solution specimens, if the RLU/CO ratio of a specimen is ≥ 1.0 and < 2.5, the specimen must be retested. If the initial retest result is positive (≥ 1.0 RLU/CO), the specimen can be reported as positive and no further retesting needs to be completed. However, if the first retest result is negative (< 1.0), then a second retest (third result) needs to be completed to generate a final result. The result of the second retest is considered the final result and is to be reported (see *Table 1: Prevalence of HPV Types in the United States* below). If there is not sufficient specimen volume to perform a retest, the result should be reported as "Quantity Not Sufficient."

Interpretation of results for HPV STD screening

HPV Result		Interpretation
Low-Risk HPV Probe	High-Risk HPV Probe	
Negative	Negative	The patient is not likely to be infected with HPV
Negative	Positive	The patient is likely to be infected with HPV
Positive	Negative	
Positive	Positive	

Note: Low-Risk HPV types (6, 11, 42, 43, or 44) are not associated with risk of cervical cancer.

The magnitude of the measured result (RLU) above the cutoff is indicative of the total amount of low-risk or high-risk HPV DNA present but this measurement has no established clinical utility.

Negative assay results do not completely rule out the presence of low-risk HPV types 6, 11, 42, 43, or 44 or high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68, particularly at very low concentrations.

The effects of age and HPV positivity are not fully known. It has been demonstrated in studies that HPV prevalence will decrease with age.[37] For information on the age-specific performance of the *digene* HC2 High-Risk HPV DNA Test versus a histological diagnosis of high-grade neoplasia, see *Table 6: Kaiser Study Data: digene* HC2 *HPV DNA Test Performance versus Consensus Histology Results (CIN2-3), Age-Specific Characteristics*.

Additional testing is recommended in any circumstance when false-positive or false-negative results could lead to adverse medical, social, or psychological consequences.

Results of this test should be interpreted only in conjunction with information available from clinical evaluation of the patient and from other procedures.

Interpretation for screening patients with ASC-US pap smear results to determine the need for referral to colposcopy

High-Risk HPV DNA-negative patients or those patients positive for Low-Risk HPV types test should be followed according to routine practice and in accordance with ACS, ASCCP, CDC, U.S. Public Health Service, and ACOG current guidelines. High-Risk HPV DNA-positive patients may be referred to colposcopy as indicated in the table below. The results of this test are not intended to prevent women from proceeding to colposcopy.

Interpretation of ASC-US specimen results

Referral Cytology Result	HPV Result		Refer to Colposcopy	Result interpretation
	Low-Risk HPV Probe	High-Risk HPV Probe		
ASC-US	Negative	Negative	No	Based on the results of the *digene* HC2 HPV DNA Test, there is a high probability[a] that a higher disease stage will not be found at colposcopy.
	Positive	Negative		
	Negative	Positive	Yes	Based on the results of the *digene* HC2 HPV DNA Test, as with repeat Pap smear, there is a low but increased probability[b] that a higher disease stage will be detected at colposcopy. However, if HPV is present, it has been documented in the medical literature that progression to high-grade disease is probable.[39, 40]
	Positive	Positive		

[a,b] The probability is equal to the assay's negative[a] or positive[b] predictive value, which is a function of prevalence for high-grade disease (see *Table 4: Comparison of digene HC2 HPV DNA Test Versus Consensus Histology*).

Interpretation of results for screening patients with LSIL or HSIL pap smear results to determine the risk of high-grade disease

The *digene* HC2 HPV DNA Test currently contains 18 RNA probes to various HPV types. The incidence of these types being detected in cervical cancer range in the United States from 0.05% for HPV 42 to 54.5% for HPV 16 (see Table 1: Prevalence of HPV Types in the United States table). These results were from specimens collected prior to colposcopy where the patient had an initial Pap smear result of LSIL or HSIL. While the assay sensitivity and specificity performance was not affected by whether the initial Pap smear result was LSIL or HSIL, the prevalence of HSIL in these two groups was different (20.1% in the LSIL group and 43.7% in the HSIL group). Therefore, in order to interpret these results, the physician must take into consideration the prevalence of HSIL in the local patient population with either an initial LSIL or HSIL Pap smear result. The digene HC2 HPV DNA Test results should not be used to deter women from proceeding to colposcopy. Refer to the current ACS, ASCCP, CDC, U.S. Public Health Service, and ACOG guidelines for clinical application of HPV test results.

Interpretation of LSIL or HSIL specimen results

Initial cytology result	HPV result		Result interpretation
	Low-Risk HPV Probe	High-Risk HPV Probe	
LSIL or HSIL	Negative	Negative	Based on the results of the *digene* HC2 HPV DNA Test, there is a high probability[a] that a severe disease will not be found at colposcopy.
	Positive	Negative	
	Negative	Positive	Based on the results of the *digene* HC2 HPV DNA Test, there is a moderate probability[b] that a severe disease will be detected at colposcopy.
	Positive	Positive	

[a,b] The probability is equal to the assay negative[a] or positive[b] predictive value, which is a function of prevalence for high-grade disease(see *Table 7: Patient Disease Status Algorithm*).

Additional testing is recommended in any circumstance when false-positive or false-negative results could lead to adverse medical, social, or psychological consequences.

Results of this test should be interpreted only in conjunction with information available from clinical evaluation of the patient and from other procedures. Refer to the Limitations of the Procedure section below.

Limitation of the Procedure

- Refer to *Rapid Capture System User Manual* for additional *Limitations of the Procedure* specific to the use of that system for high-volume sample-throughput. The performance of high-volume sample-throughput testing was approved using only the high-risk HPV types 16/18/31/33/35/39/45/51/52/56/58/59/68.
- The *digene* HC2 HPV DNA Test for human papillomavirus types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59, and 68 is not recommended for evaluation of suspected sexual abuse.
- 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.
- 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 *digene* HC2 HPV DNA Test should only be used with cervical specimens collected using the *digene* HC2 DNA Collection Device with *digene* Specimen Transport Medium (STM) or cervical specimens collected using a broom-type collection device and placed in PreservCyt Solution. Biopsy specimens may be assayed only if they are placed immediately in STM and stored at -20° C until assayed.
- The *digene* HC2 DNA Collection Device should not be used for collection of specimens from pregnant women.
- The *digene* HC2 HPV DNA Test distinguishes between two groups of HPV types: HPVs 6/11/42/43/44 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.
- Cross-reactivity may be observed in the presence of HPV type 13, because both *digene* HC2 HPV DNA Test probes cross-react with HPV 13. This cross-reactivity is not considered to be clinically relevant for anogenital specimens because HPV 13 is commonly detected in lip lesions of certain ethnic groups and rarely, if ever, detected in the anogenital tract.[38,39]

- Infection with HPV is not an indicator of cytologic HSIL or underlying high-grade CIN, nor does it imply that CIN 2-3 or cancer will develop. Most women infected with one or more high-risk HPV types do not develop CIN 2-3 or cancer.
- A negative high-risk HPV result does not exclude the possibility of future cytologic HSIL or underlying CIN2-3 or cancer. A small proportion of high-grade lesions occur in women who are high-risk HPV negative by existing technologies.[6]
- A small amount of cross-hybridization between HPV types 6 and 42 (low-risk HPV types) and the High-Risk HPV Probe 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. It has also been reported in the literature that complex probe cocktails similar to that used in this test may cause false-positive results due to cross-hybridization with HPV types 11, 40, 53, 54, 55, 66, MM4, MM7, MM8, or MM9.[40] Although several of these HPV types are rare or novel types not often encountered with high-grade disease, patients whose specimens contain high levels of these HPV DNA types may incorrectly be reported as positive for high-risk HPV types.[12,41]
- The *digene* HC2 HPV DNA Test is designed to detect high-risk HPV types including 39, 58, 59, and 68. Analytical studies conducted by QIAGEN, using cloned HPV plasmid DNA, demonstrate that the assay using High-Risk HPV Probe detects these types at levels ranging from 0.62 pg/ml to 1.39 pg/ml. This is equivalent to the detection characteristics of the other HPV types targeted by the *digene* HC2 HPV DNA Test. QIAGEN 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 (as demonstrated by Bosch, et al.), the performance characteristics of the *digene* HC2 HPV DNA Test for the detection of HPV types 39, 58, 59, and 68 has not been statistically confirmed.
- 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.

- Cross-reactivity between both *digene* HC2 HPV DNA Test probes and the plasmid pBR322 is possible. The presence of pBR322 homologous sequences has been reported in human genital specimens, and false-positive results could occur in the presence of high levels of bacterial plasmid.
- There is no known utility for HPV testing in Pap AGUS results.
- When processing PreservCyt Solution specimens, false-negative results could occur if the cell pellet is not visible after centrifugation. This observation is indicative of insufficient cellular material available to obtain a reliable test result.
- PreservCyt Solution specimens containing volumes less than 4 ml, after the ThinPrep Pap Test slides are prepared, are considered inadequate for the *digene* HC2 HPV DNA Test.
- Processing up to 36 PreservCyt specimens at a time has been validated by QIAGEN. The additional time necessary to process more than 36 specimens increases the likelihood of the cell pellets becoming dislodged during decantation of the supernatant prior to re-suspension in STM/DNR which may produce an inaccurate result.
- The denaturation step of the specimen processing procedure must be performed as directed in these instructions for use. Improper execution of the denaturation step of the *digene* HC2 HPV DNA Test Procedure may lead to false-positive results. Improper specimen vortexing, tube inversion, and agitation could result in incomplete denaturation of non-specific RNA/DNA hybrids endogenous to cervical specimens. False-positive results could occur due to contamination of the *digene* HC2 HPV DNA Test specimen with these non-specific RNA/DNA hybrids. In order to prevent possible carryover of this non-denatured cellular material, it is important that the micro-pipette tip not touch the sides of the specimen denaturation tube during transfer of the denatured specimen to the microtube or microplate well used for HPV probe hybridization.

Expected Results

HPV prevalence

The prevalence of infection by HPV type, as measured by the detection of an HPV DNA risk group, varies with the patient population. Important variables include age at first intercourse, number of sexual partners, concurrent sexually transmitted diseases, and history of abnormal Pap smears.[2,24,31,42] Also, it has been reported that the prevalence of HPV infection decreases dramatically with age.[2,37] Hence, it is not possible to define a single typical pattern of prevalence for HPV infection. *Table 1* shows the prevalence in the United States of each HPV type detected by the *digene* HC2 HPV DNA Test as reported by two independent researchers. These prevalence values are representative only of the populations tested and may vary in specific areas of the country.

Table 1. Prevalence of HPV types in the United States

HPV Type	Prevalence (%)
6	0.8[13]
11	0.8[13]
16	54.5[14]
18	9.1[14]
31	9.1[14]
33	0.2[13]
35	0.2[13]
39	*
42	0.05[13]
43	0.2[13]
44	0.4[13]
45	27.3[14]
51	0.4[13]
52	0.5[13]
56	0.2[13]
58	*
59	*
68	*

*Bosch, et al. reported that HPV types 39, 58, 59, and 68 showed worldwide prevalence of 1.6%, 2.1%, 1.7%, and 1.2% respectively; however, prevalence in the United States was not determined independently.[14]

Table 2 shows HPV prevalence results compiled from several groups of women referred to three gynecology clinics within metropolitan medical centers (high prevalence for HPV infection) for cervical abnormality and tested using the *digene* HC2 HPV DNA Test. These results demonstrate a fairly consistent pattern of positive HPV results across sites.

Table 2. Prevalence of Low-Risk and High-Risk HPV types across sites (ASC-US or more severe pap population)

Site	Number of patients	Percent of HPV positive (# pos/total #)	
		Low-Risk types	High-Risk types
1	200	12.5% (25/200)	62.0% (124/200)
2	140	13.6% (19/140)	63.6% (89/140)
3	184	14.1% (26/184)	52.7% (97/184)
Total	524	13.4% (70/524)	59.2% (310/524)

Table 3 shows the prevalence of single or combined high-risk HPV types as detected by the *digene* HC2 HPV DNA Test as reported by six independent researchers. These prevalence values are representative only of the populations tested and may vary from prevalence found in specific areas of the United States.

Table 3. Prevalence of high-risk HPV* in various populations women age over 30 years and older

Location	Study time frame	Study size	Prevalence (%)
USA Portland, OR[6,25,43]	1989–1999	13,493	9.0
Costa Rica[41,44]	1993–1995	6991	8.7
South Africa[45]	1998–1999	2925	23.4
China[46]	1999	1940	18.8
France[47]	1998–2002	2115	4.8
Germany[48]	1999–2000	7592	4.2

*Any combination of the 13 HR types detected by the High-Risk HPV Probe

Performance Characteristics

Clinical sensitivity and specificity for screening patients with ASC-US pap smear results to determine the need for referral to colposcopy

A study entitled "Utility of HPV DNA Testing for Triage of Women with Borderline Pap Smears" was conducted in 1996 under the direction of the Kaiser Foundation Research Institute and the Kaiser Permanente Medical Group. Cervical specimens for routine Pap smears and for *digene* HC2 HPV DNA Tests were obtained from women attending several Kaiser clinic facilities. Initial Pap smears were evaluated according to the Bethesda Classification. Women (15 years or older) with Pap smear results of ASC-US returned for colposcopy and biopsy. Colposcopically directed histological specimens were examined by pathologists and an initial diagnosis was made. Each histologic specimen was also reviewed by an independent pathologist and discrepancies between the initial review and the independent review were adjudicated by a third pathologist.

The *digene* HC2 HPV DNA Test was performed on the initial specimen and only the High-Risk HPV Probe was used. HPV DNA testing was performed with a prototype of the *digene* HC2 HPV DNA Test that contained probes to 11 of the 13 HPV types included in the High-Risk HPV Probe, but did not contain probes to HPV types 59 and 68. This difference would not be expected to result in significantly different performance profiles for the two assays.

digene HC2 HPV DNA Test results and histological diagnoses were available from 885 women with ASC-US Pap smears. Testing on the majority of patients was performed with specimens collected in both STM and PreservCyt Solution. Due to the similarities between the *digene* HC2 HPV DNA Test's performance characteristics for STM and PreservCyt Solution, assay performance is presented for only the PreservCyt Solution.

Table 4 shows that, among those presenting with an ASC-US referral Pap smear, the negative predictive value of the *digene* HC2 HPV DNA Test for having HSIL or greater disease at colposcopy is 99.0%.

Table 4. Comparison of *digene* HC2 HPV DNA Test versus consensus histology, ASC-US Referral Pap Population, Kaiser Study, PreservCyt Solution Specimens

	HSIL or greater at the time of colposcopy			
High-Risk HPV probe		+	-	Total
	+	66	317	383
	-	5	497	502
	Total	71	814	885

Sensitivity [TP/(TP+FN)] = 93.0% (66/71)
95% CI = 84.3 to 97.7
Specificity [TN/(TN+FP)] = 61.1% (497/814)
95% CI = 57.7 to 64.4
Disease Prevalence = 8.0% (71/885)
Positive Predictive Value = 17.2% (66/383)
Negative Predictive Value = 99.0% (497/502)

Table 5 shows theoretical positive and negative predictive values based on various prevalence results for an initial ASC-US being found to be CIN 2-3 or cancer based on High-Risk HPV Probe results.

Table 5. Theoretical positive and negative predictive values, High-Risk HPV Probe, ASC-US pap smear results

	Initial ASC-US pap smear results	
Theoretical prevalence for CIN 2–3 or cancer	Assay positive predictive value	Assay negative predictive value
5	11.2	99.4
10	21.0	98.7
15	29.7	98.0
20	37.4	97.2
25	44.3	96.3
30	50.6	95.3

Table 6 illustrates the variation between the various age groups contained in this study:

Table 6. Kaiser study data. *digene* HC2 HPV DNA Test performance versus consensus histology results (CIN2–3) age-specific characteristics

	Age < 30	Age 30–39	Age >39
n	287	233	365
Prevalence of Disease (%)	12.2	11.2	2.7
Sensitivity (%)	100.00 (35/35)	88.46 (23/26)	80.00 (8/10)
95% Confidence Interval	90.0-100	69.9-97.6	44.4-97.5
Specificity (%)	31.4 (79/252)	66.2 (137/207)	79.15 (281/355)
95% Confidence Interval	25.7-37.5	59.3-72.6	74.6-83.3
Negative Predictive Value (%)	100 (79/79)	97.86 (137/140)	99.29 (281/283)
Positive Predictive Value (%)	16.83 (35/208)	24.73 (23/93)	9.76 (8/82)

Clinical sensitivity and specificity for the determination of the risk of high-grade disease in women with LSIL or HSIL pap smears

A multicenter clinical study was conducted using the *digene* HC2 HPV DNA Test using specimens collected from several large, high-grade cervical disease and HPV prevalence hospital and medical center colposcopy clinics (three sites) in the western and southern United States. The *digene* HC2 HPV DNA Test was performed at three investigational sites not affiliated with the colposcopy clinics from which the specimens were collected. The population for this clinical study was comprised of women diagnosed as either LSIL or HSIL based on a recent Pap smear and referred for follow-up colposcopy. Of 702 patients enrolled, 327 had Pap smear results greater than ASC-US and had adequate information available; 96 of these

had a final disease status of HSIL or greater. Exfoliated cervical cell specimens were obtained with either the Cervical Brush, placed into STM, or with a broom device and rinsed in PreservCyt. Specimens were collected at the time of colposcopy. Specimens were tested with the *digene* HC2 HPV DNA Test and the results compared to the disease status determined for each patient. Disease status was based on the results of histologic evaluation; however, when histology was negative or in the absence of a histology result, disease status was determined by cytology at the time of colposcopic examination (see *Table 7*). Cytology was performed at a reference pathology laboratory and the histology was performed at the institutions performing the colposcopy. *digene* HC2 HPV DNA Test results were compared to disease status to assess the test's sensitivity, specificity, and negative and positive predictive value for detecting high-grade cervical neoplasia. Due to the similarities between the *digene* HC2 HPV DNA Test performance characteristics for STM and PreservCyt Solution, assay performance is presented for only the PreservCyt Solution.

Table 7. Patient disease status algorithm

Cytology Result	Histology Result	Disease Status
NEG	NEG or ND*	NEG
LSIL	NEG	LSIL
HSIL	NEG	HSIL
Cancer	NEG	HSIL+
NEG	LSIL	LSIL
LSIL	ND*	LSIL
LSIL	LSIL	LSIL
HSIL	LSIL	LSIL
Cancer	LSIL	LSIL
NEG	HSIL	HSIL
LSIL	HSIL	HSIL
HSIL	HSIL	HSIL
HSIL	ND*	HSIL
Cancer	HSIL	HSIL
NEG	Cancer	HSIL+
LSIL	Cancer	HSIL+
HSIL	Cancer	HSIL+
Cancer	ND*	HSIL+
Cancer	Cancer	HSIL+

*Biopsy and/or ECC not done because no abnormalities were observed upon colposcopy or histology result was not available.

Tables 8 and *9* represent the performance of the *digene* HC2 HPV DNA Test determined using 327 PreservCyt Solution specimens, 96 of which were collected from women diagnosed with

high-grade cervical disease. The comparisons were performed using all study patients with abnormal referral Pap smear results. Comparisons are shown for PreservCyt Solution specimens tested with High-Risk HPV Probe.

Tables 8 and *9* show that the *digene* HC2 HPV DNA Test with High-Risk HPV Probe demonstrated approximately 93% overall sensitivity for identifying women with high-grade neoplasia in a population referred for colposcopy on the basis of a Pap smear diagnosis of LSIL, HSIL, or equivalent. The test also demonstrated a negative predictive value of nearly 95% in this population.

No difference was observed in High-Risk HPV Probe results from STM specimens and PreservCyt Solution specimens. The following table shows the results of the High-Risk HPV Probe in this population:

Table 8. Results of High-Risk HPV Probe

	Final disease status						
Referral pap smear result	HSIL		LSIL		Negative		Total
	POS	NEG	POS	NEG	POS	NEG	
LSIL	44	4	78	33	28	37	224
HSIL	45	3	29	14	5	7	103
Total	89	7	107	47	33	44	327
	96		154		77		

Table 9. Performance characteristics. *digene* HC2 HPV DNA Test among patients having a referral pap smear or LSIL or higher and a final disease status of HSIL

	Referral pap LSIL or HSIL HSIL disease			
High-Risk HPV DNA		+	-	Total
	+	89	140	229
	-	7	91	98
	Total	96	231	327

Sensitivity [TP/(TP+FN)] = 92.7% (89/96)
95% CI = 85.6 to 97.0
Specificity [TN/(TN+FP)] = 39.4% (91/231)
95% CI = 33.1 to 46.0
Disease Prevalence for Referral LSIL to final HSIL = 21.4%
Disease Prevalence for Referral HSIL to final HSIL = 46.6%
Overall Positive Predictive Value = 38.9% (89/229)
Overall Negative Predictive Value = 92.8% (91/98)

While the specificity of the *digene* HC2 HPV DNA Test appeared to be somewhat low, a strict correlation between absence of neoplasia and a negative HPV result is not expected. HPV DNA can be present in women who have not progressed to higher-grade disease.

The following table (*Table 10*) indicates theoretical High-Risk HPV Probe-positive and -negative predictive values for an initial LSIL or HSIL being found to be HSIL or more severe disease on colposcopy.

Table 10. Theoretical positive and negative predictive values, High-Risk HPV Probe, Initial LSIL or HSIL pap smear results

	Initial ASC-US pap smear results	
Theoretical prevalence for HSIL	Assay positive predictive value	Assay negative predictive value
5	7.4	99.0
10	14.5	97.9
15	21.2	96.8
20	27.6	95.5
25	33.7	94.1
30	39.6	92.6
35	45.1	90.9
40	50.4	89.0
45	55.5	86.8
50	60.4	84.3

Analytical sensitivity

A nonclinical panel of cloned HPV plasmid DNA was tested to determine if each of the 18 HPV types is detectable by the *digene* HC2 HPV DNA Test and to determine the analytical sensitivity of the assay for each of the HPV types. Each of the 18 HPV DNA Types (6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, 58, 59. and 68) was tested in triplicate with Low-Risk HPV Probe or High-Risk HPV Probe, as appropriate (100 pg/ml, 10 pg/ml, 2.5 pg/ml, 1.0 pg/ml, 0.5 pg/ml, and 0.2 pg/ml). The mean RLU for each concentration of each HPV type was calculated and compared to the mean of the positive calibrator for the appropriate side of the assay.

The detectable limit of each HPV type is shown in *Table 11*. The detectable limits varied from 0.62 pg/ml to 1.39 pg/ml depending on the HPV type tested. The mean detectable limit of all 18 HPV DNA types was 1.09 pg/ml with a standard deviation of 0.05 pg/ml.

Table 11. Summary of *digene* HC2 HPV DNA Test detectable limits of sensitivity for each HPV DNA type

HPV DNA Type	Detectable HPV DNA Concentration (pg/ml)	Standard Deviation	95% Confidence Range
6	1.33	0.03	1.22–1.46
11	1.13	0.05	1.00–1.29
16	1.09	0.06	0.94–1.29
18	1.05	0.05	0.88–1.29
31	1.01	0.05	0.91–1.15
33	1.35	0.02	1.26–1.45
35	1.11	0.05	0.95–1.31
39	1.39	0.09	1.16–1.71
42	1.20	0.05	1.02–1.44
43	0.85	0.03	0.86–1.07
44	1.17	0.04	1.02–1.36
45	1.14	0.04	0.99–1.35
51	0.78	0.10	0.70–0.88
52	1.37	0.06	1.21–1.58
56	0.62	0.04	0.58–0.67
58	0.82	0.04	0.73–0.94
59	1.10	0.06	1.00–1.21
68	1.19	0.04	1.03–1.39
Mean (all types)	1.09	0.05	0.97–1.27

Combined-probe cocktail (CPC) probe

The same nonclinical HPV plasmid DNA panel described above was tested to determine the analytical sensitivity of each of the 18 HPV types in the *digene* HC2 HPV DNA Test following the Combined-Probe Cocktail (CPC) protocol as described in this insert. The analytical sensitivity of CPC protocol varied from 0.58 pg/ml to 1.39 pg/ml, depending on the HPV type tested. The mean detectable limit for all 18 HPV types was 0.95 pg/ml with a standard deviation of 0.07. This sensitivity is equivalent to the analytical sensitivity found for the two-probe method of the *digene* HC2 HPV DNA Test.

Equivalence between STM and PreservCyt solution specimens

Equivalence between STM and PreservCyt Solution specimens was examined for equal recovery of HPV 18 DNA from approximately 10^6 positive HeLa cells containing integrated HPV 18 genomes spiked into STM and into a negative cell pool in PreservCyt Solution. Each specimen type was processed according to its respective processing/denaturation procedures described in these instructions for use and tested with the *digene* HC2 HPV DNA Test using High-Risk HPV Probe. The results demonstrated that recovery of HPV 18 DNA from human carcinoma cells is equivalent for the two media and that the PreservCyt Solution preparation procedure does not affect the analytical sensitivity of the *digene* HC2 HPV DNA Test.

Reproducibility

A multicenter reproducibility study was performed to determine the between days, between sites, and overall reproducibility of the *digene* HC2 HPV DNA Test using a panel of HPV DNA targets and HPV-positive and HPV-negative clinical specimens.

Three external laboratories performed the testing with the same lot of *digene* HC2 HPV DNA Test kits on three different days with an identical reproducibility panel. The reproducibility panel included the following specimens: 12 denatured clinical STM specimen pools; three undenatured clinical PreservCyt Solution specimen pools; Negative Calibrator; and High- and

Low-Risk HPV Positive Calibrator at concentrations of 0.5 pg/ml, 1.0 pg/ml, 2.5 pg/ml, 5 pg/ml, and 10 pg/ml. All panel members were tested each day in triplicate using both the High-Risk HPV Probe and CPC methods. The results are shown in Table 12.

Table 12. Summary of overall statistics for multicenter reproducibility of the *digene* HC2 HPV DNA Test

Statistical Measure	High-Risk HPV Probe*	CPC*	Combined results of High-Risk HPV Probe and CPC*
Proportion of expected positives with an observed positive result	100% (99.0-100.0)	99.8% (98.92-100.0)	99.9% (99.38-100.0)
Proportion of expected negatives with an observed negative result	99.0% (97.49-99.73)	98.9% (96.79-99.77)	99.0% (97.88-99.58)
Agreement	99.5% (98.70-99.86)	99.5% (98.70-99.86)	99.5% (99.0-99.78)
Kappa	0.990	0.989	0.990

* Numbers in parentheses indicate 95% confidence intervals. Overall data are a combination of all assays at all sites.

This indicates that the reproducibility of the *digene* HC2 HPV DNA Test with clinical specimens is very good.

A second study was performed using simulated PreservCyt Solution specimens and conducted at two external laboratories and QIAGEN. Each testing laboratory performed two *digene* HC2 HPV DNA Tests (High-Risk HPV Probe only) per day on five different days. For each assay, a reproducibility panel of six simulated PreservCyt Solution specimens was individually processed and tested in quadruplicate. Each panel member was formulated by spiking cultured cells into PreservCyt Solution to yield an approximate RLU/CO value simulating two negatives (1N, 2N), two low positives (3P, 4P), one mid positive (5P), and one high positive (6P). Results are shown in *Table 13*.

Table 13. Summary of Overall Statistics for Multicenter Reproducibility for PreservCyt Solution Specimens using the High-Risk HPV Probe

Specimen	N	Mean RLU/CO	95% Confidence Interval	HPV Positive n (%)	HPV Negative n (%)
1N	120	0.17	0.01–0.33	0 (0.0)	120 (100.0)
2N	120	0.18	0.03–0.33	0 (0.0)	120 (100.0)
Total Negative	240			0 (0.0)	240 (100.0)
3P	120	4.97	3.46–6.48	120 (100.0)	0 (0.0)
4P	120	5.14	3.43–6.85	120 (100.0)	0 (0.0)
5P	120	33.1	19.47–46.73	120 (100.0)	0 (0.0)
6P	120	239.6	175.42–303.78	120 (100.0)	0 (0.0)
Total Positive	480			480 (100.0)	0 (0.0)

An additional in-house reproducibility study was performed using clinical PreservCyt Solution specimens obtained predominately from 252 women with cytology of ASC-US or greater (HPV prevalence 57%). Specimens were divided into two aliquots; each aliquot was then processed individually using the digene HC2 Sample Conversion Kit and then tested in duplicate with the High-Risk HPV Probe. As with other qualitative IVDs, variability of digene HC2 HPV DNA Test results obtained from clinical specimens is associated primarily with one or a combination of the following: 1) specimen collection; 2) specimen processing prior to testing; and 3) the testing procedure. Because the test results under comparison were obtained from the same clinical specimen, the experimental design controlled for variability due to specimen collection. The reproducibility of results obtained from two individually processed specimen aliquots from the same clinical specimen (referred to as "Between Processed Aliquots") reflects variation due to the combination of PreservCyt specimen conversion processing and the digene HC2 HPV DNA Test procedure. In contrast, the reproducibility of replicate results obtained from the same processed specimen aliquot (referred to as "Within Processed Aliquot") reflects variation from the digene HC2 HPV DNA Test procedure alone. The results are shown in Table 14.

Table 14. Summary of High-Risk HPV Probe Reproducibility PreservCyt Specimens, ASC-US or Greater Cytology

Reproducibility	Positive Agreement (n/N) 95% CI	Negative Agreement (n/N) 95% CI	Overall Agreement (n/N) 95% CI
Within a Processed Aliquot	97.59 (283/290) 95.09–99.02	94.39 (202/214) 90.41–97.07	96.23 (485/504) 94.18–97.72
Between Processed Aliquots	98.62 (285/289) 96.49–99.62	94.88 (204/215) 91.03–97.42	97.02 (489/504) 95.14–98.32

Because each specimen in the study generated four test results, there was insufficient volume remaining to allow for retesting of specimens in the defined Cutoff Region Retest Area; therefore, Table 14 presents initial data only. In Table 15, the results from this study are tabulated where only results outside the 1.0 to 2.5 RLU/CO Cutoff Retest Region are considered in the analysis. An assay user employing the Cutoff Region Retest Algorithm given in the Interpretation of Specimen Results section of these instructions for use would be expected to obtain results between those seen in Tables 14 and 15.

Table 15. Summary of digene HC2 HPV DNA Test (High-Risk HPV Probe only) Reproducibility of Test Results <1.0 or ≥2.5 RLU/CO PreservCyt Specimens, ASC-US or Greater Cytology

Reproducibility	Positive Agreement (n/N) 95% CI	Negative Agreement (n/N) 95% CI	Overall Agreement (n/N) 95% CI
Within a Processed Aliquot	99.26 (268/270) 97.35–99.91	99.51 (202/203) 97.29–99.99	99.37 (470/473) 98.16–99.87
Between Processed Aliquots	99.62 (265/266) 97.92–99.99	99.03 (204/206) 96.54–99.88	99.36 (469/472) 98.15–99.87

A multi-center clinical study was also conducted to estimate the additional contribution of cervical specimen sampling to digene HC2 HPV Test result variability. These results are summarized in Table 16. Paired PreservCyt Solution specimens were taken from each patient, processed separately using the digene HC2 Sample Conversion Kit, and then tested separately. Paired STM specimens were also collected and tested separately. Specimens were collected from female patients attending an OB/GYN clinic, colposcopy clinic, STD clinic, hospital, or family planning center. Four geographically diverse sites within the United States collected the PreservCyt specimens, and the STM specimens were all collected from a separate population from multiple clinics in metropolitan San Diego. Testing was performed at four accredited U.S. laboratories. Results for each specimen type were interpreted as recommended, i.e., PreservCyt Solution specimen testing employed a Cutoff Region Re-test Algorithm in the 1.0 to 2.50 RLU/CO range, while the initial test results were compared for STM specimens.

Note: These data do not equate to clinical false-positive or false-negative results due to the nature of the paired study design, which assesses duplicate specimen testing agreement.

Table 16. Clinical High-Risk HPV DNA Paired Specimen Test Reproducibility

Specimen Type	High-Risk HPV Prevalence	Positive Agreement (n/N) 95% CI	Negative Agreement (n/N) 95% CI	Overall Agreement (n/N) 95% CI
STM (initial)	29.2%	92.15 (270/293) 88.45–94.96	97.89 (695/710) 96.54–98.81	96.21 (965/1003) 94.84–97.31
PreservCyt (with Retest algorithm)	19.0%	88.37 (190/215) 83.31–92.33	97.53 (910/933) 96.20–98.35	95.82 (1099/1148) 94.40–96.83

These results reflect variability associated with specimen collection, in addition to the variability due to specimen processing and the assay procedure. Further inspection of these results

revealed that result variability was concentrated in the assay cutoff retest region. When only specimens yielding results outside the cutoff region are included in the analysis, positive agreement for PreservCyt Solution specimens increases to 94.0% while negative agreement increases to 99.4%; while similarly for STM specimens, the positive and negative agreement values increase to 97.5% and 99.6%, respectively.

Cross-Reactivity

Cross-reactivity panel

A battery of bacteria, viruses, and plasmids commonly found in the female anogenital tract, as well as a collection of cutaneotropic HPV types for which clones were available, were assayed to determine if cross-reactivity would occur with the HPV probes used in the *digene* HC2 HPV DNA Test. All microorganisms were assayed at concentrations of 10^5 and 10^7 organisms per ml. Purified DNA of viruses and plasmids were assayed at a concentration of 4 ng per ml.

Below is a list of the bacteria tested. All bacteria tested negative in the *digene* HC2 HPV DNA Test.

Acinetobacter anitratus	Mycoplasma hominis
Acinetobacter lwoffi (ATCC 17908)	Mycoplasma hyorhinis
Bacteroides fragilis (ATCC 25285)	Neisseria gonorrhoeae (ATCC 19424)
Bacteroides melaninogenicus	Neisseria lactamica (NRL 2118)
Candida albicans (ATCC 14053 or 10231)	Neisseria meningitidis (ATCC 13077)
Chlamydia trachomatis	Neisseria sicca (ATCC 29256)
Enterobacter cloacae	Peptostreptococcus anaerobius
Escherichia coli (HB101)*	Proteus vulgaris (ATCC 21117, 8427, 33420)
Escherichia coli	Serratia marcescens
Fusobacterium nucleatum	Staphylococcus aureus (Cowan strain)
Gardnerella vaginalis	Staphylococcus epidermidis
Haemophilus ducreyi	Streptococcus faecalis (ATCC 14508)
Klebsiella pneumoniae	Streptococcus pyogenes (ATCC 27762)
Lactobacillus acidophilus	Treponema pallidum
Mobiluncus curtisii	Trichomonas vaginalis
Mobiluncus mulieris	Ureaplasma urealyticum

* Both the *E. coli* strain used to grow plasmids (HB101) and a clinical isolate of *E. coli* were assayed.

Below is a list of the viral or plasmid DNA or human serum tested:

Adenovirus 2	Human Papillomavirus type 1
Cytomegalovirus	Human Papillomavirus type 2
Epstein-Barr Virus	Human Papillomavirus type 3
Hepatitis B surface antigen-positive serum	Human Papillomavirus type 4
Herpes Simplex I	Human Papillomavirus type 5
Herpes Simplex II	Human Papillomavirus type 8
Human Immunodeficiency Virus (HIV, RT DNA)	Human Papillomavirus type 13
Simian Virus type 40 (SV40	Human Papillomavirus type 30
	pBR322

The only viruses or plasmids that showed cross-reactivity in the *digene* HC2 HPV DNA Test were HPV type 13 and plasmid pBR322. HPV type 13 DNA reacted with the Low-Risk HPV Probe only. HPV 13 is commonly detected in lip lesions of certain ethnic groups, but has not been detected in the anogenital tract.[38] Thus, the cross-reactivity observed between HPV 13 and Low-Risk HPV Probe would not be expected to cause a clinically confusing result for anogenital specimens. Cross-reactivity between pBR322 and Low-Risk and High-Risk HPV Probes is not unexpected because it is difficult to remove all of the vector pBR322 DNA when isolating the HPV insert. The presence of pBR322 homologous sequences has been reported in human genital specimens, and false-positive results could occur in the presence of high levels of bacterial plasmid. However, 298 clinical specimens testing positive with the Low-Risk and High-Risk HPV Probes showed that no positive results were due to pBR322 when tested with a pBR322 probe. Thus, the likelihood of *digene* HC2 HPV DNA Test false-positive result due to homologous pBR322 sequences in clinical specimens appears to be low.

Cross-hybridization

Each of the 18 HPV types was tested with both Low-Risk and High-Risk HPV Probes at concentrations of 4 ng/ml of HPV DNA. All of the HPV targets were expected to be positive with the appropriate probe group whereas none of the specimens were expected to be positive with the opposite probe group. This study demonstrated that there is a small amount of cross-hybridization between HPV types 6 and 42 (low-risk HPV types) and the high-risk probe group. Specimens with high levels (4 ng/ml or higher) of HPV 6 or HPV 42 DNA may be positive for both probe groups. The clinical significance of this is that patients with 4 ng/ml or higher of HPV 6 or HPV 42 DNA may be referred to colposcopy. In addition, the High-Risk HPV Probe has been shown to cross-react with HPV types 40, 53, and 66. These types are rare, and there is insufficient evidence to establish the exact correlation between infection with these types and development of high-grade disease.

Effect of blood and other substances on STM specimens

The effect of blood and other potentially interfering defined or undefined substances was evaluated in the *digene* HC2 HPV DNA Test. Whole blood, douche, anti-fungal cream, and contraceptive jelly (agents that may commonly be found in cervical specimens) were added to STM-negative and STM-positive specimens (clinical specimen pools and non-clinical specimens) at concentrations that may be found in cervical specimens. No false-positive results were observed with any of the four agents at any concentration. However, a false-negative result may be reported in clinical specimens with HPV DNA levels close to that of the positive cutoff for the assay (1 pg/ml) if high levels of anti-fungal cream or contraceptive jelly were present. However, it is very unlikely that a clinical specimen will consist almost entirely of one of these substances because the cervix is routinely cleared prior to obtaining specimens for Pap smear and for HPV testing.

Effect of blood and other substances on PreservCyt solution specimens

The effect of blood and other potentially interfering defined or undefined substances potentially present in PreservCyt Solution clinical specimens was evaluated in the *digene* HC2 HPV DNA Test. Whole blood, douche, anti-fungal cream, and contraceptive jelly (agents that may commonly be found in cervical specimens) were added to PreservCyt-Solution-negative and -positive clinical specimen pools at concentrations that may be found in cervical specimens. No false-positive or false-negative results were observed with any of the four agents at any concentration. Furthermore, substances inherent in some clinical specimens do not inhibit the detection of the HPV DNA by the *digene* HC2 HPV DNA Test.

Reproducibility of *digene* HC2 HPV DNA Test with clinical specimens collected in STM

The reproducibility of the *digene* HC2 HPV DNA Test with clinical specimens collected in STM was determined in a study using 20 clinical pools (10 positive and 10 negative) prepared by combining previously denatured and tested cervical brush specimens collected in STM. Specimens were tested in replicates of four on each of five days for a total of 20 replicates per specimen. Testing was performed using the Combined-Probe Cocktail method. Mean, standard deviation and 95% confidence interval (CI) about the mean were calculated for each specimen within day and over five days and are shown in *Table 17* below.

Table 17. Mean RLU/CO with Confidence Intervals and Percent Positive (Descending Order by Mean RLU/CO)

No.	Spec. ID	Mean RLU/CO	CI	% Positive
1	10	3.18	3.02–3.35	100 (20/20)
2	20	1.43	1.36—1.50	100 (20/20)
3	11	1.25	1.20–1.28	100 (20/20)
4	12	1.21	1.15–1.27	100 (20/20)
5	15	1.20	1.14–1.25	100 (20/20)
6	13	1.07	1.01–1.11	80 (16/20)
7	16	1.06	1.01–1.09	75 (15/20)
8	17	1.04	1.00–1.06	80 (16/20)
9	14	0.98	0.92–1.02	45 (9/20)
10	18	0.92	0.87–0.96	20 (4/20)
11	19	0.72	0.68–0.75	0 (0/20)
12	7	0.40	0.33–0.46	0 (0/20)
13	4	0.38	0.35–0.39	0 (0/20)
14	9	0.37	0.32–0.41	0 (0/20)
15	1	0.35	0.32–0.36	0 (0/20)
16	2	0.35	0.31–0.37	0 (0/20)
17	8	0.32	0.29–0.34	0 (0/20)
18	3	0.30	0.27–0.31	0 (0/20)
19	6	0.27	0.24–0.30	0 (0/20)
20	5	0.26	0.23–0.28	0 (0/20)

For the five specimens with a mean RLU/CO at 20% or more above the cutoff (Nos. 1–5), 100 of 100 replicates (100.0%) were positive. For the five specimens with a mean RLU/CO within 20% above or below the assay cutoff (Nos. 6–10), 60 of 100 (60%; 95% CI = 49.7-69.6) of the replicates were positive and 40 of 100 (40%) were negative. For the 10

specimens with the mean RLU/CO at more than 20% below the assay cutoff, 200 of 200 replicates (100%) were negative.

Thus, specimens with a mean RLU/CO of 20% or more above the cutoff were positive 100% of the time, while specimens with a mean RLU/CO of 20% or more below the cutoff were negative 100% of the time, indicating that specimens at 20% or more away from the cutoff can be expected to yield consistent results. Specimens close to the cutoff yielded approximately equal numbers of positive and negative results. These data demonstrate that STM specimens yield reproducible results in the *digene* HC2 HPV DNA Test.

Reproducibility of PreservCyt solution specimens in the *digene* HC2 HPV DNA Test

The reproducibility of clinical specimens in PreservCyt Solution in the *digene* HC2 HPV DNA Test was determined in a study using 24 mock specimens at a concentration spanning a range of HPV DNA concentrations. Specimens consisted of PreservCyt Solution and white blood cells, with and without HPV 16 plasmid-containing bacteria.

Specimens were tested in replicates of four on each of five days, for a total of 20 replicates per specimen. On each of the five days of the study, an 8-ml aliquot from each specimen was processed and tested according to the *digene* HC2 Sample Conversion Kit instructions for use instructions using High-Risk HPV Probe only. Mean, standard deviation, and 95% confidence interval (CI) were calculated for each specimen within day and over all five days and replicates. The mean RLU/CO, confidence interval about the mean, and the percent of positive replicates is shown below in *Table 18* for each specimen, in descending order based on the mean RLU/CO.

Table 18. Mean RLU/CO with Confidence Intervals and Percent Positive (Descending Order by Mean RLU/CO)

No.	Spec. #	Mean RLU/CO	CI	% Positive
1	21	3.51	3.19–3.83	100 (20/20)
2	12	1.58	1.48–1.69	100 (20/20)
3	13	1.42	1.32–1.52	100 (20/20)
4	17	1.38	1.23–1.53	90 (18/20)
5	18	1.36	1.23–1.48	95 (19/20)
6	15	1.32	1.16–1.49	85 (17/20)
7	23	1.17	1.06–1.27	75 (15/20)
8	16	1.14	1.07–1.20	75 (15/20)
9	20	1.10	0.96–1.21	85 (17/20)
10	19	1.06	0.95–1.17	45 (9/19)
11	22	1.05	0.99–1.10	70 (14/20)
12	11	1.04	0.96–1.11	65 (13/20)
13	14	0.94	0.86–1.01	25 (5/20)
14	24	0.77	0.73–0.81	0 (0/20)
15	3	0.28	0.25–0.30	0 (0/20)
16	1	0.27	0.24–0.30	0 (0/20)
17	7	0.27	0.25–0.30	0 (0/20)
18	2	0.27	0.25–0.28	0 (0/20)
19	5	0.26	0.24–0.28	0 (0/20)
20	4	0.24	0.22–0.25	0 (0/20)
21	9	0.23	0.21–0.25	0 (0/20)
22	8	0.22	0.18–0.27	0 (0/20)
23	10	0.22	0.20–0.25	0 (0/20)
24	6	0.19	0.17–0.21	0 (0/20)

For the six specimens with a mean RLU/CO at 20% or more above the cutoff (Nos. 1–6), 114 of 120 replicates (95.0%) were positive. For the seven specimens with a mean RLU/CO within 20% above or below the assay cutoff (Nos. 7–13), 88 of 139 (63.3%; 95% CI = 54.3 -70.9) of the replicates were positive and 51 of 139 (36.6%), 41 of 80 (51.3%; 95% CI = 39.8–62.6) of the replicates were positive and 39 (48.7%) were negative. For the 11 specimens with the mean RLU/CO at more than 20% below the assay cutoff, 220 of 220 replicates (100%) were negative.

Thus, specimens with a mean RLU/CO of 20% or more above the cutoff were positive greater than 95% of the time, while specimens with a mean RLU/CO of 20% or more below the cutoff were negative 100% of the time, indicating that specimens at 20% or more away from the cutoff can be expected to yield consistent results. Specimens close to the cutoff yielded approximately equal numbers of positive and negative results. These data demonstrate that PreservCyt Solution specimens yield reproducible results in the *digene* HC2 HPV DNA Test.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Sufficient for n sample preparations
	For in vitro diagnostic use
	Lot number
	Catalog number
	Global Trade Item Number
	Use by
	Temperature limitation
	Consult instructions for use
	Material Number
	Manufacturer
	USA Only: For Prescription Use Only

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
Improper or no color change observed during denaturation.		
a.)	Denaturation Reagent not added, or Denaturation Reagent not prepared properly.	Verify that the Denaturation Reagent contains the Indicator Dye and is a dark purple color. Verify that Denaturation Reagent was added to the specimen by measuring the specimen volume (1.5 ml is expected). If the volume indicates that Denaturation Reagent was not added, make the appropriate addition, mix and proceed with the assay if the proper color change is then observed.
b.)	Specimen contains blood or other materials that mask the color change.	The exact color change described is not expected with these types of specimens; assay test results should not be adversely affected.
c.)	Specimen pH may be unusually acidic.	If neither of the other causes applies, the specimen may be unusually acidic, and the expected color change will not occur. Collect a new specimen prior to the application of acetic acid to the cervix because improper specimen pH will adversely affect the test results.
Quality Controls give incorrect results		
a.)	Incorrect software protocol chosen for test (e.g., used LR protocol for HR method)	If the software protocol is incorrect for the test being performed, the plate should be read again within 30 minutes after Detection Reagent 2 addition and with the correct protocol.
b.)	Reverse placement of QC1-LR and QC2-HR	Retest Specimens.

Improper color change observed during hybridization.

a.)	Inadequate mixing of probe cocktail with denatured calibrators, quality controls, and/or specimens; or, Probe cocktail not added; or, incorrect volume of reagent added	Shake hybridization microplate or microtube rack for an additional 2 minutes. If there are wells that still remain purple, add an additional 25 µl of the appropriate Probe Cocktail and mix well. If, upon probe addition and remixing, the proper color change does not occur, and the specimen did not contain blood or other materials, retest the specimen.
b.)	Specimen contains blood or other materials that mask the color change.	The exact color change described is not expected with these types of specimens; assay test results should not be adversely affected.
c.)	Specimen had < 1000 µl STM.	Check the volume of the original specimen. Volume should be 1350 µl ± 20 µl (after removing 75 µl each for Low-Risk and High-Risk HPV Probes). If volume is < 1350 µl, original specimen contained < 1000 µl STM. Obtain a new specimen.

Assay fails validation criteria. No signal observed in Calibrator, Quality Controls, or in specimens.

a.)	No Probe added to Probe Diluent.	Prepare Probe Cocktails as described in these instructions for use. Label tubes carefully.
b.)	Probe contaminated with RNase during preparation.	Use aerosol-barrier pipette tips when pipetting probe and wear gloves.
c.)	Inadequate mixing of Probe and Probe Diluent.	After adding Probe to Probe Diluent, mix very thoroughly by vortexing at high speed for at least 5 seconds. A visible vortex must be produced.
d.)	Inadequate mixing of diluted Probe and denatured specimen.	After adding Probe Cocktail and specimen to each Hybridization Microwell or microtube, shake on Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. Check for color change from purple to yellow in every tube or well.
e.)	Incorrect time or temperature during hybridization step.	Hybridize for 60 ± 5 minutes at 65 ± 2°C. Check temperature of Microplate Heater I or hybridization water bath. Ensure the Microplate Heater I is set to heat specimens to correct temperature and is preheated for 60 minutes prior to use. Ensure that water level is adequate to heat specimens to correct temperature. Water baths should be calibrated periodically.

f.)	Inadequate mixing during capture step.	Shake on the Rotary Shaker I for 60 ± 5 minutes at 20°–25°C as described in these instructions for use. Verify shaker speed by calibration as outlined in the *Shaker Speed Calibration* section of *Rotary Shaker I User Manual*.
g.)	Switched Probes/Probe Cocktails/Hybridization Tubes.	Prepare Probe Cocktails carefully and label Probe Cocktail tubes accordingly. Be careful to add the correct Probe to the correct set of Hybridization Tubes or microwells. Label Probe Cocktail tubes, Hybridization Tubes and/or racks to minimize the potential for switches.
h.)	Failure to add correct amount of Detection Reagent 1 or to incubate for specified time.	Pipette 75 µl Detection Reagent 2 into each well using an eight-channel pipettor. Incubate 20°–25°C for 15–30 minutes.
i.)	Luminometer malfunction or incorrect programming.	Refer to the maintenance/service and troubleshooting sections in the applicable *digene* assay analysis software User Manual for further instructions, or call QIAGEN Technical Services.

Elevated RLU values in Calibrators, Quality Controls, and/or specimens (≥ 200 RLUs in many or all wells). Assay may fail validation criteria.

a.)	Denaturation Reagent not added; or, incorrect volume of reagent added; or, inadequate mixing of Denaturation Reagent with Calibrators, Quality Controls, or specimens.	Verify that the repeating pipettor is delivering accurately prior to adding Denaturation Reagent. Calibrated pipettors are essential. Add a half-volume of Denaturation Reagent to each tube and mix well. To avoid false-positive results, make sure liquid washes entire inner surface of tube. Calibrators, Quality Controls, and specimens should turn purple after addition of Denaturation Reagent.
b.)	Light leak in the luminometer Door not sealed Seal around door broken	Perform a background reading (raw data measurement) of the luminometer by reading an empty microplate. A reading of greater than 50 RLUs indicates that a light leak may exist. Refer to the maintenance/service and troubleshooting sections in the applicable *digene* assay analysis software User Manual for further instructions, or call QIAGEN Technical Services.
c.)	Contamination of Detection Reagent 2 or capture microwells by Detection Reagent 1 or exogenous alkaline phosphatase.	Refer to Contamination Check in this Troubleshooting section.
d.)	Contaminated Wash Buffer.	Refer to Contamination Check in this Troubleshooting section.
e.)	Contaminated Automated Plate Washer.	Refer to Contamination Check in this Troubleshooting section.

f.)	Inadequate washing of capture microwells after Detection Reagent 1 incubation.	Wash microwells thoroughly with Wash Buffer six times, filling wells to overflowing each time or using Automated Plate Washer. There should be no residual pink liquid visible in the wells after washing. See *Automated Plate Washer User Manual* for instructions on testing for contamination or malfunctions.
g.)	Detection Reagent 1 contamination of microwells.	Ensure all work surfaces are clean and dry. Use care when using Detection Reagent 1. Avoid aerosols.
h.)	Blotting hybridization solution on same area of Kimtowels Wiper.	Do not reblot on previously used area of Kimtowels Wiper.
i.)	Used incorrect blotting towels	Use Kimtowels Wiper or equivalent low-lint paper towel for blotting.
j.)	Quality control material used as Positive Calibrator. Assay fails validation Calibrator/Negative Calibrator ratio > 15	Ensure correct placement of Positive Calibration and Quality Control materials.

Low PC/NC ratios or high number of low positive specimens with ratios <2.0 (> 20%). Assay may fail validation criteria.

a.)	Inadequate specimen preparation.	Add the appropriate volume of Denaturation Reagent and mix thoroughly by vortexing. To avoid false-positive results, make sure liquid washes entire inner surface of tube with both the manual and MST Vortexer methods (for the manual vortexer method, invert tube one time). For PreservCyt Solution specimens, ensure proper mixing and resuspension of the cell pellet is completed prior to denaturation incubation. Consult *digene* HC2 Sample Conversion Kit instructions for use for protocol details. A distinct color change from clear to dark purple should be seen. Incubate for 45 ± 5 minutes at 65 ± 2°C.
b.)	Probe inadequately mixed or insufficient Probe added to assays.	Prepare Probe Cocktails as described. Mix thoroughly by vortexing ensuring that a visible vortex is produced. Probe Cocktails must be added to tubes with a positive displacement pipettor or a multichannel pipettor to ensure accurate delivery.
c.)	Inadequate volume of diluted Probe added to each Hybridization microwell or microtubes.	Verify that the pipettor is delivering accurately prior to adding Probe Cocktail to Hybridization Microplate or microtubes. Twenty-five µl of diluted Probe should be added to the denatured specimen at the bottom of each microwell or microtube. Verify that the eight-channel pipettor is delivering accurately prior to adding probe cocktail to hybridization wells. Color change should be from dark purple to yellow upon addition and thorough mixing of Probe Cocktail. PreservCyt Solution specimens should turn pink instead of yellow.
d.)	Loss of Detection Reagent 1 activity.	Store Detection Reagent 1 at 2°–8°C. Use before the expiration date on the kit outer box label.

e.)	Insufficient capture.	The capture step should be performed using the Rotary Shaker I set at 1100 ± 100 rpm for 60 ± 2 minutes. Validate shaker speed by calibration.
f.)	Inadequate washing.	Wash microwells thoroughly with Wash Buffer six times, filling the wells to overflowing each time or using the Automated Plate Washer.
g.)	Contaminated Wash Buffer.	If Detection Reagent 2 is not contaminated, check Wash Buffer for contamination. Pipette 10 µl Wash Buffer into 75 µl Detection Reagent 2 in a blank capture microwell. Cover and incubate 15 minutes at 20°–25°C. Read microwell on the luminometer. Readings above 200 RLUs indicate contamination. See *Reagent Preparation and Storage* section for instructions on cleaning and maintenance of Wash Apparatus. See *Automated Plate Washer User Manual* for instructions on testing for contamination or malfunctions.

Series of positive specimens with RLU values approximately the same.

a.)	Contamination of capture microwells during assay manipulation.	Cover Capture Microplate during all incubations. Avoid exposing tubes to aerosol contamination while performing the assay. Wear powder-free gloves during manipulations.
b.)	Detection Reagent 2 contamination.	Be careful not to contaminate the stock when pipetting Detection Reagent 2 into capture microwells. Avoid contamination of Detection Reagent 2 by aerosols from Detection Reagent 1 or from laboratory dust, etc.
c.)	Automated Plate Washer malfunction.	See *Automated Plate Washer User Manual* for instructions on testing for contamination or malfunctions.

Wide %CVs between replicates.

a.)	Inaccurate pipetting.	Check pipettor to ensure that reproducible volumes are being delivered. Calibrate pipettors routinely.
b.)	Insufficient mixing.	Mix thoroughly at all steps. Vortex prior to denaturation incubation and after adding Probe Cocktail. Ensure that a visible vortex is produced.
c.)	Incomplete transfer of liquid from hybridization microwells or microtubes to capture microwells.	Take care during transfer step from hybridization microwells or microtubes to capture microwells to ensure reproducible volumes are transferred.
d.)	Improper washing conditions.	Wash microwells thoroughly with Wash Buffer six times, filling to overflowing each time or using Automated Plate Washer and proper Automated Plate Washer protocols.
e.)	Detection Reagent 1 contamination of microwells.	Ensure all work surfaces are clean and dry. Use care when using Detection Reagent 1. Avoid aerosols.

False-positive results obtained from known negative specimens.

a.)	Detection Reagent 2 contaminated.	Be careful not to cross-contaminate specimens as you aliquot Detection Reagent 2 between specimens. If only using part of a kit, aliquot the volume needed for that assay into a clean, disposable reagent reservoir prior to filling the pipettor.
b.)	Detection Reagent 1 contamination of microwells	Wash microwells thoroughly with Wash Buffer six times, filling to overflowing each time or using Automated Plate Washer. There should be no residual pink liquid visible in the microwells after washing.
c.)	Contamination of pipette tip with undenatured material during transfer of denatured specimen to the microtube or microplate well used for HPV probe hybridization.	The denaturation step of the specimen processing procedure must be performed as directed in these instructions for use. Improper specimen vortexing, tube inversion, and agitation can result in incomplete denaturation of non-specific RNA/DNA hybrids endogenous to cervical specimens. When using PreservCyt Solution specimens in particular, these hybrids are likely to be present on the inside walls of the specimen denaturation tube. In order to prevent possible carryover of this non-denatured cellular material, the micro-pipette tip must not touch the sides of the specimen denaturation tube during transfer of the denatured specimen to the microtube or microplate well used for HPV probe hybridization.
d.)	During decanting and blotting of the Capture Microplate, the microplate was blotted on the same area of the Kimtowels Wipers or equivalent low-lint paper towels.	Do not blot on area that has been previously used as cross-contamination could occur.
e.)	Inadequate specimen preparation.	Add the appropriate volume of Denaturation Reagent and mix thoroughly by vortexing. To avoid false-positive results, make sure liquid washes entire inner surface of tube with both the manual and MST Vortexer methods (for the manual vortex method, invert tube one time). For PreservCyt Solution specimens, ensure proper mixing and resuspension of the cell pellet is completed prior to denaturation incubation. Consult *digene* HC2 Sample Conversion Kit instructions for use for protocol details. For all specimens, a distinct color change to dark purple should be seen. Incubate for 45 ± 5 minutes at 65 ± 2°C.
f.)	Improper washing conditions.	Wash microwells thoroughly with Wash Buffer six times, filling the wells to overflowing each time or using the Automated Plate Washer and proper Automated Plate Washer protocols.

Elevated Negative Calibrator RLU values (> 200 RLUs). Remainder of assay performs as expected.

g.)	Detection Reagent 2 was incubated at a temperature greater than 20-25°C.	Repeat the test and ensure that Capture and Detection steps are incubated at 20°–25°C.
h.)	Detection Reagent 2 was incubated longer than 30 minutes.	Read plate after 15 minutes of incubation (and no later than 30 minutes of incubation) at 20°–25°C.

i.)	Detection Reagent 2 or Wash Buffer was contaminated with alkaline phosphatase or Detection Reagent 1.	Check aliquoted Detection Reagent 2 for contamination by pipetting 75 µl into a blank capture microwell. Incubate 20°–25°C for 15 minutes and read on the luminometer. Readings above 200 RLUs indicate Detection Reagent 2 contamination. Take care when pipetting Detection Reagent 2. Wear gloves and avoid touching tips to any work surfaces. Repeat troubleshooting procedure on the master vial of Detection Reagent 2, and if not contaminated, repeat assay using this material. If contaminated, obtain a new kit and repeat assay. If Detection Reagent 2 is not contaminated, check the Wash Buffer for contamination. Pipette 10 µl of Wash Buffer into 75 µl of Detection Reagent 2 into a blank capture microwell. Cover and incubate 15 minutes at 20°–25°C. Read microwell on the luminometer. Readings above 200 RLUs indicate Wash Buffer contamination. See *Reagent Preparation and Storage* section for instructions on cleaning and maintenance of Wash Apparatus.

Contamination check

Reagent Evaluated	Contamination Check Procedure	Interpretation of Results
Detection Reagent 2	**Note:** Take care when pipetting Detection Reagent 2 to avoid contamination. Wear gloves and avoid touching pipette tips on any work surfaces. ● Pipette 75 µl of the aliquoted, residual and/or original vial of Detection Reagent 2 into a blank Capture Microplate well. ● Incubate 20-25°C for 15 minutes. Avoid direct sunlight. ● Read in the Microplate wells in the luminometer. **Note:** Testing the Detection Reagent 2 in replicates of 3 provides optimal assessment of performance.	● The Detection Reagent 2 Control should be < 50 RLUs. ● If Detection Reagent 2 values are < 50 RLUs, the Detection Reagent 2 can be used to repeat the assay. ● If contaminated (>50 RLUs), obtain a new kit and repeat assay.
Wash Buffer Apparatus and/or Water Source	● Pipette 75 µl of Detection Reagent 2 into 3 separate Capture Microplate wells. ● Label wells 1-4. ● Well 1 serves as the Detection Reagent 2 control. ● Pipette 10 µl of Wash Buffer from the wash bottle into well 2. ● Allow Wash Buffer to flow through the washer tubing. ● Pipette 10 µl of Wash Buffer from the tubing into well 3. ● Obtain an aliquot of the water used to prepare Wash Buffer. Pipette 10 µl of the water into well 4. ● Incubate 20-25°C for 15 minutes. Avoid direct sunlight. ● Read the Microplate wells in the luminometer.	● The Detection Reagent 2 Control (well 1) should be < 50 RLUs. ● Compare the RLU value from wells 2, 3 and 4 to the Detection Reagent 2 control RLU value (well 1). The individual RLU values for wells 2, 3 and 4 should not exceed 50 RLUs of the Detection Reagent 2 control RLU value (well 1). ● Values exceeding 50 RLUs of the Detection Reagent 2 control indicate contamination. See *Reagent Preparation and Storage* section for instructions on cleaning and maintenance of Wash Apparatus.

Reagent Evaluated	Contamination Check Procedure	Interpretation of Results
Automated Plate Washer	• Pipette 75 µl of Detection Reagent 2 into 5 separate Capture Microplate wells. • Label wells 1-5. • Well 1 serves as the Detection Reagent 2 control. • Pipette 10 µl of Wash Buffer from the plate washer bottle labeled *Wash* into well 2. • Pipette 10 µl of the rinse liquid from the plate washer bottle labeled *Rinse* into well 3. • Depress the Prime key on the Plate Washer key pad, allowing wash buffer to flow through the lines. • Pipette 10 µl of the wash buffer from the trough into well 4. • Press the Rinse key on the Plate Washer key pad, allowing the rinse liquid to flow through the lines. • Pipette 10 µl of Wash Buffer from the trough into well 5. • Cover and incubate 15 minutes at 20-25°C. Avoid direct sunlight. • Read the Microplate wells in the luminometer.	• The Detection Reagent 2 Control (well 1) should be < 50 RLUs. • Compare the RLU value from wells 2, 3, 4 and 5 to the Detection Reagent 2 control RLU value (well 1). The individual RLU values for wells 2, 3, 4 and 5 should not exceed 50 RLUs of the Detection Reagent 2 control RLU value (well 1). • Values exceeding 50 RLUs of the Detection Reagent 2 control indicate contamination of the Plate Washer. • See Automated Plate Washer User Manual, Decontamination Procedure section.

Ordering Information

Product
digene HC2 HPV DNA Test
digene HC2 DNA Collection Device
digene HC2 Sample Conversion Kit
digene Wash Buffer Concentrate
Hologic PreservCyt Solution
Microtubes
Microtube Rack
Plate Sealers
Extra-long Pipette Tips
Specimen Collection Tube Rack
Specimen Collection Tube Screw Caps
Disposable Reagent Reservoirs
Specimen Collection Tubes
Microplate Lids
Multi-Specimen Tube Rack and Lid
DuraSeal Film Tube Sealer
Tube Sealer Dispenser and Sealer Cutter
Hybridization Microplate
Microplate well strips

Luminometer

PC System

Printer Cable

Printer

digene Hybrid Capture 2 System Software (with *digene* Hybrid Capture 2 System Software, *digene* Hybrid Capture 2 System Assay Protocols for HPV, LumiCheck Plate Software)

Hybrid Capture System Rotary Shaker I

Hybrid Capture System Microplate Heater I

Hybrid Capture System Automated Plate Washer

Hybrid Capture System Multi-Specimen Tube (MST) Vortexer 2

Wash Apparatus

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user manual. QIAGEN kit instructions for use and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
R4, 01/2020	• Updated GHS information to be compliant with regulations • Added Symbols section • Removed Patents and added updated Trademarks and Limited License Agreement • Updated to Sample to Insight Branding

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