HC *Hybrid Capture® 2*

HC2 High-Risk HPV DNA Test

Digene Catalog Number 5101-1296

For *In Vitro* Diagnostic Use

A Nucleic Acid Hybridization Microplate Assay with Signal Amplification for the Chemiluminescent Detection of Human Papillomavirus (HPV) Types 16,18,31,33,35,39,45,51,52,56,58,59 and 68 in Cervical Specimens

Reagents for 96 Tests

To be used in conjunction with:

Digene Cervical Sampler™
Digene Specimen Transport Medium
Cytyc ThinPrep® Pap Test™ PreservCyt® Solution

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Table of Contents

NAME AND INTENDED USE

The *HC2 High-Risk HPV DNA Test* using *Hybrid Capture®* 2 (HC2) technology is a nucleic acid hybridization assay with signal amplification using microplate chemiluminescence for the qualitative detection of thirteen high-risk types of human papillomavirus (HPV) DNA in cervical specimens. The HPV types detected by the assay are the high-risk types 16/18/31/33/35/39/45/51/52/56/58/59/68. The *HC2 High-Risk HPV DNA Test* cannot determine the specific HPV type present.

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

Cervical specimens which may be tested with the *HC2 High-Risk HPV DNA Test* include the following:

- Specimens collected with the *Digene Cervical Sampler*
- Biopsies collected in *Digene Specimen Transport Medium*
- Specimens collected using a broom type collection device and placed in Cytyc PreservCyt® Solution (refer to the *Digene Sample Conversion Kit* package insert for complete details).

The use of this test is indicated:

1. To screen patients with ASCUS (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.

2. In women 30 years and older the HC2 High-Risk HPV DNA Test can be used with Pap to adjunctively screen to assess the presence or absence of high-risk HPV types. This information, together with the physician's assessment of cytology history, other risk factors, and professional guidelines, may be used to guide patient management.

WARNING

- The *HC2 High-Risk HPV DNA Test* is not intended for use as a screening device for Pap normal women under age 30 and is not intended to substitute for regular Pap screening.
- There is insufficient evidence to indicate whether a single WNL Pap result with concurrent negative high risk HPV result confers low risk similar to consecutive annual, technically adequate WNL Pap results.
- Detection of HPV using HC2 does not differentiate HPV types or infection with more than one type, and cannot evaluate persistence of any one type.
- The use of this test has not been evaluated for the management of women with prior cytologic or histologic abnormalities, hysterectomy, who are postmenopausal, or who have other risk factors (e.g., HIV+, immunocompromised, DES exposure, history of STI).

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

Another Digene kit, the Digene *HC2 HPV DNA Test (Catalog Number 5101-1096)*, which detects both high-risk (using the kit's High-Risk Probe) and some low-risk HPV types (using the kit's Low-Risk Probe A **should not** be used as an adjunct for screening, because low-risk types are not associated with risk of cervical cancer. Only the *HC2 High-Risk HPV DNA Test* should be used as an adjunct for screening.

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[3,44]. 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[40,44,49,53,59]. 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.[70,71]

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 HPVs[8,44,52]. HPV types 31, 33, and 35 have been demonstrated to have an intermediate association with cancer[14,44]. 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.[42,44] These five HPV types together account for about 80% of cervical cancers[21,36,44]. 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[17,32-37,44].

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.[21,31,44] Prospective studies (age 16-60 years) have shown that 15-28% of HPV DNA positive women developed cytologic (Pap smear) interpretations of squamous intraepithelial lesions (SIL) suggestive of CIN 1-3 or cancer within 2 years compared to only 1-3% of HPV DNA negative women .[22,23,49] In particular, the risk of progression for HPV types 16 and 18 was greater (approximately 40%) than for other HPV types[22,49,50,52,59]. Most SIL was low-grade.

Very few HPV DNA positive women develop cytologic high-grade SIL (HSIL) indicating underlying CIN 2-3 or cancer.[68] The absolute risk of developing an incident cytologic abnormality following an HPV infection with types detected by HC2 has not been adequately described, and is known to vary in different populations.[59]

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[44-46, 49-50, 52-57], 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 HR HPV negative appear to be at low risk for having or developing cervical precancerous lesions.[47,50,51,53]

A negative *HC2 HR HPV Test* result with a concurrent normal Pap result implies low risk at a single point in time for the development of cervical neoplasia and is therefore clinically meaningful for assessing risk; however there are insufficient data to establish a definitive time period over which this lower risk is clinically relevant.

PRINCIPLE OF THE PROCEDURE

The *HC2 High-Risk 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 which 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 indicates the presence of high-risk HPV DNA sequences in the specimen. An RLU measurement less than the Cutoff Value indicates the absence of the specific high-risk HPV DNA sequences tested or HPV DNA levels below the detection limit of the assay.

REAGENTS AND MATERIALS PROVIDED

5101-1296	**Catalog Number**
	96 Tests
1 x 0.35 ml	**Indicator Dye:** Contains sodium azide.
1 x 50 ml	**Denaturation Reagent:** Dilute sodium hydroxide (NaOH) solution.
1 x 7 ml	**Probe Diluent:** Buffered solution with sodium azide.
1 x 150 µl	**High-Risk HPV Probe:** HPV 16/18/31/33/35/39/45/51/52/56/58/59/68 RNA probe cocktail in buffered solution (red cap).
1 x 2.0 ml	**Negative Control:** Carrier DNA in Specimen Transport Medium (STM) with sodium azide.
1 x 1.0 ml	**High-Risk HPV Calibrator:** 1.0 pg/ml cloned HPV 16 DNA and carrier DNA in STM with sodium azide (red cap).
1 each	**Capture Microplate:** Coated with goat polyclonal anti-RNA:DNA hybrid antibodies.
1 x 12 ml	**Detection Reagent 1:** Alkaline phosphatase-conjugated antibodies to RNA:DNA hybrids in buffered solution with sodium azide.
1 x 12 ml	**Detection Reagent 2:** CDP-Star® with Emerald II (chemiluminescent substrate).
1 x 100 ml	**Wash Buffer Concentrate:** Contains sodium azide.

NOTE: Positive Assay Controls are not supplied with this kit. However, control material is available from Digene.

MATERIALS REQUIRED BUT NOT SUPPLIED

Hybrid Capture System Equipment[2]

DML 2000™ Instrument and PC System
Rotary Shaker I with adjustable speed setting
Microplate Heater I
Automated Plate Washer I or Wash Apparatus
Multi-Specimen Tube (MST) Vortexer I, Rack, and Rack Lid[4] (optional)
Tube Sealer dispenser and cutting device (optional, used with the MST Vortexer I)
EXPAND-4 Pipettor™ [3] and stand (optional)

Other Equipment

65 ± 2°C water bath of sufficient size to hold either 1 MST Vortexer I rack (36 x 21 x 9 cm) or 2 specimen racks (each rack 28 x 12.8 x 7.5 cm)
Microcentrifuge (optional for centrifuging probe vials to obtain maximum probe volume)
Vortex mixer with cup attachment
Single-channel micropipettor; variable settings for 20 - 200 µl volumes
Repeating positive displacement pipettor such as Eppendorf® or equivalent
8-Channel pipettor
Specimen Collection Tube Rack[1] (to fit specimen collection tubes)
Timer
Power Surge Protector

Accessories

Digene Cervical Sampler™[1]
Positive assay controls[1] (*HC2 HPV DNA Test Panel;* see "Quality Control" section for additional details)
Hybridization Microplate[1]
Microplate Lids[1]
Extra-long pipette tips for removal of specimen[1]
Screw Caps[1]
Disposable reagent reservoirs[1]
Duraseal™ Tube Sealer Film[1] (optional, used only with the MST Vortexer I)
Microtubes[1,5]
Microtube racks[1,5]
Plate Sealers[1,5]
Disposable bench cover
Lint-free Paper towels
Powder-free gloves
Lint-free tissues
Sodium hypochlorite solution, 5% (or household bleach)
Disposable aerosol-barrier pipette tips for single-channel pipettor (20 to 200 µl)
Disposable tips for Eppendorf® repeating pipettor (25 and 500 µl)
Disposable tips for 8-channel pipettor (25 to 200 µl)
5-ml and/or 15-ml snap-cap round bottom polypropylene tubes (for Probe dilution)
Kimtowels® wipers or equivalent
Disposable 5-ml serological pipette or single-channel pipette tips capable of 1000 µl volume (for Probe Diluent transfer)
Empty Microplate Strip (available from Costar Model #2581, optional for use with Automated Plate Washer I to fill empty columns before washing)
Parafilm® or equivalent[5]

PreservCyt Specimen Processing needed Equipment and Accessories

Digene Sample Conversion Kit[1] (for PreservCyt specimen processing)
Swinging Bucket Centrifuge capable of reaching 2900 x g and holding 10-ml conical polypropylene centrifuge tubes
Sarstedt 10-ml Conical tubes with Caps (Cat. # 62 9924-283)
Disposable tips for Eppendorf repeating pipettor (50 and 100 µl)

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

[2] Only this luminometer and specific models of other equipment indicated have been validated for use with the HC2 High-Risk HPV DNA Test. Equivalent equipment must be validated by Digene prior to use (See the Ordering Information section of this package insert for specific details).

[3] 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.

[4] MST Vortexer I and related accessories are used to vortex Cervical Sampler Specimens only. The MST Vortexer I cannot be used to vortex PreservCyt specimens. See Sample Conversion Kit package insert for PreservCyt processing protocol details.

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

WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use.

Safety Precautions

1. 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 NCCLS Approved Guideline M29-A, *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.*

2. Do not pipette by mouth.

3. Do not smoke, eat, or drink in areas where reagents or specimens are handled.

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

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

   Solid Wastes: Autoclave.

   Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0% (1:5 dilution of household bleach). Allow 30 minutes for decontamination before disposal[24,25].

6. SPILLS: Non-base-containing spills should be wiped thoroughly with a 5% sodium hypochlorite solution (full-strength household bleach). Base-containing spills should be neutralized, wiped dry, and then the spill areas should be wiped with a 5% sodium hypochlorite solution.

   The wiped area should be covered with absorbent material, saturated with a 5% sodium hypochlorite solution and allowed to stand for at least 10 minutes. A glass or plastic cover or tray can be used to reduce exposure to fumes.

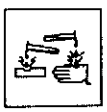
   All wiping materials should be treated as hazardous waste.

Safety And Health Risk Information

The materials below have been assessed according to the requirements of EC Directives 67/548/EEC and 88/379/EEC as amended and CHIP2 and 96 as amended.

> Caution: The Probe Diluent may cause reversible eye irritation. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

The Wash Buffer Concentrate contains sodium azide and is classified per applicable European Community (EC) directives as: Toxic (T). The following are appropriate risk (R) and safety (S) phrases.
R25: Toxic if swallowed
R32: Contact with acids liberates very toxic gas.
S35: This material and its container must be disposed in a safe way.
S36/37/39: Wear suitable protective clothing, gloves, and eye/face protection.
S45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).



The Denaturation Reagent contains sodium hydroxide and is classified per applicable European Community (EC) directives as: Corrosive (C). The following are the appropriate risk (R) and safety (S) phrases.
R35: Causes severe burns.
S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S35: This material and its container must be disposed in a safe way.
S36/37/39: Wear suitable protective clothing, gloves, and eye/face protection.
S45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Handling Precautions

1. For *in vitro* diagnostic use.

2. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

3. Do not use the reagents beyond the expiration date on the outer box label.

4. The HC2 High-Risk HPV DNA Test Procedure, Quality Control, and the Interpretation of Specimen Results must be followed closely to obtain reliable test results.

5. 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.

6. These components have been tested as a unit. **Do not** interchange components from other sources or from different lots.

7. 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.**

8. 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, since exogenous alkaline phosphatase may react with Detection Reagent 2 producing false-positive results.**

9. Protect Detection Reagent 2 from prolonged exposure to direct light. Use Reagent immediately after aliquoting and avoid direct sunlight.

10. 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.

11. Multi-channel pipetting should be performed using the reverse pipetting technique for dispensing Detection Reagents 1 and 2. Check each pipette tip on the multi-channel pipettor for proper fit and filling.

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

13. Cervical brush for use with non-pregnant women only.

14. Allow 60 minutes for the Microplate Heater I to equilibrate to temperature from a cold start. Not allowing for this warm-up period could result in melting of the Hybridization Microplate. Consult *Microplate Heater I Operator's Manual* for details.

REAGENT PREPARATION AND STORAGE

1. Upon receipt, store the kit at 2-8°C. The Wash Buffer Concentrate may be stored at 15-25°C, if desired.

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

3. All reagents are ready-to-use except Denaturation Reagent, High-Risk HPV Probe, and Wash Buffer.

Reagent	Preparation Method
Denaturation Reagent	**PREPARE FIRST:** • Add 5 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 3 months when stored at 2-8°C. Label it with the new expiration date. If the color fades, add 5 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.

Reagent	Preparation Method
High-Risk HPV Probe Mix **(Prepared from High-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 High-Risk HPV probe. A visible vortex must form in the liquid during the mixing step. Incomplete mixing may result in reduced signal.** • Centrifuge the vial of **High-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 run are desired, the total number of tests per kit may be reduced due to limited Probe and Probe Diluent volumes. • 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 High-Risk HPV Probe in Probe Diluent to prepare Probe Mix. No. of Tests/Strips — Volume Probe Diluent* — Volume Probe* 96/12 — 3.50 ml — 140.0 µl 72/9 — 2.60 ml — 105.0 µl 48/6 — 1.75 ml — 70.0 µl 24/3 — 0.875 ml — 35.0 µl 1 test — 0.040 ml — 1.6 µl *These values include the recommended extra volume. • Pipette High-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 "High-Risk HPV Probe Cocktail." **Unused Probe Mix should be discarded.**

Reagent	Preparation Method
Wash Buffer	**PREPARE DURING CAPTURE STEP:** **For the Automated Plate Washer I**, 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 I 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 Wash Buffer Concentrate when preparing.** Amount of Wash Buffer Concentrate / Amount of DI Water Amount of DI Water / Final Volume of Wash Buffer 33.3 ml / 966.7 ml / 1 L 66.6 ml / 1,933.4 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 non-use.** **Prior to each assay run, make sure the waste reservoir is empty and the rinse reservoir is filled with deionized water.** See Automated Plate Washer I Operator's 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 deionized water once every three months to prevent possible contamination from alkaline phosphatase present in bacteria and molds.

Volumes for Ready-to-Use Reagents

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 8-channel pipettor is not being used, a repeating pipettor may be substituted. In this case, aliquots of the Reagent should be made into an appropriately sized polypropylene tube. No. of Tests/Strips / Volume Detection Reagent 1 and 2 96/12 / contents of bottle 72/9 / 7.0 ml 48/6 / 5.0 ml 24/3 / 3.0 ml 1 test / 0.125 ml

29

SPECIMEN COLLECTION AND HANDLING

The types of cervical specimens recommended for use in the *HC2 High-Risk 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 HC2 High-Risk HPV DNA Test's performance characteristics with other specimen types and collection devices is unknown**. Cervical specimens must be collected prior to the application of acetic acid or iodine if colposcopic examination is being performed.

Cervical Brushes*

The *HC2 High-Risk HPV DNA Test* is designed for use with specimens collected and transported using the *Digene Cervical Sampler* (Digene Cervical Brush and Specimen Transport Medium). Specimens may be held for up to two weeks at room temperature, after which specimens can be stored an additional week at 2-8°C. If the assay will be performed more than 3 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 Specimen Transport Medium to retard bacterial growth and to retain the integrity of DNA. It is **not intended** to preserve viability of organisms or cells.

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 three 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 up to 5 mm in cross-section may also be analyzed with the *HC2 High-Risk HPV DNA Test.* The biopsy specimen must be placed immediately into 1.0 ml of Specimen Transport Medium 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 Cytyc PreservCyt Solution

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

There must be at least 4 ml of PreservCyt Solution remaining for the HC2 High-Risk HPV DNA Test. Samples with less than 4 ml after the Pap Test has been prepared may contain insufficient material and could be falsely negative in the HC2 High-Risk HPV DNA Test.

PreservCyt Solution specimens may be held for up to three weeks at temperatures between 4°C and 37°C, following collection and prior to processing for the *HC2 High-Risk HPV DNA Test.* PreservCyt Solution specimens cannot be frozen. To process these specimens, refer to the *Sample Conversion Kit* package insert (Catalog No. 5100-1400).

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

1. *Cover caps with Parafilm prior to shipping specimens previously frozen. Specimens may be shipped frozen or ambient.*
2. *When removing specimens from the freezer for testing, replace caps immediately with Screw Caps (catalog no. 5080-1000).*

TEST PROCEDURE

Specimens may contain infectious agents and should be handled accordingly.

Setup

1. Allow 60 minutes for the Microplate Heater I to equilibrate to temperature from a cold start. Consult *Microplate Heater I Operator's 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 to 30 minutes.

3. Create a plate layout using the Digene Hybrid Capture System Version 2 (DHCS v.2) Software or the Digene Qualitative Software.

4. Place Control, Calibrators, and specimens to be tested in a test tube rack, in the same order in which they will be tested. **The Negative Control and High-Risk Calibrator must be tested FIRST.** Negative Control (NC) and High-Risk HPV Calibrator (HRC) and specimens should be run in an 8-microwell column configuration. See *Example Layout* below.

EXAMPLE LAYOUT FOR A RUN OF 24 MICROWELLS:

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

5. NC and HRC are tested in triplicate with High-Risk HPV Probe Cocktail, the Calibrator and Controls positions in the microplate are determined by Digene software (See *DML 2000 Instrument and Version 2 Software User Manual* or the *Digene Qualitative Software* for further details), Specimens may be tested once.

Denaturation

Notes:

- **Caution:** Denaturation Reagent is corrosive. Use care and wear powder-free gloves when removing tape seal from bottle and when handling.
- **Important:** Some cervical specimens may contain blood or other biological material, which may mask the color changes upon addition of Denaturation Reagent and Probe Mix. Specimens that exhibit a dark color prior to the addition of Denaturation Reagent may not give the proper color changes at these steps. In these cases, failure to exhibit the proper color change will not affect the results of the assay.
- Do not remove 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, Controls, and specimens may be prepared up through the denaturation step and stored at 2-8°C overnight, or at -20°C for up to 3 months. A maximum of 4 freeze/thaw cycles may be performed with a maximum of 2 hours at room temperature during each thaw cycle. Mix well before using.
- To avoid false-positive results, it is critical that all Calibrator, Control, and specimen material come into contact with Denaturation Reagent. Mixing after Denaturation Reagent addition is a critical step; **if using the Multi-Specimen Tube (MST) Vortexer I, 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, 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, lab personnel should still adhere to practical universal precautions.

1. Remove and discard caps from Calibrator, Control, and specimens.

2. Pipette Denaturation Reagent with Indicator Dye into each Calibrator, Control, or specimen using a repeating or adjustable pipettor. Take care not to touch the sides of the tube or cross-contamination of specimens could occur. The volume of Denaturation Reagent needed is equivalent to half the sample volume. The exact volume for each type of calibrator and specimen is listed in the table below (for PreservCyt specimens, refer to Digene Sample Conversion Kit package insert for denaturation).

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

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

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

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

Multi-Specimen Tube (MST) Vortexer I Method

Notes:
- MST Vortexer I can be used **only** with specimens collected with the Digene Cervical Sampler in Specimen Transport Medium (STM). Specimens collected in PreservCyt **must** be processed according to instructions provided in the Digene Sample Conversion Kit package insert.
- Cervical Sampler specimens mixed using the MST Vortexer I **must** be hybridized using the hybridization microplate and Microplate Heater I method.

a) Cover the Control/Calibrator/Specimen tubes with Duraseal film by pulling the film over the tubes in the rack.
b) Place the rack lid over the film-covered tubes and lock into place with the two side clips. Cut the film with the cutter device.
c) Place the rack on the MST Vortexer I and secure the rack with the clamp. Verify speed setting is at 100 (maximum speed) and turn the vortexer power switch to the "on" position. Vortex the tubes for 10 seconds.

Manual/Individual Tube Vortexing Method

a) Recap the Calibrator, Control and specimen tubes with clean screw caps.
b) Mix each tube thoroughly by vortexing individually, at high speed, for 5 seconds. **There must be a visible vortex of liquid inside each tube during mixing such that the liquid washes the entire inner surface of the tube.** *The Calibrators, Controls and specimens should turn purple.*
c) Invert specimen tube one time to wash the inside of the tube, cap and rim.
d) Return tube to rack.

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 controls, Calibrators and specimens should turn purple.

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

Note: For PreservCyt specimen processing, see *Digene Sample Conversion Kit* package insert.

Hybridization Method Using Hybridization Plate and Microplate Heater I

Notes:
- High-Risk HPV Probe Mix is viscous. Care should be taken to ensure thorough mixing and that the required amount is completely dispensed into each microwell. See *Reagent Preparation* section.
- Specimens collected with the Digene Cervical Sampler in specimen transport medium (STM) and processed using the MST Vortexer I method can be hybridized utilizing the Microplate Heater I method **only**.
- If the denatured specimen has been stored at –20°C, allow the specimen to thaw to room temperature, and thoroughly vortex the specimen before proceeding with hybridization.
- Preheat Microplate Heater I to 65 ± 2°C for 60 minutes prior to use.

1. Obtain and label a Hybridization Microplate. Aliquot the prepared and thoroughly vortexed Probe Mix into a reagent reservoir. Carefully pipette 25 µl of the High-Risk Probe Mix into the bottom of each well of the Hybridization Microplate using an 8-channel pipettor or a repeating pipettor. Dispense the volume of probe into the bottom of each hybridization well, preventing back splashing. Avoid touching the sides of the wells. Place the plate lid on the microplate for the duration of the denaturation incubation.

2. Remove Controls, Calibrators and specimens from the water bath after the incubation. If the Multi-Specimen Tube Vortexer I is being used, vortex the entire rack for a minimum of 5 seconds on the maximum speed setting. Alternatively, vortex each tube individually for at least 5 seconds just prior to removing aliquots.

3. Remove the plate lid. Pipette 75 µl of each Control, Calibrator or specimen into the **bottom** of the Hybridization Microplate well following the created plate layout. 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 Controls, Calibrators or specimens. It is not necessary to remove the specimen collection device from the specimen transport tube. Denatured specimens may be capped with screw caps and stored with specimen collection devices remaining in the tubes.

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

4. Cover the Hybridization Microplate with a plate lid. Shake the Hybridization Microplate on Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. *The Controls, Calibrators and specimens should turn yellow after shaking.* Wells that remain purple may not have received the proper amount of Probe Mix. Add an additional 25µl of Probe Mix to samples that remain purple and shake again. If wells remain purple after following this procedure, specimens should be retested.

Notes:
- After shaking, PreservCyt 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.

5. Incubate in a preheated and equilibrated to 65 ± 2°C Digene Microplate Heater I for 60 ± 5 minutes. Create a plate layout file using the Digene Hybrid Capture System (DHCS) v.2 or Digene Qualitative Software if this has not been completed earlier.

Hybridization Method Using Microtubes and Water Bath

Notes:
- The processing of specimens collected with the Digene Cervical Sampler in Specimen Transport Medium (STM) using the MST Vortexer I method for mixing and the water bath method for hybridization **has not been validated**. Specimens collected with the Digene Cervical Sampler in STM and processed using the MST Vortexer I method can be hybridized utilizing the Microplate Heater I method only.
- If the denatured specimen has been stored at –20°C, allow the specimen to thaw to room temperature, and thoroughly vortex the specimen before proceeding with hybridization.

1. Place the required number of clean hybridization microtubes into the microtube rack.
2. Pipette 25 µl of High-Risk Probe Mix into each microtube using a repeating pipettor (for the Eppendorf, use a 1.25 ml tip and a pipettor setting of 1). Holding the repeating pipettor vertically, insert the tip of the pipettor approximately 1/4" into the center of the tube. Dispense the volume of probe such that the probe strikes the microtube near the bottom. Dispense the volume to all tubes. Gently tap the rack to assure that all of the probe mixture falls to the bottom of the microtubes. Inspect the rack from underneath to verify that all wells have received the appropriate amount of Probe Mix and that it is at the bottom of each tube. If necessary, repeat probe addition into the next set of hybridization microtubes using a clean pipette tip.
3. Remove Control, Calibrators and specimens from the water bath after incubation.
4. Vortex each tube individually for at least 5 seconds just prior to removing aliquots. Pipette 75 µl of each Control, Calibrator, or specimen into the bottom of appropriate hybridization microtube following the created

plate layout. Use a clean extra-long pipette tip for each transfer to avoid cross-contamination of Controls, Calibrators, or specimens. It is not necessary to remove the specimen collection device from the specimen transport tube. Denatured specimens may be stored with the specimen collection device remaining in the tube.

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

5. Cover the microtubes with a plate sealer. Place rack cover on top of rack. Shake the microtube rack on Rotary Shaker set at 1100 ± 100 rpm for 3 ± 2 minutes. *The Control, Calibrators 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 samples that remain purple and shake again. If tubes remain purple after following this procedure, specimens should be retested.

Note: After shaking PreservCyt specimens should turn pink instead of yellow.

6. Incubate in a 65 ± 2°C water bath for 60 ± 5 minutes. Assure 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.

Hybrid Capture

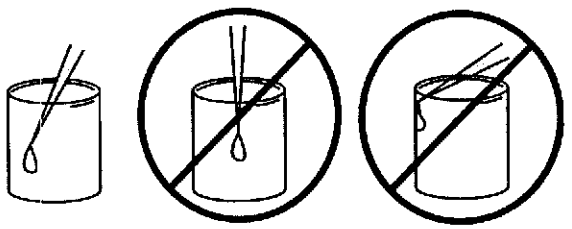
1. Remove all but the required number of Capture Microplate wells from the plate frame. Return the unused microwells to the original bag and reseal. With a marker, number each column 1, 2, 3. . . . and label the microplate with an appropriate identifier. The samples will be added to the wells according to the example layout shown below and/or the template previously prepared:

Example Layout for a Run of 24 Microwells			
Row	Column		
	1	2	3
A	NC	Spec. 3	Spec. 11
B	NC	Spec. 4	Spec. 12
C	NC	Spec. 5	Spec. 13
D	HRC	Spec. 6	Spec. 14
E	HRC	Spec. 7	Spec. 15
F	HRC	Spec. 8	Spec. 16
G	Spec. 1	Spec. 9	Spec. 17
H	Spec. 2	Spec. 10	Spec. 18

2. Carefully remove Hybridization Microplate or microtube rack containing Calibrators, Controls and specimens from Microplate Heater I. Immediately remove Plate Lid and place it on clean surface, or immediately remove Plate Sealer and discard.

3. Transfer the entire contents (approximately 100 µl) of the Control, Calibrator and specimen microwells to the bottom of the corresponding capture microwell using an 8-channel pipettor. Use new pipette tips for each column transferred and allow each pipette tip to drain well to ensure complete sample transfer. If desired, the pipettor may be steadied by resting the **middle** of the pipette tips on the top edge of the capture microwells (see *Diagram 1*).

Diagram 1: Correct Pipetting



4. Cover microplate with the plate lid or 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 I, check Rinse and Waste reservoirs during this incubation. See Reagent Preparation section.

6. When the capture step is complete, remove the Capture Microplate from the shaker 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 plate**; blot by tapping firmly 2-3 times on clean Kimtowels absorbent paper. 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 8-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 procedure below. Wipe tips on Reagent reservoir to remove excess Reagent before delivery to 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 or cross-contamination of specimens could occur. Refer to Diagram 1 shown earlier.

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

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

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

c) Immerse tips into the DR1 solution.

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

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

f) Refill tips and repeat until all wells are filled. Fill 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 plate lid or clean Parafilm and incubate at 20-25°C for 30-45 minutes.

Washing

Wash the capture plate using one of the two methods below.

Automated Plate Washer I Method

Note:
- **Always keep the Automated Plate Washer I on.** Ensure that the Rinse Reservoir is filled and the Waste Reservoir is empty. The Automated Plate Washer I will routinely rinse the system for cleaning.

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

1. Remove plate lid and place plate on Automated Plate Washer I platform.

2. Verify that the power is on, and that the display reads "Digene Wash."

Note: If only a partial strip of capture wells are being used, empty microplate wells will need to be placed in capture plate to complete the column prior to washing. See Accessories Section for ordering information.

3. Select the number of strips to be washed by pressing the "Rows" key and then "+" or "-" to adjust. Press "Rows" key to return to "Digene Wash."

4. Press "Start/Stop" to begin.

5. The 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 I is finished washing, it will read "Digene Wash Ready."

6. Remove the microplate from the 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 absorbent paper on top of the plate and carefully inverting. Before inverting, ensure that the paper is in contact with the entire surface area of the plate. Allow the plate to drain for 1-2 minutes. Blot well on clean Kimtowels. Carefully discard the used Kimtowels to avoid alkaline phosphatase contamination of later steps.

2. Using the Wash Apparatus, hand wash the plate 6 times. Each well is washed to overflowing to remove conjugate from the tops of the wells. Washing begins at well A1 and continues in a serpentine fashion to the right and downward. After all wells have been filled, decant liquid into sink with a strong downward motion. The second wash is started at well H12 moving in a serpentine motion to the left and upward. This sequence of 2 washes is repeated 2 more times for a total of 6 washes per well.

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

4. Plate should appear white, and no pink residual liquid should remain in the microwells.

Signal Amplification

Notes:

- Use a 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 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 consistent.
- Take care not to touch the sides of the microwell or splash reagent back onto tips because cross-contamination of specimens could occur (see *diagram 1*).

1. Carefully pipette 75 µl of Detection Reagent 2 into each well of the Capture Microplate using an 8-channel pipettor and the reverse pipetting technique as previously described. *All microwells should turn a yellow color.* Verify that all wells have been filled accurately by observing the intensity of the color. All wells should have similar intensity.
2. Cover microplates with a plate lid or clean Parafilm, and incubate at 20-25°C for 15 minutes. Avoid direct sunlight.
3. Read the microplate on the DML 2000 Instrument immediately after 15 minutes of incubation (and no later than 30 minutes of incubation).
4. If the DML 2000 Instrument is used to read the results, assay specific software will allow the entry of pertinent run information directly into the spreadsheet. Alternatively, record the lot number, date and operator name for each test run on the data sheet provided and on the Luminometer printout.
5. If a full microplate was not used, remove used microwells from the microplate holder, rinse the holder thoroughly with deionized water, dry and reserve for next assay.

ASSAY CALIBRATION 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 cut-off value. The *HC2 High-Risk HPV DNA Test* requires calibration with each run, therefore, it is necessary to verify each run using the following criteria. This verification procedure is not intended as a substitute for internal quality control testing.

1. Negative Control

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

2. Calibrator

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

   The assay calibration verification described above for the Negative Control and Calibrator is performed automatically by the DHCS v.2 and the Digene Qualitative Software and printed on the data analysis report. **The DHCS v.2 Software automatically verifies the High-Risk Calibrator %CV is ≤ 15%.** However, the Digene Qualitative Software (previous version) will **NOT** invalidate the assay unless the %CV is >25% for the High-Risk

Calibrator. **Therefore the user must manually verify that the %CV calculated by the luminometer software is ≤15% and proceed as indicated for Situation 1 in the table below. If the %CV of the Calibrator replicates falls between 15 and 25%, refer to the instructions in Situation 2 or 3 in the table below and proceed with the indicated "User Action."**

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 (n-1) of the two remaining replicate RLU values by the mean of the two remaining replicate RLU values (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 %CV's, recalculating the assay cutoff, or recalculating the RLU/cutoff of the specimens, please call Digene 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 assay runs performed with the were compiled. The results showed that the average %CV for these 152 runs was 8.1%. Considering all 3 replicates of the Calibrator per test run, Calibrator reproducibility of greater than 15%CV was observed for only 17 out of 152 runs (11.2%), with 10 out of these 17 test runs resulting in %CV between 15-25% (Situation 2). For the 17 test runs 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 test run's %CV remained greater than 15%, invalidating the test run. The %CVs of the remaining 151 test runs were calculated for an average %CV of 6.0.

3. The Calibrator mean ($HRC\bar{x}$) and Negative Control mean ($NC\bar{x}$) results are used to calculate the $HRC\bar{x}/NC\bar{x}$ ratio. This ratio must meet the following criteria to verify the assay calibration **before the specimen results can be interpreted**:

Assay Calibration Verification Acceptable Ranges
$HRC\bar{x} / NC\bar{x} \geq 2.0$

4. Calculate the HRC$\bar{x}$/NC$\bar{x}$ ratio. If the ratio is ≥ 2.0, proceed to the next step. If the ratio is <2.0, **the assay calibration is invalid and must be repeated.** All patient specimens should be repeated within the run.

Note: Acceptable ranges for the Negative Control and Calibrator have been established only for the DML 2000 Instrument. To be acceptable for use with the HC2 High-Risk HPV DNA Test, other luminometers must meet the assay calibration verification criteria described above. Absolute RLU values on these luminometers may be different from the DML 2000 Instrument and must be established by the laboratory. The HC2 HPV DNA Test Panel described in the "Quality Control" section below may be used to confirm that the luminometer will measure the HC2 High-Risk HPV DNA Test results correctly.

CUTOFF CALCULATION

Once an assay has been validated according to the criteria stated above, the Cutoff Value for determining positive specimens is the HRC$\bar{x}$.

Example Cutoff Calculation:

	NC RLU Values	HRC RLU Values
	97	312
	101	335
	91	307
Mean Value	96	318
%CV	4.9	4.7
HRC$\bar{x}$/NC$\bar{x}$	NA	3.31

Therefore, Positive Cutoff Value is (HRC$\bar{x}$) = 318

All specimen RLU values should be converted into a ratio to the appropriate Cutoff Value. For example, all assays should be expressed as Specimen RLU/Cutoff Value.

Note: RLU/CO values and positive/negative results for all specimens tested are reported in the DML 2000 data analysis report.

QUALITY CONTROL

Since positive quality control samples are not supplied with the *HC2 High-Risk HPV DNA Test* kit, alternate control material is needed. To facilitate internal quality control testing, Digene offers a *HC2 HPV DNA Test Panel* consisting of six samples (one HPV negative sample and five samples containing High-Risk and Low-Risk HPV types in varying concentrations), ranging from low levels detectable around the assay cutoff to high levels of HPV. For the purpose of internal quality control, it is recommended that a negative sample (Target 1) and at least two positive high-risk HPV type samples be selected. In order to monitor the ability of the *HC2 High-Risk HPV DNA Test* to reproducibly detect HPV at levels around the assay cutoff, it is recommended that the low-level control (Target 3) be tested. Testing of the intermediate- and high-level specimens (Targets 4 and 6) will serve to monitor for substantial reagent failure only. These can be tested with each new lot of kits and, if necessary, at 3-month intervals over the life of the kit. Refer to the HC2 HPV DNA Test Panel package insert for additional information.

The expected results for each specimen included in the HC2 HPV DNA Test Panel (Targets 1 to 6) are summarized below.

Target No.	***HPV Level***	***HPV Type***	***Expected Result*** *(Target RLU $\bar{x}$ /Cutoff Value)*
			High-Risk HPV Probe
1	Negative	None	<1
2	Low	Low risk (HPV 6)	<1
3	Low	High risk (HPV 16)	≥2
4	Intermediate	High risk (HPV 18)	≥8
5	High	Low risk (HPV 43)	<1
6	High	High risk (HPV 56)	≥80

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

The specimens contained in the *HC2 HPV DNA Test Panel* are cloned HPV DNA targets and not derived from wild-type HPV. This is the same material used for the Calibrators supplied with the *HC2 High-Risk HPV DNA Test.* Please confirm that different lots of the specimens are utilized for internal quality control testing, as recommended by NCCLS Document C24-A[41].

This control material will not act as an appropriate control for the specimen transport medium. Please refer to the *HC2 HPV DNA Test Panel* package insert for additional information regarding these specimens.

The samples contained in the *HC2 HPV DNA Test Panel* may be used for internal quality control or users may develop their own internal quality control material, as defined by NCCLS C24-A[41]. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations. Please refer to NCCLS C24-A for additional guidance on appropriate internal quality control testing practices.

41

INTERPRETATION OF SPECIMEN RESULTS

1. Specimens with RLU/Cutoff Value ratios ≥ 1.0 are considered positive.

2. Specimens with RLU/Cutoff Value ratios < 1.0 are considered negative. High-risk HPV DNA sequences are either absent or the HPV DNA levels are below the detection limit of the assay.

3. If the RLU/HRC $\bar{\chi}$ ratio of a specimen is close to but less than 1.0 and high-risk HPV infection is suspected, alternate testing methods and/or a repeat specimen should be considered.

4. Since this assay only detects high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, other low-risk HPV types may be present in the specimen.

Table 1
Interpretation of HC2 High Risk Results

RLU/Cutoff ratio	High-Risk HPV HC2 Result	Result Report	Interpretation
<1.0	Negative	HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68 not detected.	PAP WNL: Very low likelihood of underlying CIN 2-3 or cancer; results do not preclude future HPV infection or cytologic abnormalities with underlying CIN 2-3 or cancer.
			PAP ASCUS: Low likelihood of underlying CIN 2-3 or cancer; results are not intended to prevent women from proceeding to colposcopy.
			PAP LSIL: Reduced likelihood that CIN 2-3 or cancer will be found at colposcopy compared with HC2 positive LSIL.
			PAP HSIL: Expected to be uncommon result, representing possible error in HC2 or cytology.
≥1.0	Positive	HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68 detected.	PAP WNL: Low likelihood of underlying high grade CIN; HPV infection may be transient, resolving or persistent.
			PAP ASCUS/LSIL: Low but increased likelihood that underlying high grade CIN will be detected at colposcopy. Medical literature suggests that progression to high grade disease is possible.[39, 40]
			PAP HSIL: High likelihood that CIN 2-3 or cancer will be detected at colposcopy

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

Negative assay results do not completely rule out the presence of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68, particularly at very low concentrations. Refer to "Relative False-Positive and False-Negative Rate" discussion under the **Performance Characteristics** section for additional information.

The effects of age and HPV positivity are not fully known. It has been demonstrated in studies that HPV prevalence will decrease with age[38]. For information on the age-specific performance of the *Digene High-Risk HPV DNA Test* versus a histological diagnosis of high-grade neoplasia, please refer to Table 8 (page 27) of this insert.

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.

Results of this test are not intended to prevent women from proceeding to colposcopy or from continuing regular cervical cancer screening. This test is not intended for use in women with normal cytology who are under age 30.

Diagnostic Algorithm

This algorithm is used to interpret the results of the *HC2 HR HPV DNA Test* in conjunction with Pap test results as an aid in determining appropriate patient management. Results should be interpreted only in conjunction with information available from clinical evaluation of the patient including other procedures, patient history and demographics.

Table 2
Diagnostic Algorithm

	High-Risk HPV	
Cytology	**Positive**	**Negative**
Normal (Age 30 and over)	Follow up in accordance with accepted screening guidelines for cytologically-normal women with risk factors for cervical cancer.[a,b]	Follow up according to routine screening.guidelines[a,c]
ASCUS	Refer to ACS, ASCCP, CDC, US Public Health Service or ACOG current guidelines	
LSIL or HSIL	Refer to ACS, ASCCP, CDC, US Public Health Service or ACOG current guidelines	

[a] At the discretion of the physician, in accordance with ACS, ASCCP, CDC, US Public Health Service and ACOG current guidelines.

[b] The medical literature indicates that although the risk of developing CIN 2-3 and cancer is increased when HR HPV is present, most infections are transient and are not indicative of underlying CIN 2-3 or cancer.

[c] A negative *HC2 HR HPV Test* result with a concurrent normal Pap result implies low risk at a single point in time for the development of CIN 2-3 or cancer and is therefore clinically meaningful for assessing risk; however there are insufficient data to establish a definitive time period over which this lower risk is clinically relevant.

LIMITATIONS OF THE PROCEDURE

1. The HC2 *High-Risk HPV DNA Test* for human papillomavirus types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 is not recommended for evaluation of suspected sexual abuse.

2. 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.

3. A negative result does not exclude the possibility of HPV infection since very low levels of infection or sampling error may cause a false-negative result. Also, this test does not detect DNA of HPV low-risk types (6, 11, 42, 43, 44 and many other low-risk types).

4. The *HC2 High-Risk HPV DNA Test* should only be used with cervical cytologic specimens collected using the *Digene Cervical Sampler* and *Digene Specimen Transport Medium* or cervical cytologic specimens collected using a broom-type collection device and placed in Cytyc ThinPrep Pap Test PreservCyt Solution. Biopsy specimens may be assayed only if they are placed immediately in *Digene Specimen Transport Medium* and stored at -20°C until assayed.

5. 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.

6. A negative result does not exclude the possibility of future cytologic HSIL or underlying CIN 2-3 or cancer. A small proportion of high-grade lesions occur in women who are HPV negative by existing technologies.[59]

7. A small amount of cross-hybridization between HPV types 6 and 42 (low risk HPV types) and the high-risk Probe exists. Specimens with high levels (4 ng/ml or higher) of HPV 6 or HPV 42 DNA may be positive. 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, 53, 54, 55, 66, MM4, MM7, MM8, or MM9[43]. 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 in the *HC2 High-Risk HPV DNA Test.*[42,72]

8. The *Digene High-Risk HPV DNA Test* is designed to detect high-risk HPV types including 39, 58, 59, and 68. Analytical studies conducted by Digene, using cloned HPV plasmid DNA, demonstrate that this assay 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 High-Risk HPV DNA Test. Digene was able to validate the detection of these HPV types in only a limited number of clinical specimens. Due to the low prevalence of these types in the general population (as demonstrated by Bosch et. al.), the performance characteristics of the Digene High-Risk HPV DNA Test for the detection of HPV types 39, 58, 59, and 68 has not been statistically confirmed.

9. 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.

10. Cross-reactivity between the HC2 high-risk HPV DNA Test probe and the plasmid pBR322 is possible. The presence of pBR322 homologous sequences has been reported in human genital samples and false-positive results could occur in the presence of high levels of bacterial plasmid.

11. There is no known utility for HPV testing in Pap AGUS results.

EXPECTED RESULTS

High-Risk 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[44,48,50,57]. Also, it has been reported that the prevalence of HPV infection decreases dramatically with age.[38,44] Hence, it is not possible to define a single typical pattern of prevalence for HPV infection. Table 3 shows the prevalence in the United States of each high-risk HPV type detected by the *HC2 High-Risk 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 4 shows high-risk 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 HC2 High-Risk HPV DNA Test. These results demonstrate a fairly consistent pattern of HPV positivity across sites.

Table 3
Prevalence of Specific High-Risk HPV types in the United States
(Restricted to High-Risk HPV-Positive Specimens)

HPV Type	Prevalence (%)
16	54.5[36]
18	9.1[36]
31	9.1[36]
33	0.2[21]
35	0.2[21]
39	*
45	27.3[36]
51	0.4[21]
52	0.5[21]
56	0.2[21]
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 U.S was not determined independently [36].

Table 4
Prevalence of High Risk HPV Types Across Sites
ASCUS or More Severe Pap Population

Site	Number of Patients	Percent of HPV Positive (n) High-Risk Types
1	200	62.0% (124/200)
2	140	63.6% (89/140)
3	184	52.7% (97/184)
Total	524	59.2% (310/524)

Table 5 shows the prevalence of single or combined high-risk HPV types as detected by the *HC2 High-Risk HPV DNA Test* as reported by five 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 5
Prevalence of High-Risk HPV* in Various Populations
Women Age 30 years and Older

Location	Time Frame	Study Size	Prevalence (%)
USA Portland, OR[59,60,68]	1989-1999	13,493	9.0
Costa Rica[61,72]	1993-1995	6991	8.7
South Africa[62]	1998-1999	2925	23.4
China[64]	1999	1940	18.8
France[67]	1998-2002	2115	4.8

*Any combination of the 13 HR types detected by the *HC2 HR HPV Test*

PERFORMANCE CHARACTERISTICS

Clinical Sensitivity and Specificity for Screening Patients with ASCUS 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 smear and for HC2 HPV testing were obtained from women attending several Kaiser clinic facilities. Initial Pap smears were evaluated according to current Kaiser standards. Women (15 years or older) with Pap smear results of ASCUS 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.

HC2 HPV testing was performed on the initial specimen. HPV DNA testing was performed with a prototype of the HC2 High-Risk HPV DNA Test that contained probes to 11 of the 13 HPV types included in the HC2 High-Risk HPV DNA Test, 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.

HC2 High-Risk HPV DNA Test results and histological diagnoses were available from 885 women with ASCUS (atypical cells of undetermined significance) Pap smears. Testing on the majority of patients was performed with specimens collected in both STM and PreservCyt specimen transport medium. Due to the similarities between the HC2 HPVs performance characteristics for STM and PC, assay performance is presented for only the PC specimen transport medium.

Table 6 shows that among those presenting with an ASCUS referral Pap smear, the negative predictive value of the HC2 High-Risk HPV DNA Test for having HSIL or greater disease at colposcopy is 99.0%.

Table 6
Comparison of HC2 High-Risk HPV DNA Test versus Consensus Histology
ASCUS Referral Pap Population
Kaiser Study
PC Specimens

		CIN 2-3 or cancer at the time of colposcopy		
		+	-	**Total**
HC2 High Risk HPV	+	**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)
Assay's Positive Predictive Value = 17.2% (66/383)
Assay's Negative Predictive Value = 99.0% (497/502)

The following table (Table 7) shows theoretical positive and negative predictive values based on various prevalences for an initial ASCUS being found to be CIN 2-3 or cancer based on HC2 High-Risk HPV DNA Test results.

Table 7
Theoretical Positive and Negative Predictive Values
HC2 High-Risk HPV DNA Test
ASCUS Pap Smear Results

	Initial ASCUS Pap smear result	
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

The following table (Table 8) illustrates the variation between the various age groups contained in this study:

Table 8
Kaiser Study Data
HC2 High-Risk HPV DNA Test Performance versus Consensus Histology Results (CIN 2-3)
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)

In Women 30 Years and Older, Screening Performance of the HC2 High-Risk HPV DNA Test as an Adjunct to the Pap Test to Help Guide Patient Management.

Test Performance in Clinical Samples

Although no clinical trial was performed specifically to support the use of HC2 High-Risk HPV Test as an adjunct to the Pap test, compared with Pap test alone, consistent data obtained from multiple cross-sectional and prospective cohort studies conducted with a variety of cell sampling methods and utilizing Digene's Hybrid Capture HPV tests and several research-use testing methods provide strong evidence that a negative HPV DNA test implies very low risk of prevalent or incipient CIN 2-3 or cancer when Pap results are normal (WNL).[59-69,73]

Relative False-Positive and False-Negative Rate

In a study conducted by Digene and the National Cancer Institute (NCI) involving 209 samples, a research use type-specific HPV PCR (NCI) was utilized as the reference method to determine the apparent false-positive and false-negative rates of the HC2 High-Risk HPV DNA Test for the detection of high risk types of HPV. The NCI PCR method was optimized and had demonstrated a reasonably acceptable level of agreement between the HC2 High-Risk HPV DNA Test and the PCR method. The NCI PCR result was used as the sole determinant for the presence of HPV DNA. The study used archived (not randomly selected) samples. Of the 209 samples, the proportion of PCR negative results that were positive by HC2 High-Risk HPV DNA Test was 31/56, resulting in a relative false-positive rate of 55.4% (95% CI 41.5—68.7). Conversely, the proportion of PCR positive results that were negative by HC2 was 5/153, resulting in a relative false-negative rate of 3.3% (95% CI 1.07—7.46) (see Table 9).

When calculating the relative false-positive and false-negative rates as described above, PCR was considered to be 100% sensitive and specific since it was used as the standard for HPV detection. However, calculating a false-positive rate or a false-negative rate making these assumptions is flawed as it is known that PCR may have inherent variability and may not be 100% sensitive or specific. This is due to the potential for inhibition and other factors. PCR methods for HPV are especially complex due to the large numbers of different types and the interfering effects that may occur when there is more than one HPV type in a given specimen. Hence, the terms "relative" false-positive rate and "relative" false-negative rate are used to acknowledge the limitations of the estimates.

Taking these limitations into consideration, we have calculated a relative false-positive rate for the HC2 High-Risk HPV DNA Test determined by the proportion of HC2 positive samples (179) that were negative by PCR alone (31). This relative false-positive rate is not constant, but instead depends on the proportion of PCR negative specimens included in the study. When the data were analyzed interpreting the results in this manner, the relative false-positive rate of the HC2 High-Risk HPV DNA Test was determined to be 17% (31/179) (95% CI 12-23%) based on 26.8% of the specimens evaluated being PCR negative. In other words, 17% of the HC2 HPV positive results were not confirmed by the PCR test. If the proportion of PCR negative specimens evaluated had been larger, this relative false-positive rate may have been larger. We have also calculated a relative false-negative rate defined as the proportion of HC2 negatives that were positive by PCR. From Table 9, this estimate is 17% (5/30) (95% CI 6-35%).

Table 9
Analytical Detection of HPV DNA Comparing the
HC2 High-Risk HPV DNA Test to HPV Type-specific PCR

		PCR		
		Positive	Negative	Total
HC2 HPV	Positive	148	31	179
	Negative	5	25	30
	Total	153	56	209

[a]The reference method for this analysis is PCR. The values presented in the table above have been adjusted to account for the fact that PCR is probably not 100% sensitive or specific, and reflect a sample in which 26.8% of the specimens evaluated were PCR negative. No analysis of false positive and negative rates for individual genotypes is available.

Relative False positive rate: 17% (31/179) (95% CI 12-23%)
Relative False negative rate: 17% (5/30) (95% CI 6-35%)

Analytical Sensitivity

A nonclinical panel of cloned HPV plasmid DNA was tested to determine if each of the 13 HPV types are detectable by the HC2 High-Risk HPV DNA Test and to determine the analytical sensitivity of the assay for each of the HPV types. Each HPV target concentration (100 pg/ml, 10 pg/ml, 2.5 pg/ml, 1.0 pg/ml, 0.5 pg/ml, and 0.2 pg/ml targets of each of the 13 HPV DNA types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) was run in triplicate. The mean signal (in Relative Light Units, RLU) for each concentration of each HPV type was calculated and compared to the HRC $\bar{x}$.

The detectable limit of each HPV type is shown in Table 10. The detectable limits varied from 0.62 pg/ml to 1.39 pg/ml depending on the HPV type tested. All HPV types were detectable at an estimated level of 1.08 pg of HPV DNA target per 1 ml of specimen. The mean detectable limit of all 13 HPV DNA types was 1.08 pg/ml with a standard deviation of 0.05 pg/ml.

Table 10
Summary of HC2 High-Risk HPV DNA Test Detectable Limits of Sensitivity for Each Detectable HPV DNA Type

HPV DNA Type	Detectable HPV DNA Concentration (pg/ml)	Standard Deviation	95% Confidence Range
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
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)	1.08	0.05	0.95 - 1.25

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 their respective processing/denaturation procedures described in this package insert and tested with the HC2 High-Risk HPV DNA Test. 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 HC2 High-Risk HPV DNA Test.

Reproducibility

A multicenter reproducibility study was performed to determine the between days, between sites, and overall reproducibility of the HC2 High-Risk 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 HC2 High-Risk 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 specimen pools; Negative Control; and Positive High-Risk Calibrator at concentrations of 1 pg/ml, 0.5 pg/ml, 2.5 pg/ml, 5 pg/ml and 10 pg/ml. All panel members were tested each day in triplicate. The results are shown in Table 10

Table 11
Summary of Overall Statistics for Multicenter
Reproducibility of the HC2 High-Risk HPV DNA Test.

Statistical Measure	High Risk HPV Probe[a]
Proportion of expected positives with an observed positive result	100% (99.0-100.0)
Proportion of expected negatives with an observed negative result	99.0% (97.49-99.73)
Agreement	99.5% (98.70-99.86)
Kappa	0.990

[a] Numbers in parentheses indicate 95% confidence intervals. Overall data are a combination of all runs at all sites.

This indicates that the reproducibility of the HC2 High-Risk HPV DNA Test with clinical specimens is very good.

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 HC2 High-Risk 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 HC2 High-Risk HPV DNA Test.

Acinetobacter anitratus
Acinetobacter lwoffi (ATCC 17908)
Bacteroides fragilis (ATCC 25285)
Bacteroides melaninogenicus
Candida albicans (ATCC 14053 or 10231)
Chlamydia trachomatis
Enterobacter cloacae
Escherichia coli (HB101)*
Escherichia coli
Fusobacterium nucleatum
Gardnerella vaginalis
Haemophilus ducreyi
Klebsiella pneumoniae
Lactobacillus acidophilus
Mobiluncus curtisii
Mobiluncus mulieris
Mycoplasma hominis
Mycoplasma hyorhinis
Neisseria gonorrhoeae (ATCC 19424)
Neisseria lactamica (NRL 2118)
Neisseria meningitidis (ATCC 13077)
Neisseria sicca (ATCC 29256)
Peptostreptococcus anaerobius
Proteus vulgaris (ATCC 21117, 8427, 33420)
Serratia marcescens
Staphylococcus aureus (Cowan strain)
Staphylococcus epidermidis
Streptococcus faecalis (ATCC 14508)
Streptococcus pyogenes (ATCC 27762)
Treponema pallidum
Trichomonas vaginalis
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
Cytomegalovirus
Epstein-Barr Virus
Hepatitis B surface antigen-positive serum
Herpes Simplex I
Herpes Simplex II
Human Immunodeficiency Virus (HIV, RT DNA)
Simian Virus type 40 (SV40)
Human Papillomavirus type 1
Human Papillomavirus type 2
Human Papillomavirus type 3
Human Papillomavirus type 4
Human Papillomavirus type 5
Human Papillomavirus type 8
Human Papillomavirus type 13
Human Papillomavirus type 30
pBR322

The only plasmid that showed cross-reactivity in the HC2 High-Risk HPV DNA Test was pBR322. Crossreactivity between pBR322 and HC2 High-Risk HPV DNA Test Probe 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 samples, and false-positive results could occur in the presence of high levels of bacterial plasmid. However, 298 clinical samples testing positive with the HC2 High-Risk HPV DNA Test, showed that no positive results were due to pBR322 when tested with a pBR322 probe. Thus, the likelihood of HC2 High-Risk HPV DNA Test false-positive result due to homologous pBR322 sequences in clinical specimens appears to be low.

Cross-Hybridization

Eighteen different HPV types (high and low risk) were tested with the HC2 High-Risk HPV DNA Test at concentrations of 4 ng/ml of HPV DNA. All of the high-risk HPV targets were positive with High-Risk HPV Probe. This study also showed that there is a small amount of crosshybridization between HPV types 6 and 42 and the High-Risk HPV Probe. Patient specimens with high levels (4 ng/ml or higher) of HPV 6 or HPV 42 DNA may be falsely positive with the High-Risk HPV DNA Test. The clinical significance of this is that patients with 4 ng/ml or higher of HPV 6 or HPV 42 DNA may be unnecessarily referred to colposcopy.

The HC2 High-Risk HPV DNA Test has also been shown to crossreact 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 HC2 High-Risk 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 positive samples (clinical specimen pools and non-clinical samples) 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 since the cervix is routinely cleared prior to obtaining specimens for Pap smear and for HPV testing.

Effect of Blood and Other Substances on PreservCyt Specimens

The effect of blood and other potentially interfering defined or undefined substances potentially present in PreservCyt clinical specimens was evaluated in the HC2 High-Risk 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 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 HC2 High-Risk HPV DNA Test.

Reproducibility of HC2 High-Risk HPV DNA Test with Clinical Specimens Collected in STM

The reproducibility of the HC2 High-Risk HPV DNA Test with clinical specimens collected in STM was determined in a study using 20 clinical pools (ten positive and ten 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 a combined probe cocktail consisting of the HC2 High-Risk HPV DNA Test probe and low-risk HPV type probes. Means, standard deviation and 95% confidence intervals about the mean (CI's) were calculated for each specimen within day and over five days and results are shown in Table 12 below. The reproducibility of the assay would not be expected to differ when using only the high-risk HPV type probe in this kit.

Table 12
Mean RLU/PC with Confidence Intervals and Percent Positive
(Descending Order by Mean RLU/PC)

No.	Spec. ID	Mean RLU/PC	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/PC 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/mean PC 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/mean PC at more than 20% below the assay cutoff, 200 of 200 replicates (100%) were negative.

Thus, specimens with a mean RLU/PC of 20% or more above the cutoff were positive 100% of the time, while specimens with a mean RLU/PC 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 HC2 High-Risk HPV DNA Test.

Reproducibility of PreservCyt Solution Specimens in the *HC2 High-Risk HPV DNA Test*

The reproducibility of PreservCyt Solution specimens in the HC2 High-Risk 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 Sample Conversion Kit* package insert instructions. Means, standard deviations, and 95% confidence intervals (CIs) were calculated for each specimen within day and over all five days and replicates. The mean RLU/PC, confidence interval about the mean, and the percent of positive replicates are shown below in Table 13 for each specimen, in descending order based on the mean RLU/PC.

Table 13
Mean RLU/PC with Confidence Intervals and Percent Positive
(Descending Order by Mean RLU/PC)

No.	Spec #	Mean RLU/PC	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/PC 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/PC 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%) were negative. For the 11 specimens with the mean RLU/PC at more than 20% below the assay cutoff, 220 of 220 replicates (100%) were negative.

Thus, specimens with a mean RLU/PC of 20% or more above the cutoff were positive greater than 95% of the time, while specimens with a mean RLU/PC 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 specimens yield reproducible results in the HC2 High-Risk HPV DNA Test .

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45. **NOTE: References will be renumbered 1 to end.**

HC2 High-Risk HPV DNA Test
Troubleshooting Guide

Observation	Probable Causes	Solutions
Improper or no color change observed during denaturation.	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.
	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.
	Specimen pH may be unusually acidic.	If neither of the other causes apply, 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 since improper specimen pH will adversely affect the test results.
Improper color change observed during hybridization.	Inadequate mixing of Probe Cocktail with denatured 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 which 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.
	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.
	Specimen had < 1000 µl STM.	Check the volume of the original specimen. Volume should be 1425 µl ± 20 µl (after removing 75 µl for High-Risk HPV Probe). If volume is < 1425 µl, original specimen contained < 1000 µl STM. Obtain a new specimen.

HC2 High-Risk HPV DNA Test
Troubleshooting Guide

Observation	Probable Causes	Solutions
Assay fails validation criteria. No signal observed in Calibrator or in specimens.	No Probe added to Probe Diluent.	Prepare Probe Cocktail as described in the package insert. Label tube carefully.
	Probe contaminated with RNase during preparation.	Use aerosol-barrier pipette tips when pipetting probe and wear gloves. Dilute probe in sterile containers. Only use clean, new disposable reagent reservoirs.
	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.
	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.
	Incorrect time or temperature during hybridization step.	Hybridize for 60 ± 5 minutes at 65 ± 2°C. Check temperature of Microplate Heater I or water bath. Ensure the Heater/water bath is set to heat specimens to correct temperature and is preheated for 60 minutes prior to use.
	Inadequate mixing during capture step.	Shake on a rotary shaker for 60 ± 5 minutes at 20-25°C as described in the package insert. Verify shaker speed by calibration, as outlined in the Shaker Speed Calibration section of the *Rotary Shaker I Operator's Manual.*
	Failure to add correct amount of Detection Reagent 1 or to incubate for specified time.	Pipette 75 µl Detection Reagent 1 into each well using an 8-channel pipettor. Incubate 20-25°C for 30 to 45 minutes.
	Failure to add correct amount of Detection Reagent 2 or to incubate for specified time.	Pipette 75 µl Detection Reagent 2 into each well using an 8-channel pipettor. Incubate 20-25°C for 15 to 30 minutes.
	Luminometer malfunction or incorrect programming.	Refer to *DML 2000 Instrument and Version 2 Software User Manual* or *Digene Qualitative Software User Manual* (Maintenance & Troubleshooting section) for further instructions, or call Digene Technical Services.

Observation	Probable Causes	Solutions
Elevated RLU values in Control, Calibrator and/or specimens (≥ 500 RLUs in many or all wells). Assay may fail validation criteria.	Denaturation Reagent not added; or, incorrect volume of reagent added; or, inadequate mixing of Denaturation Reagent with specimens or controls.	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, Controls and specimens should turn purple after addition of Denaturation Reagent.
	Light leak in the luminometer. Door not sealed. Seal around door broken.	Check background reading of the Luminometer by reading an empty microplate. A reading of greater than 50 RLUs indicates that a light leak exists. Refer to the *DML 2000 Instrument and Version 2 Software User Manual* or *Digene Qualitative Software User Manual* (Maintenance & Troubleshooting section) for further instruction, or call Digene Technical Services.
	Contamination of Detection Reagent 2 or capture microwells by Detection Reagent 1 or exogenous alkaline phosphatase.	Check aliquoted DR2 for contamination by pipetting 75 µl into a blank capture microwell. Incubate 20-25°C for 15 minutes and read in the luminometer. Readings above 200 RLUs indicate DR2 contamination. Take care when pipetting DR2. Wear gloves and avoid touching tips to any work surfaces. Repeat troubleshooting procedure on the master vial of DR2, and if not contaminated, repeat assay using this material. If contaminated, obtain a new kit and repeat assay.
	Contaminated Wash Buffer.	If DR2 is not contaminated, check the Wash Buffer for contamination. Pipette 10 µl of Wash Buffer into 75 µl of DR 2 into a blank capture well. Cover and incubate 15 minutes at 20-25°C. Read microwell on luminometer. Readings above 200 RLUs indicate Wash Buffer contamination. See Reagent Preparation for instructions on cleaning and maintenance of Wash Apparatus. See Automated Plate Washer Operator's manual for instructions on testing for contamination or malfunctions.
	Contaminated Automated Plate Washer I	See *Automated Plate Washer I Operator's Manual, Decontamination Procedure.*
	Inadequate washing of capture microwells after Detection Reagent 1 incubation.	Wash microwells thoroughly with Wash Buffer 6 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 Operator's manual for instructions on testing for contamination or malfunctions.
	Detection Reagent 1 contamination of microwells.	Ensure all work surfaces are clean and dry. Use care when using Detection Reagent 1. Avoid aerosols.
	Blotting hybridization solution on same area of Kimtowels.	Do not reblot on previously used area.
	Used incorrect blotting towels.	Use Kimtowels for blotting.

HC2 High-Risk HPV DNA Test
Troubleshooting Guide

Observation	Probable Causes	Solutions
Low PC/NC ratios or high number of low positive specimens with ratios <2.0 (> 20%). Assay may fail validation criteria.	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 I methods (for the manual vortexer method, invert tube one time). For PreservCyt Specimens ensure proper mixing and resuspension of the cell pellet is completed prior to denaturation incubation. Consult *Sample Conversion Kit* package insert for protocol details. A distinct color change from clear to dark purple should be seen. Incubate for 45 ±5 minutes at 65 ±2°C
	Probe inadequately mixed or insufficient Probe added to assays.	Prepare Probe Cocktail as described. Mix thoroughly by vortexing ensuring that a visible vortex is produced. Probe Cocktail must be added to tubes with a positive displacement pipettor or multichannel pipettor to ensure accurate delivery.
	Inadequate volume of diluted Probe added to each Hybridization microwell or microtubes.	Verify that the repeating pipettor is delivering accurately prior to adding Probe Cocktail to Hybridization Microplate or microtubes. 25 µl of diluted Probe should be added to the bottom of each microwell or microtube. Color change should be from dark purple to yellow upon addition and thorough mixing of Probe Cocktail. PreservCyt specimens should turn pink instead of yellow.
	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.
	Insufficient capture.	The capture step should be performed using a rotary shaker set at 1100 ± 100 rpm for Digene Rotary Shaker. Validate shaker speed by calibration.
	Inadequate washing.	Wash microwells thoroughly with Wash Buffer 6 times, filling the wells to overflowing each time or using Automated Plate Washer I.
	Contaminated Wash Buffer.	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 luminometer. Readings above 200 RLUs indicate contamination. See Reagent Preparation for instructions on cleaning and maintenance of Wash Apparatus. See Automated Plate Washer I Operator's manual for instructions on testing for contamination or malfunctions.

HC2 High-Risk HPV DNA Test
Troubleshooting Guide

Observation	Probable Causes	Solutions
Series of positive specimens with RLU values approximately the same.	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.
	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.
	Automated Plate Washer malfunction.	See Automated Plate Washer Operator's manual for instructions on testing for contamination or malfunctions.
Wide %CVs between replicates.	Inaccurate pipetting.	Check pipettor to assure that reproducible volumes are being delivered. Calibrate pipettors routinely.
	Insufficient mixing.	Mix thoroughly at all steps. Vortex prior to denaturation incubation and after adding Probe Cocktail. Ensure that a visible vortex is produced.
	Incomplete transfer of liquid from hybridization microwells or microtubes to capture microwells.	Take care during transfer step from Hybridization Microplate or microtubes to capture microwells to ensure reproducible volumes are transferred.
	Improper washing conditions.	Wash microwells thoroughly with Wash Buffer 6 times, filling to overflowing each time or using Automated Plate Washer and proper Automated Plate Washer protocols.
	Detection Reagent 1 contamination of microwells.	Ensure all work surfaces are clean and dry. Use care when using Detection Reagent 1. Avoid aerosols.

29

HC2 High-Risk HPV DNA Test
Troubleshooting Guide

Observation	Probable Causes	Solutions
False-positive results obtained from known negative specimens.	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 reagent reservoir prior to filling the pipettor.
	Detection Reagent 1 contamination of microwells.	Wash microwells thoroughly with Wash Buffer 6 times, filling to overflowing each time or using Automated Plate Washer. There should be no residual pink liquid visible in the microwells after washing.
	Blotting on same area of Kimtowels over several rows.	Do not blot on area that has been previously used.
	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 I methods (for the manual vortexer method, invert tube one time). For PreservCyt Specimens, ensure proper mixing and resuspension of the cell pellet is completed prior to denaturation incubation. Consult *Sample Conversion Kit* package insert 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.
	Improper washing conditions.	Wash microwells thoroughly with Wash Buffer 6 times, filling the wells to overflowing each time or using Automated Plate Washer and proper Automated Plate Washer protocols.
Elevated Negative Control RLU values (> 200 RLUs). Remainder of assay performs as expected.	Detection Reagent 2 was incubated at a temperature greater than 20-25°C.	Rerun the test, and ensure that Capture and Detection steps are incubated at 20-25°C.
	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.
	Detection Reagent 2 or Wash Buffer was contaminated with alkaline phosphatase or Detection Reagent 1.	Check aliquoted DR2 for contamination by pipetting 75 µl into a blank capture microwell. Incubate 20-25°C for 15 minutes and read in the luminometer. Readings above 200 RLUs indicate DR2 contamination. Take care when pipetting DR2. Wear gloves and avoid touching tips to any work surfaces. Repeat troubleshooting procedure on the master vial of DR2, and if not contaminated, repeat assay using this material. If contaminated, obtain a new kit and repeat assay. If DR2 is not contaminated, check the Wash Buffer for contamination. Pipette 10 µl of Wash Buffer into 75 µl of DR 2 into a blank capture microwell. Cover and incubate 15 minutes at 20-25°C. Read microwell on luminometer. Readings above 200 RLUs indicate Wash Buffer contamination. See Reagent Preparation for instructions on cleaning and maintenance of Wash Apparatus.

63

Hybrid Capture® 2
High-Risk HPV DNA Test
Ordering Information

Reagents

HC2 High-Risk HPV DNA Test (96 tests)
HC2 HPV DNA Test Panel
Digene Cervical Sampler
Digene Sample Conversion Kit
Specimen Transport Medium (30 ml)
Wash Buffer Concentrate
Cytyc ThinPrep® Pap Test™ (Cytyc telephone: 800-442-9892)

Accessories

Hybridization Microplates
Microplate Lids
Extra-long Pipette Tips
Specimen Collection Tube Rack
Screw Caps
Disposable Reagent Reservoirs
Multi-Specimen Tube Rack and Lid (optional)
Tube Sealer Dispenser and Sealer Cutter (optional: use with MST Vortexer I)
Duraseal™ Film Tube Sealer (optional: use with MST Vortexer I)
Microtubes
Microtube Rack
Plate Sealers

Equipment

DML 2000 Instrument
PC System
Printer Cable
Inkjet Printer
Digene Software Package
Multi-Specimen Tube Vortexer I (optional)
Microplate Heater I
Rotary Shaker I
Automated Plate Washer I (optional)
Wash Apparatus
EXPAND-4 Pipettor (optional)
Pipettor Power Supply (optional)
EXPAND-4 Pipettor Stand (optional)

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This product and its method of use are covered by one or more of the following patents:

U.S. HPV Patent Nos.	Foreign HPV Patent Nos.	U.S. Hybrid Capture Patent Nos.	Other Patents
4,849,331	EP 294,659	4,732,847	"Use of CDP-Star® is covered by one or more of:
4,849,332	JP 1047383	4,865,980	
4,849,334	EP 019200B1	4,581,333	U.S. Patent Nos.
4,908,306	EP 0591376B1	5,605,800	
5,411,857	CA 1,339,729	5,955,262	4,931,569
5,643,715	EP 0370625B1	6,228,578B1	5,145,772
5,712,092	JP 3076578		5,326,882
5,876,922	JP 89/38944		5,538,847
5,952,487			
5,958,674			licensed from Tropix, Inc./Applied Biosystems."
5,981,173			
6,107,086			

Registered trademark acknowledgments:
Kimtowels: Kimberly-Clark Corporation
Eppendorf: Eppendorf-Netheler-Hinz
Parafilm: American Can Co.
PreservCyt and ThinPrep: Cytyc Corporation
Windows: Microsoft
CDP-Star: Tropix, Inc.

Trademark acknowledgements:
ThinPrep Pap Test: Cytyc Corporation
DuraSeal: Diversified Biotech, Inc.

Summary of HC2 High-Risk HPV DNA Test

Important: *It is important that one be thoroughly familiar with the detailed procedure before using this summary.*

	Procedure	
DENATURE (For PreservCyt specimens, see Sample Conversion Kit package insert)	**Manual Vortex Method** Label Hybridization Microtubes. Prepare Denaturation Reagent. ↓ Pipette Denaturation Reagent (volume is equivalent to half the specimen volume) into controls and specimens. Vortex each specimen and control individually for 5 seconds at high speed (see package insert for details). Check that all tubes show a purple color. ↓ Incubate at 65 ±2°C for 45 ±5 minutes. ↓ Prepare HPV Probe Mix. ↓ ↓ ↓	**Multi-Specimen Tube (MST) Vortexer I Method** Label Hybridization Plate. Prepare Denaturation Reagent. ↓ Pipette Denaturation Reagent (volume is equivalent to half the specimen volume) into controls and specimens. Check that all tubes show a purple color. ↓ Cover rack with film and lid. ↓ Vortex for 10 seconds. ↓ Incubate at 65 ±2°C for 45 ±5 minutes. ↓ Prepare HPV Probe Mix. ↓
HYBRIDIZE	**Water bath Method** Pipette 25 µl High-Risk HPV Probe Cocktail into Hybridization microtubes. Mix denatured specimen well, and pipette 75 µl into tubes. Cover microtubes with a plate sealer and shake on Rotary Shaker I at 1100 ± 100 rpm for 3 ± 2 minutes. *Check that all tubes show yellow color.* Incubate at 65 ±2°C for 60 ± 5 minutes. Prepare Capture Microplate. ↓ ↓	**Microplate Heater I Method** Pipette 25 µl High-Risk HPV Probe Mix into Microplate wells. ↓ Mix denatured specimen well, and pipette 75 µl into Microplate wells. ↓ Cover microplate with a plate lid and shake on Rotary Shaker I at 1100 ±100 rpm for 3 ±2 minutes. *Check that all wells show yellow color. (PreservCyt will turn pink.)* ↓ Incubate at 65 ±2°C for 60 ±5 minutes. Prepare Capture Microplate. ↓
CAPTURE	Transfer contents from each Hybridization Plate Well or microtube to corresponding well in Capture Microplate using an 8-channel pipettor. Cover with a plate lid or plate sealer. Shake at 1100 ±100 rpm at 20-25°C for 60 ±5 minutes. Prepare Wash Buffer. ↓ Decant and blot Capture Microplate (see package insert for details). ↓	
CONJUGATE	Pipette 75 µl Detection Reagent 1 into each well of Capture Microplate. Cover Capture Microplate with a plate lid or Parafilm. **Incubate at 20-25°C for 30 - 45 minutes. Wash plate using desired method.** ↓	
WASH	**Manual Washing Method** Decant and blot Capture Microplate (see package insert for details). ↓ Wash 6 times. ↓ Blot on absorbent pad. ↓	**Automated Plate Washer I Method** Place plate on washer and press "START/STOP" to begin. ↓ Go to next step. ↓ ↓ ↓
GENERATE SIGNAL	Pipette 75 µl Detection Reagent 2 into each well of Capture Microplate. **Cover with a plate lid or plate sealer. Incubate at 20-25°C for 15 minutes.** ↓	
READ	Read Capture Microplate on DML 2000 Instrument. ↓ Validate assay and interpret specimen results.	

Hybrid Capture® 2
High-Risk HPV DNA Test
Data Sheet

Date:	**Tech:**	**Room Temp:**
Assay:	**Kit Lot #:**	**Exp Date:**

	Calibrator RLUs			Mean RLUs	%CV	Ratio
NC						
Calibrator						Calibrator $\bar{x}$ /NC $\bar{x}$ =
Cutoff Value =Calibrator $\bar{x}$						Cutoff

Assay Validation:

1. Does the Negative Control result demonstrate a %CV ≤ 25%? ___Yes ___No
   - If "yes" → proceed to step 2.
   - If "no" → discard the outlier furthest from the mean and recalculate the mean.
     Are each of the 2 RLU values within 25% of the mean for the Calibrators? ___Yes ___No
     - If "yes" → proceed to step 2.
     - If "no" → **assay is invalid and must be repeated.**

2. Does the Calibrator result demonstrate a %CV ≤ 15%? ___Yes ___No
   - If "yes" → proceed to step 3.
   - If "no" → discard the outlier furthest from the mean and recalculate the mean.
     Are each of the 2 RLU values within 25% of the mean for the Calibrators? ___Yes ___No
     - If "yes" → proceed to step 3.
     - If "no" → **assay is invalid and must be repeated.**

3. Is the NC $\bar{x}$ ≤ 250 RLU values? ___Yes ___No
   - If "yes" → proceed to step 4.
   - If "no" → **assay is invalid and must be repeated.**

4. Calculate the assay validation ratio: Calibrator $\bar{x}$ /NC $\bar{x}$ for the Calibrator. **The ratio should be ≥ 2.0**
   - Is the assay validation ratio within the acceptable range? ___Yes ___No
     - If "yes" → proceed with cutoff calculations.
     - If "no" → **assay is invalid and must be repeated.**

Cutoff Calculation: Positive Cutoff Value = Calibrator $\bar{x}$

Example
Calibrator RLU Values: 312 + 335 + 307 = 954
954 ÷ 3 = 318 = Calibrator $\bar{x}$

Positive Cutoff Value = (Calibrator $\bar{x}$) = 318

To facilitate *HC2 High-Risk HPV DNA Test* Results Interpretation, users should normalize their specimen RLU data against the Positive Cutoff. To do this, the specimen RLU value should be divided by the Positive Cutoff Value. Once the data are normalized, ratios ≥ 1.0 are considered "Positive" for HPV DNA. Ratios < 1.0 are considered Negative for HPV DNA.

Assuming a Positive Cutoff of 318 RLUs, specimen RLU values, ratios and results interpretation are as follows:

Specimen RLU Value	Ratio (RLU/Pos Cutoff Value)	Interpretation
2467	7.76	Positive
299	0.94	Negative
148	0.47	Negative

Hybrid Capture® 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
B	NC RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
C	NC RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
D	CALIBRATOR RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
E	CALIBRATOR RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
F	CALIBRATOR RLU	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.
G	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2 3. 4.
H	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.	1. 2. 3. 4.

1. Specimen ID Number 2. Specimen RLU Value 3. RLU/CO Ratio 4. Interpretation (Positive or Negative)

87