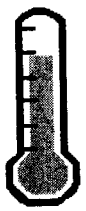

HiFi® DNA

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HiFi DNA Tech, LLC
160 Hawley Lane
Trumbull, CT 06611
Fax 203 385-3832

March 7, 2007

Document Mail Center (HFZ-215)
Center for Devices and Radiological Health
Regulations Staff (attention: Ms. Heather Rosecrans)
Food and Drug Administration
1350 Piccard Drive
Rockeville, Maryland 20857 Via Fed Ex delivery 8604 0044 1113

RE: Reclassification Petition - Human Papillomavirus (HPV) DNA Nested Polymerase Chain Reaction (PCR) Detection Device (K063649)

Dear Sir/Madam:

This submission is a request for reclassification of the new device for "Human Papillomavirus (HPV) DNA Nested Polymerase Chain Reaction (PCR) Detection" (K063649) into a Class II category according to Section 513(f), 21 CFR § 860 Subpart C. The information was prepared in accordance with 21 CFR 860.123 and contains the following in triplicate:

(A) Reclassification Petition

(B) FDA Form 3427, Supplemental Data Sheet – Attachment 1 on inside of cover (A)

(C) FDA Form 3429, General Device Classification Questionnaire- Attachment 2 on inside of cover (A)

(D) Attachment 3: Volume A and Volume B as part of 21 CFR § 860.123(a)(6)

(E) Copies of 94 source documents marked as "Appendix [21CFR §860.123(a)(9)"

If you have any questions or require further information regarding this submission, please contact me by telephone at 203 876-4258 (direct line) or via facsimile at 203 385-3832.

Sincerely,

Sin Hang Lee, M. D.
President, HiFi DNA Tech, LLC
Tel. 203 876-4258 (direct line to medical office)
Fax 203 385-3832

2007P-0210

CCP 1

RECLASSIFICATION PETITION

FOR

Human Papillomavirus (HPV) DNA

Nested Polymerase Chain Reaction (PCR) Detection

Under Section 513(f), 21 CFR § 860 Subpart C

(Formerly K063649)

HIFI DNA TECH, LLC

MARCH 7, 2007

TABLE OF CONTENTS

I. Introduction

In accordance with Section 513(f) of the Federal Food, Drug, and Cosmetic Act ("FFDCA"), the petitioner, HiFi DNA Tech, LLC, is requesting that Human Papillomavirus (HPV) DNA Nested Polymerase Chain Reaction (PCR) *In Vitro* Devices for detection of HPV DNA in alcohol-preserved cells of liquid-based cervicovaginal lavage samples and for preparation of clinical materials suitable for further nucleic acid-based HPV genotyping be reclassified from Class III to Class II by the U.S. Food and Drug Administration ("FDA"). This reclassification will facilitate introduction of the next generation of HPV *in vitro* test devices into clinical laboratories by the industry, consistent with a pledge made by the former FDA Commissioner Mark B. McClellan, M.D., Ph.D. that "FDA is committed to bringing safe and effective new technologies to the market quickly" when the agency announced approving expanded use of the Digene HC2 HPV test on March 31, 2003 [1].

Over the past 20 years, it is gradually realized among the medical scientists that a sensitive and specific HPV test which can detect a small quantity of HPV DNA in clinical materials and can provide accurate genotyping information of the HPV detected is important for following patients with persistent HPV infection and for evaluation of prevention strategy for the individual sexually active women who consider immunization with type-specific HPV vaccines for protection against HPV infections.

It is now generally accepted that persistent infection of the uterine cervix caused and maintained by a "high-risk" HPV is the pivotal tumor promoter in cancer induction. In its news release, P03-26, dated March 31, 2003 the FDA has officially stated that the FDA-approved HC2 High-Risk HPV DNA Test manufactured by Digene Corporation is "a laboratory test to detect the presence in women of HPV, one of the most common sexually transmitted infections" [1]. In the prior years since 1988, the FDA had historically considered this *in vitro* device and similar systems to be intended for use in identifying and typing HPV infection as devices to stratify women at risk for cervical cancer, thus customarily assigned them to class III by the OIVD [2, 3]. The change in the view of the FDA toward the same HPV DNA test kit produced by the same manufacturer (Digene Corporation) over a period of 15 years from 1988 to 2003, from the view of a test to stratify women at risk for cervical cancer to that of a test for the detection of a virus causing one of the most common sexually transmitted infections, indicates that the agency has already pursued a *de facto* down-classification of the HPV DNA test based on "new information" since the risk-based classification of 21 CFR §860.3 (c) was promulgated. The FDA news release of March 31, 2003 acknowledges that "most infections (by HPV) are short-lived and not associated with cervical cancer", in recognition of the advances in medical science and technology since 1988. In other words, since 2003 the scientific staff of the FDA no longer considers HPV infection to be a high-risk disease when writing educational materials for the general public whereas the regulatory arm of the agency is still bound by the old classification scheme that had placed HPV test as a test to stratify risk for cervical cancer in regulating the industry.

On December 8, 2006 the petitioner submitted to the FDA a premarket notification (510K) titled "Human Papillomavirus DNA Nested Polymerase Chain Reaction Detection Kit" (K063649), identifying as the predicate device the Digene's HC2 High-Risk HPV DNA Test which was approved for expanded use by the FDA on March 31, 2003 as a virology test for a very common

sexually transmitted infection. The OIVD of the FDA cited the 1988 approval order for Digene's Virapap as the regulatory mechanism for classifying this new device into a class III device [3], which would require premarket approval. This petition presents science-based evidence to help bridge the gap between the scientific understanding and the regulatory control of the FDA to justify an official down-classification of the HPV DNA test to a class II device so that the manufacturers will be allowed to bring their safe and effective new technologies for HPV testing to the market timely through the 510k system in consistence with the least burdensome principles of the Food and Drug Administration Modernization Act (FDAMA) of 1997.

The PCR technology patent (U.S. Patent 4,683,195) was issued on July 28, 1987 and the PCR technology was not widely available to any clinical or research laboratory until after Hoffmann-La Roche purchased the patent rights from Cetus Corporation in 1991. Therefore, PCR-based *in vitro* devices were not in existence prior to the Medical Device Amendments of 1976 to the FFDCA. By regulation, HPV DNA PCR detection devices would have to be automatically classified into Class III unless FDA in response to a petition reclassified them into Class I or Class II under section 513(f)(2) or 513(f). The petitioner submitted a Petition on January 18, 2007 requesting review of a new device for the detection of HPV DNA through the route of Evaluation of Automatic Class III Designation under Section 513(f)(2) of the FDCA (the Act), but has been advised to withdraw the 513(f)(2) application and submit this 513(f) petition instead.

II. Specification of the device [21 CFR § 860.123(a)(1)]

The classification name for the *in vitro* device requested to be down-classified to Class II is "Human Papillomavirus (HPV) DNA Polymerase Chain Reaction (PCR) Detection Device" or "HPV DNA PCR Detection Device" intended to be used for detection of HPV DNA in clinical samples. It is a nucleic acid-based technology designed to amplify and detect a minute quantity of HPV DNA in alcohol-preserved cells in liquid-based cervicovaginal lavage samples. The device is to be used for qualitative tests to determine if a clinical sample harbors HPV DNA. The principle is to use consensus PCR primers to perform repeated cycle enzymatic amplification of a highly conserved target segment of the HPV genomic DNA between two chosen primer binding sites so that a selected region of the HPV genome is copied repeatedly and exponentially for detection analyses and for genotyping. The sensitivity of the PCR device is capable of amplifying not less than 10 copies of purified HPV type 16 genomic DNA to generate specific PCR products or amplicons suitable for HPV genotyping by direct DNA sequencing or by other equivalent nucleic acid-based typing techniques for further validation.

III. Action requested [21 CFR § 860.123(a)(2)]

It is requested that PCR-based HPV DNA *in vitro* amplification devices for detection of HPVs in alcohol-preserved cells of liquid-based cervicovaginal lavage samples and for preparation of clinical materials suitable for further nucleic acid-based HPV genotyping be reclassified from Class III to Class II devices.

IV. Supplemental data sheet [21 CFR § 860.123(a)(3)]

A completed supplemental data sheet (FDA For 3427) is submitted as Attachment 1.

V. Classification questionnaire [21 CFR § 860.123(a)(4)]

A completed product classification questionnaire (FDA For 3429) is submitted as Attachment 2.

VI. Statement of the basis for disagreement with the present classification status [21 CFR § 860.123(a)(5)]

The basis of this reclassification request is that the present regulatory classification of HPV DNA tests as devices intended for use in identifying and typing HPV infection to stratify women at risk for cervical cancer, thus assigned to class III, requiring submission and approval of PMAs [2], is no longer appropriate because continued designation of low-to-moderate risk HPV DNA test devices as class III devices contradicts the current understanding of HPV infection and its relationship to the development of cervical cancer. Based on new scientific information published in the past 15 years, it is now generally agreed that identifying and typing HPV infection does not bear a direct relationship to stratification of the risk for cervical cancer. Most acute infections caused by HPV are self-limiting [1, 4-7]. It is the persistent HPV infection that may act as a tumor promoter in cancer induction [8-11]. Identifying and typing HPV is an important tool for following patients with persistent HPV infection. Repeated sequential transient HPV infections, even when caused by "high-risk" HPVs, are characteristically not associated with high risk of developing squamous intraepithelial lesions, a precursor of cervical cancer.

A woman found to be positive for the same strain (genotype) of HPV on repeated testing is highly likely suffering from a persistent HPV infection and is considered to be at high risk of developing precancerous intraepithelial lesions in the cervix. It is the persistent infection, not the virus, that determines the cancer risk.

The FDA has accepted the above interpretation of current medical science, as reflected in its March 31, 2003 announcement on approval of the Digene HC2 High-Risk HPV DNA Test while making the following public statements on record [1]:

"The FDA today approved expanded use of a laboratory test to detect the presence in women of human papillomavirus (HPV), one of the most common sexually transmitted infections."

"The HPV DNA test does not test for cancer, but for the HPV viruses that can cause cell changes in the cervix. If left untreated, these changes can eventually lead to cancer in some women."

"Most women who become infected with HPV are able to eradicate the virus and suffer no apparent long-term consequences to their health. But a few women develop a persistent infection that can eventually lead to pre-cancerous changes in the cervix."

"...most infections are short-lived and not associated with cervical cancer."

The present regulatory control of all HPV DNA *in vitro* tests as class III devices should be reviewed and the present classification status of this type of devices should be subjected to reclassification according to 21 CFR §860.3 (c) to conform with the current interpretation by the FDA of medical science on this subject, in the spirit of the Federal Food, Drug, and Cosmetic Act promulgated by the agency to bring safe and effective new technologies to the market timely in a fashion consistent with the least burdensome principles of the FDAMA of 1997.

VII. Full statement of reasons, together with supporting data satisfying the requirements of 860.7, why the device should not be classified into its present classification and how the proposed classification will provide reasonable assurance of the safety and effectiveness of the device. [21 CFR § 860.123(a)(6)]

History of the HPV DNA Nested PCR Application

On October 30, 2006, the undersigned, Sin Hang Lee, M.D., a practicing pathologist wrote a letter [12] to Dr. Steven I. Gutman, Director, Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD), Center for Devices and Radiological Health (CDRH), FDA, enclosing the manuscript of a scientific report titled "Human Papillomavirus Genotyping by DNA Sequencing-The Gold Standard HPV Test for Patient Care" [13] which was submitted to a professional journal to be considered for publication. The purpose of the letter was to inform the FDA that a more sensitive and more specific device is being introduced for detection of HPV in clinical samples and for preparation of materials for HPV genotyping and to request advice and guidance from the agency for making this device available to hospital laboratories at the point of care. With this letter and manuscript, the FDA was informed of the need for a new generation of HPV testing based on new information available because:

1) A sensitive HPV detection device that can provide accurate genotyping information is needed for following patients with persistent infection that is now recognized to be the tumor promoter in cancer induction.

2) A PCR-based HPV detection device with provision for accurate HPV genotyping is more urgently needed now because vaccination with Gardasil™ of the women who are already sero-positive and PCR-positive for vaccine-relevant genotypes of HPV has been found to increase the risk of developing high-grade precancerous lesions by 44.6%, according to an FDA VRBPAC Background Document: Gardasil™ HPV Quadrivalent Vaccine. May 18, 2006 VRBPAC Meeting. www.fda.gov/ohrms/dockets/ac/06/briefing/2006-4222B3 [14].

Without a response from Dr. Gutman or from the OIVD, the petitioner submitted a 510k application (K063649) on December 7, 2006, identifying Hybrid Capture® 2(hc2) High-Risk HPV DNA Test (Digene hc2) manufactured by Digene Corporation, 1201 Clopper Road, Gaithersburg, MD 20878 as the predicate device.

A letter dated January 9, 2007 from the FDA in response to the K063649 submission, signed by Sally A. Hojvat, M.Sc., Ph.D., Director, Division of Microbiology Devices, OIVD [3], stating: "We have determined that your type of device is classified as a class III device by the approval order for the VRAPAP Human Papillomavirus DNA detection Kit dated December 23, 1988" and "Section 515(a)(2) of the Act requires a class III device to have an approved PMA before it can be legally marketed, unless the device is reclassified."

On January 18, 2007, the petitioner submitted a Request for Evaluation of Automatic Class III Designation under Section 513(f)(2) of the FDCA (the Act), but was advised by the Office of Device Evaluation on February 22, 2007 to withdraw the 513(f)(2) submission and resubmit this petition under Section 513(f).

Historically, HPV testing [15] was introduced to compensate for the poor sensitivity and specificity of the Pap smear cytology often used as a diagnostic tool for borderline precancerous lesions. The only FDA approved Digene Hybrid Capture 2 (HC2) assay is commonly used to determine if a cervicovaginal lavage sample harbors "high-risk" HPVs [16], as an adjunctive test for evaluation of the cytologically borderline cases [17-19]. However, it is now recognized that persistent infection of a "high-risk" HPV, not the HPV virus itself, is the pivotal promoter in causing cervical precancerous lesions and cancer [7-10]. Most of HPV infections, even caused by "high-risk" genotypes, are transient with normal Pap cytology in sexually active young women [1, 3-6]. In 93% of the initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [20]. The median duration of positivity detectable by PCR for a specific HPV type in these young women is 168 days [21]. Multiple "high-risk" HPV infections do not constitute a higher risk for the development of cervical neoplasia when compared with single high-risk HPV infection [22]. For the development and maintenance of a high-grade squamous intraepithelial lesion (SIL), the risk is greatest in women positive for the same genotype of HPV on repeated testing [7-9]. Viral load is not a useful parameter to predict high-grade SIL [23]. High-grade SIL is often associated with a viral DNA load lower than that observed in less severely affected cells [24].

<u>The need of a Class II Reclassification for PCR-based HPV DNA detection devices</u>

In view of the recent advances in the understanding of the relationship between persistent HPV infection and cervical neoplasia, a sensitive and specific method to detect and accurately genotype HPV is needed for following patients with persistent infection. The HC2 test cannot be converted to a genotyping assay and is associated with a significant number of false-negative and false-positive results when compared with other more stringent PCR-based HPV genotyping assays [25-29]. It is reported to generate 25% false-negative results in cases with biopsy-proven high-grade SIL even when all these biopsies have been proven to contain high-risk HPV DNA by PCR [30].

The introduction of the type-specific Gardasil™ HPV vaccines among the sexually active women also requires genotype monitoring of the HPV infections before and after immunization to develop prevention strategy for the individual patients. Based on a "Background Document" submitted to the FDA by Merck & Co., Inc. [14], injection of HPV vaccines into women who have concurrent vaccine-relevant HPV type infections may increase the risk, by 44.6%, of developing high-grade precancerous lesions in the cervix. Therefore, it would be prudent to perform a sensitive HPV detection assay with accurate genotype determination on the patients to be vaccinated if prior HPV infection is suspected.

PCR-amplification of target HPV genomic DNA followed by nucleic acid-based HPV genotyping is a well characterized highly sensitive and highly specific research tool for generating scientific data used in epidemiological studies and in assisting development of type-specific vaccines against HPV infections. An incomplete list of publications in the world literature includes the following:

Author (s)	1st PCR primers	Nested PCR primers	Validation by	Ref.
Manos	MY09/MY11	none	In situ hybridization	1989 [31]
Snijders	GP5/GP6	none	Hybridization, RFLP	1990 [32]
Van den Brule	GP5/GP6	none	Hybridization, RFLP	1990 [33]
Bernard	MY09/MY11	none	DNA sequencing	1994 [34]
Jacobs	GP5+/GP6+	none	Hybridization, RFLP	1995 [35]
De Roda Husman	GP5+/GP6+	none	Hybridization	1995 [36]
Stewart	MY09/MY11	none	DNA sequencing	1996 [37]
Qu	GP5+/GP6+ & MY09/MY11	none	Hybridization	1997 [38]
Jacobs	GP5+/GP6+	none	Hybridization	1997 [39]
Cope	MY09/MY11	none	Hybridization	1997 [25]
Feoli-Fonseca	MY09/MY11	none	DNA sequencing	1998 [40]
Feoli-Fonseca	MY09/MY11	none	DNA sequencing	1998 [41]
Feoli-Fonseca	GP5/GP6 MY09/MY11	GP5/GP6	DNA sequencing	1998 [42]
Feoli-Fonseca	GP5/GP6	none	DNA sequencing	1999 [43]
Jacobs	GP5+/GP6+ & MY09/MY11	none	Hybridization	1999 [44]
Vernon	MY09/MY11	none	Hybridization/DNA Sequencing	2000 [26]
Nelson	MY09/MY11	GP5+/GP6+	T-Sequencing Ladder	2000 [45]
Giuliano	MY09/MY11	none	Hybridization	2001 [46]
Laconi	GP5+/GP6+	none	Hybridization	2001 [47]
Andersson	GP5+/GP6+	none	DNA Sequencing/RFLP	2003 [48]
Johnson	MY09/MY11	GP5+/GP6+	DNA Sequencing	2003 [27]
Asato	GP5,6/MY09,11	none	DNA Sequencing	2004 [49]
Speich	MY09/MY11	GP5+/GP6+	DNA Sequencing	2004 [28]
Gharizadeh	MY09/MY11	GP5+/GP6+	Pyrosequencing	2005 [50]
Evans	GP5+/GP6+	none	Hybridization	2005 [51]
Kim	MY11/GP6	none	Hybridization/DNA Sequencing	2006[52]
Guo	MY09/MY11 and GP5+/GP6+	none	Real time PCR	2006 [53]

The above list shows a trend of using PCR technology as a research tool for HPV detection and genotyping in the world scientific community since 1989. PCR-based HPV DNA tests have not been introduced into the clinical laboratories for assisting patient management in the U.S.A. due to the present FDA regulatory control by which this "type of device is classified as a class III device by the approval order for the VIRAPAP Human Papillomavirus DNA detection Kit dated December 23, 1988 [2, 3]. The regulatory arm of the FDA has resorted to invoking an approval order issued before the HPV PCR technology was developed to block all PCR-based HPV DNA detection assays by assigning them a class III status, requiring PMA submission for their approval. The burden put on the industry in fulfilling the requirements for a PMA submission to clear a qualitative HPV DNA detection assay at the FDA is enormous and is illustrated by the following Overview of IVD Regulation issued by the OIVD/CDRH published on-line [54]:

Office of In Vitro Diagnostic Device Evaluation and Safety

Overview of IVD Regulation [54]

What is a Premarket Approval (PMA)?

Studies Required to Demonstrate Safety and Effectiveness

For most PMAs, sponsors identify surrogate endpoints and establish the device performance (clinical sensitivity and specificity or agreement) with relation to the identified endpoints in corollary studies using randomly collected clinical studies.

Limitations to FDA Review

There are several limitations to FDA's review of PMA applications:

- *Lack of a "gold standard" against which to judge performance;*
- *Bias may occur in the collection of data to establish safety and effectiveness, through problems in the study design or conduct;*
- *It can be challenging to determine the minimum performance required for approval.*

Based on the current understanding of the nature of HPV infection and its relationship to risk of cancer development, it is extremely difficult to design a truly science-based protocol to conduct the required studies in compliance with the above IVD regulation for evaluation of an HPV DNA detection device. The reasons are as follows:

1) HPV detection is a virology test. Like any virology test, it should be as sensitive and as specific as possible for the detection of a target virus. The result is to be validated according to principles based on the science of microbiology. There are no "consensus"

or generally accepted surrogate endpoints or *device performance (clinical sensitivity and specificity or agreement) with relation to the identified endpoints.* Most of HPV infections, even caused by "high-risk" genotypes, are transient with normal Pap cytology in sexually active young women [4-7, 20, 21]. In 93% of initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [20]. The median duration of positivity detectable by PCR for a specific HPV type in these young women is 168 days [21]. The FDA also agrees on public record [1] that *"Most women who become infected with HPV are able to eradicate the virus and suffer no apparent long-term consequences to their health. But a few women develop a persistent infection that can eventually lead to pre-cancerous changes in the cervix." and "...most infections are short-lived and not associated with cervical cancer."* There is no precedent for using clinical sensitivity and specificity or agreement or cytopathological finding as an endpoint for the detection of causative agents in infection. The traditional required answer for a clinical microbiology test is whether there is evidence of a pathogenic microbe or virus in the clinical sample being examined. It is a "positive" or "negative" qualitative test. Since most of the HPVs detected are associated with normal Pap cytology in clinically asymptomatic women and will clear up spontaneously without causing a detectable pathology, any surrogate endpoint using clinical sensitivity and specificity or agreement or cytopathological finding for evaluating device performance is open to challenge.

2) The limitations to FDA's review of PMA applications for HPV device performance are numerous. But there is an undisputable "gold standard" *against which to judge performance* for all clinical virology tests. That is the science of microbiology that should be the basis for evaluating all *in vitro* clinical microbiology device submissions under PMAs or 510Ks. To not rely on accepted "gold standard" or to knowingly demand evaluation of a device when there is no "gold standard" for evaluation puts a great burden on the industry because it is highly "burdensome" to conform to an undefined arbitrary standard set up by regulators.

3) As stated in the Overview of IVD Regulation, the minimum performance required for PMA approval is difficult to determine. This is particularly true in a case like HPV DNA detection if the performance is evaluated by standards other than principles generally accepted in the science of microbiology. Most small innovative manufacturers do not have a large public relation staff to lobby for an acceptable minimum performance agreeable to all parties.

As a result, few or no manufacturers are willing to invest in PMA submissions in order to introduce a PCR-based technology for HPV DNA detection. Assigning a class III classification and requiring PMA application for a new HPV DNA detection device can only serve to suffocate new technologies that may compete with the outdated inaccurate FDA-endorsed Digene HC2 assay. The major reason for which the PCR-based HPV DNA detection device should not be classified into its present class III classification is to remove the regulatory roadblock for the FDA to allow the introduction of "safe and effective new technologies to the market quickly" as promulgated in a statement made by former FDA commissioner Mark B McClellan, M.D. Ph.D. [1].

The Legal Basis for Reclassification

This reclassification petition is submitted under section 513 (f) of 21CFR 860 Subpart C that provides the legal basis for reclassification of *in vitro* devices as follows.

Sec. 860.120 General.

(a) Sections 513(e) and (f), 514(b), 515(b), and 520(l) of the act provide for reclassification of a device and prescribe the procedures to be followed to effect reclassification. The purposes of subpart C are to:

(1) Set forth the requirements as to form and content of petitions for reclassification;

(2) Describe the circumstances in which each of the five statutory reclassification provisions applies; and

(3) Explain the procedure for reclassification prescribed in the five statutory reclassification provisions.

(b) The criteria for determining the proper class for a device are set forth in 860.3(c). The reclassification of any device within a generic type of device causes the reclassification of all substantially equivalent devices within that generic type. Accordingly, a petition for the reclassification of a specific device will be considered a petition for reclassification of all substantially equivalent devices within the same generic type.

(c) Any interested person may submit a petition for reclassification under section 513(e), 514(b), or 515(b). A manufacturer or importer may submit a petition for reclassification under section 513(f) or 520(l). The Commissioner may initiate the reclassification of a device classified into class III under sections 513(f) and 520(l) of the act.

21 CFR §860.3 (c) provides following definitions of three categories of regulatory control for medical devices:

(1) Class I means the class of devices that are subject to only the general controls authorized by or under sections 501 (adulteration), 502 (misbranding), 510 (registration), 516 (banned devices), 518 (notification and other remedies), 519 (records and reports), and 520 (general provisions) of the act. A device is in class I if (i) general controls are sufficient to provide reasonable assurance of the safety and effectiveness of the device, or (ii) there is insufficient information from which to determine that general controls are sufficient to provide reasonable assurance of the safety and effectiveness of the device or to establish special controls to provide such assurance, but the device is not life-supporting or life-sustaining or for a use which is of substantial importance in preventing impairment of human health, and which does not present a potential unreasonable risk of illness of injury.

(2) Class II means the class of devices that is or eventually will be subject to special controls. A device is in class II if general controls alone are insufficient to provide reasonable assurance of its safety and effectiveness and there is sufficient information to establish special controls, including the promulgation of performance standards, post-market surveillance, patient registries, development and dissemination of guidance documents (including guidance on the submission of clinical data in premarket notification submissions in accordance with section 510(k) of the act), recommendations, and other appropriate actions as the Commissioner deems necessary to provide such assurance. For a device that is purported or represented to be for use in supporting or sustaining human life, the Commissioner shall examine and identify the special controls, if any, that are necessary to provide adequate assurance of safety and effectiveness and describe how such controls provide such assurance.

(3) Class III means the class of devices for which pre-market approval is or will be required in accordance with section 515 of the act. A device is in class III if insufficient information exists to determine that general controls are sufficient to provide reasonable assurance of its safety and effectiveness or that application of special controls described in paragraph (c)(2) of this section would provide such assurance and if, in addition, the device is life-supporting or life-sustaining, or for a use which is of substantial importance in preventing impairment of human health, or if the device presents a potential unreasonable risk of illness or injury.

Reasons for a Class II classification of the device

The petitioner requests that the *in vitro* diagnostic device described in the K063649 application and in the present petition be classified as a Class II device according to 21 CFR §860.3 (c). The reasons for a class II classification are that general controls alone are insufficient to provide reasonable assurance of its safety and effectiveness and there is sufficient information to establish special controls to provide such assurance and that the device is not life-supporting or life-sustaining or for a use which is of substantial importance in preventing impairment of human health, and which does not present a potential unreasonable risk of illness or injury.

These reasons are further elaborated in the following paragraphs (a-l):

(a) The subject device is not intended to be a stand-alone diagnostic assay. As stipulated in "The indications for use" in the premarket notification, K063649 and in the Special Controls section of this petition (*see below*), this device is used to screen the cervicovaginal specimens from patients which have already been diagnosed by qualified licensed pathologists, who are American board-certified MD specialists, as "atypical squamous cells of undetermined significance" (ASCUS) based on the standard Pap smear cytology examination. Or in women 30 years and older, in conjunction with genotyping by direct automated DNA sequencing, the

device, at the discretion of and when ordered by a physician, can be used with Pap smear to adjunctively screen to assess the presence or absence of high-risk HPV types. If an HPV genomic DNA is detected, the nested PCR products resulting from the use of this device can provide materials suitable for HPV genotyping by direct automated DNA sequencing to determine by the ordering physician the need for referral to colposcopy. This information, together with the physician's assessment of cytology history, other risk factors, and professional guidelines, may be used to guide patient management. The use of the information is not intended to prevent women from proceeding to colposcopy.

As stipulated in the K063649 application and reiterated in this petition, the indications for the use of the subject device are:

(1) To screen patients with ASCUS (atypical squamous cells of undetermined significance) Pap smear results and to provide materials suitable for human papillomavirus (HPV) genotyping by direct automated DNA sequencing 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 HPV DNA Nest PCR Test™ in conjunction with genotyping by direct automated DNA sequencing 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.

The "indications for use of the device" as an assay of adjunctive nature to be performed in parallel to the Pap smear cytology examination of the same specimen by a physician pathologist, is a reasonable built-in assurance of its safety and effectiveness because the subject device will never generate a stand-alone diagnosis of human disease. As described in the section of Specimen Collection & Preparation for the assay procedure, the specimen used for the HPV DNA Nest PCR Test™ is a small aliquot, about 1 mL, of the alcohol-preserved liquid-based cell suspensions in the cervicovaginal lavage samples collected in the Cytyc or Surepath vials for cytological examinations. The test result derived from the use of the device is always an adjunct to the diagnostic Pap smear cytology report issued by a pathologist.

(b) As stipulated in the K063649 submission and as part of the Special Controls reiterated in this petition, the device package will carry the following language to specify the conditions for sales and for use:

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

HPV DNA Nest PCR Test™ is for professional use within the confines of a licensed high-complexity laboratory, as defined by the Clinical Laboratory Improvement Amendments (CLIA) of 1988.

WARNING

The Nest PCR 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 (within normal limits) Pap result with concurrent negative HPV Nest PCR test confers low risk similar to consecutive annual, technically adequate WNL Pap results.

The use of this test has not been evaluated for the management of women with prior cytological or histological abnormalities, hysterectomy, who are postmenopausal, or who have other risk factors (e.g. HIV+, immunocompromised, DES exposure, history of STI).

The HPV Nest PCR Test™ is designed to augment existing methods for the detection of cervical disease and for following persistent HPV infections 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.

HPV Nest PCR Test™ results should not be used as the sole basis for clinical assessment and treatment of patients.

Positive results of HPV Nest PCR Test™ should be confirmed by genotyping with DNA sequencing, or be considered inconclusive if no genotyping results can be obtained by DNA sequencing.

***A Pap test, HPV genotyping by DNA sequencing and associated testing materials are not included in the test kit and must be obtained separately.**

(c) For promulgation of performance standards as a Special Control, the sales conditions of this device will stipulate that the users of this device will validate all positive nested PCR products by HPV genotyping with direct automated DNA sequencing with the HPV sequences confirmed by on-line BLAST algorithm, The Basic Local Alignment Search Tool sequence alignment analysis program maintained by the National Institutes of Health, or by an alternative HPV genotyping method which has been properly validated, or by an HPV genotyping method which has been approved by the FDA.

(d) Another Special Control for the use of this device in a clinical laboratory is the requirement of adding a PCR water control tube for every 20 specimens to rule out the possibility of cross contamination between samples, in addition to the general negative and positive controls as required for other *in vitro* diagnostic devices.

(e) As a Special Control measure, the petitioner will maintain a division of HPV DNA genotyping by direct automated DNA sequencing to provide needed technical assistance to those laboratories which are using this device. The experience of the users with the device

and the problems encountered will be recorded on file. Periodic reports will be made to the FDA if required to assure the safety and effectiveness of the device.

(f) The device is not life-supporting. The device is designed to detect a minute quantity of nucleic acid molecules of a sexually transmitted virus, HPV, in women's cervicovaginal lavage. The patients are all in general good health and ambulatory. These patients do not need a life-supporting nucleic acid-based test for cervicovaginal HPV. The device is not intended to be used as a life-supporting device.

(g) The device is not life-sustaining since the patients as qualified in "The indications for use" are all in general good health and ambulatory. The device is only used as an adjunct to the Pap smear invariably performed on ambulatory women patients.

(h) The device is not for a use which is of substantial importance in preventing impairment of human health since the device is an adjunctive assay, used in conjunction with a cytopathological examination that will detect atypical cells which may be cancerous or indicative of a potential early development of cancer. The cytopathological examination or the Pap smear performed by board-certified pathologists with or without the use of this adjunctive device is a diagnostic assay and is of substantial importance in preventing impairment of human health. The subject device that is a nucleic acid-based virology test is not a stand-alone cytopathological diagnostic test to screen cancer or to diagnose cancer or to stratify the risk of developing cancer. It is used only adjunctively in conjunction with some of the cytology Pap smear assays. Therefore, the subject device is not of substantial importance in preventing impairment of human health by definitions of the Classification.

(i) The device does not present a potential unreasonable risk of illness or injury when appropriate special controls in addition to general control requirements to provide reasonable assurance of safety and effectiveness for its intended uses are adopted. Special Controls may include those as proposed above and listed in under Special Controls section of this petition as well as others recommended by the FDA.

(j) HPV infection, as determined by its natural disease history, is a low-risk or moderate-risk pathologic condition which may cause cervicitis, epithelial metaplasia, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), if the HPV cervicitis is persistent. HSIL and LSIL are usually detected and diagnosed by certified pathologists on Pap smear cytology. When the Pap smear cytology does not meet certain morphological criteria for the pathologists to make a firm diagnosis of HSIL or LSIL (both considered potentially precancerous conditions) an uncertain diagnosis of "Atypical Squamous Cells of Undetermined Significance", or ASCUS, is rendered, according to the Bethesda System [55]. In the natural history of "dysplasia", a small percentage of the HSIL or LSIL cases will progress into clinical cervical cancer [56]. The impairment of human health, namely the precancerous lesions and cancer of the uterine cervix, is not directly caused by the HPV itself, but by the abnormal growth, or uncontrollable proliferation, of the epithelial cells in response to the persistent HPV infection. These abnormal or atypical cells are the mutants of squamous epithelial cells that may lead to the formation of cancer, a long

process with the end result being influenced by many other factors, including the immunity status of the host and numerous environmental carcinogens.

One infective agent substantially equivalent or comparable to HPV in its capacity of initiating human carcinogenesis is the bacterium, *Helicobacter pylori* (*H. pylori*), which may cause gastritis and chronic ulcer that may lead to stomach malignancy (cancer or lymphoma), a serious impairment of human health. The relationship between H. pylori infection and gastric cancer is well established. [57, 58]

In the pathogenesis of the disorders initially caused by these two infective agents, it is the inflammation, the chronic persistent inflammation that acts as a tumor promoter in cancer induction [59]. Both *H. pylori* and HPV cause inflammation in or around a lining epithelium that may lead to proliferation of the epithelial cells, epithelial cell metaplasia, dysplasia and cancer, in the stomach [60] and in the uterine cervix [56], respectively. However, in most patients, the pathologic process of both of these diseases does not progress to the stage of high-grade dysplasia, nor cancer in the stomach or the cervix. Only 10%-15% of individuals infected with *H. pylori* develop peptic ulcer disease, and the risk of gastric cancer is estimated to be approximately 1%-3% [60]. The percentage of patients infected by HPV who eventually progress into overt cervical cancer is even lower because a high percentage of "dysplasia" cases regress to normal cytology on long-term follow-ups [56].

The general acceptance of *H. pylori* as an infective agent in gastric carcinogenesis is evidenced by the fact that the Nobel Assembly at Karolinska Institute recently awarded the Nobel Prize in Physiology or Medicine for 2005 jointly to Barry J. Marshall and J. Robin Warren for their discovery of "the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" and "Malignancies associated with *Helicobacter pylori* infection"[61]. The NCI in its *General Information on Gastric Cancer* , Health Professional Version last modified on 11/30/2006 [62], also listed *Helicobacter pylori* gastric infection as the leading risk factor. The estimated deaths due to stomach cancer were 11,430 in the United States in 2006.

For comparison, the NCI listed the total deaths due to cervical cancer in the United States were about 3,700 in 2006 [63].

Since the annual deaths (about 11,430) due to gastric cancer which is related to the infection caused by *H. pylori* are about 3 times the number of deaths (about 3,700) due to cervical cancer related to infections caused by HPV in the United States, it is reasonable to conclude that *H. pylori* infection is associated with a higher level of risk than HPV infection in causing fatal cancer. Logically, an *in vitro* diagnostic device for preventing the development of gastric cancer is of **more** substantial importance in preventing impairment of human health than an *in vitro* diagnostic device for preventing the development of cervical cancer if the *"for a use which is of substantial importance in preventing impairment of human health"* provision under 21 CFR §860.3 (c)(3) is invoked as the criteria for risk-based classification of the subject device. The number of annual human deaths can be considered the undisputable measure in grading and comparing the degrees of impairment of human health initiated by these two causative agents, namely *H. pylori* and HPV.

Based on records published on-line, the FDA has customarily considered *Helicobacter pylori* as a low-risk pathogen in reviewing 510k applications for clearance of several *in vitro* diagnostic devices for *Helicobacter pylori* infections as class II or class I devices. One of such examples is the approval letter dated June 10, 2003 for an Immunoassay, *H. Pylori* Test, K024360 [64], although it is well known that *H. pylori* infections might eventually lead to stomach cancer that causes more human deaths than cervical cancer in the United States. An *in vitro* diagnostic device for an infection that causes significantly less human deaths than the infection caused by *H. pylori* cannot be considered to be of more substantial importance in preventing impairment of human health than a class II or a class I device if the "risk-based" classification rule of medical devices is applied. Therefore, that HPV plays an important role in the initiation of persistent infection which may lead to carcinogenesis of the uterine cervix does not constitute a regulatory basis for mandatory assignment of an HPV nucleic acid *in vitro* device into the Class III category especially when the device is intended to be used as an adjunct to the Pap smear diagnostic cytology test performed by a physician pathologist.

(k) As reported in the world's literature, detectable HPV infection has been shown to be most common in young women and the infection is often transient with normal cervicovaginal cytology.[4-7]. In 93% of initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [20]. Although the prevalence varies among regions, it generally reaches a peak of about 20% in women aged 20-24, with subsequent decline to approximately 8% to 10 % among women over age 30 [65]. Of the small percent of women whose HPV infection leads into formation of intraepithelial lesions, the majority likely will develop only LSIL which usually regresses or does not progress, particularly in women under age 35. Progression to detectable, precancerous lesions can take as long as 10 years. One study estimates that the risk of progression from a moderate SIL to high SIL and to overt precancerous lesion is 32% within 10 years [56]. These data further substantiate the assertion that in most cases HPV infection is a self-limiting disease. The virus, HPV itself, only poses a low risk to the impairment of human health unless there is evidence of persistent infection maintained by a "high-risk" HPV genotype. For the development and maintenance of HSIL, the risk is greatest in women positive for the same genotype of HPV on repeated testing. Sequential, multiple infections, even caused by different so-called "high-risk" HPV genotypes, are characteristically not associated with high risk of cancer development [8-10]. This self-limiting nature of HPV infection probably accounts for the fewer cervical cancer deaths than stomach cancer deaths in the U.S. while the prevalence rate of HPV infection in the population is much higher than that of *H. pylori* infection.

(l) The summary of the current scientific data by the National Cancer Institute (NCI) in its official document labeled FactSheet on HPV, reviewed and updated on 06/08/2006 [66], further supports the conclusions that even the so-called "high-risk" HPV genotypes pose only a low risk to the impairment of human health and that HPV assays are adjunctive or additional in nature to the Pap test and biopsy, the two pivotal *in vitro* tests to screen and diagnose precancerous cervical conditions. The relevant paragraphs in this NCI document supporting these conclusions are quoted as follows.

"Most HPV infections occur without any symptoms and go away without any treatment over the course of a few years. However, HPV infection sometimes persists for many years, with or without causing cell abnormalities."

"Some types of HPV are referred to as "low-risk" viruses because they rarely develop into cancer. HPV types that are more likely to lead to the development of cancer are referred to as "high-risk." Both high-risk and low-risk types of HPV can cause the growth of abnormal cells, but generally only the high-risk types of HPV may lead to cancer. Sexually transmitted, high-risk HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and possibly a few others. These high-risk types of HPV cause growths that are usually flat and nearly invisible, as compared with the warts caused by types HPV–6 and HPV–11. It is important to note, however, that the majority of high-risk HPV infections go away on their own and do not cause cancer."

"Having many sexual partners is a risk factor for HPV infection. Although most HPV infections go away on their own without causing any type of abnormality, infection with high-risk HPV types increases the chance that mild abnormalities will progress to more severe abnormalities or cervical cancer. Still, of the women who do develop abnormal cell changes with high-risk types of HPV, only a small percentage would develop cervical cancer if the abnormal cells were not removed. Studies suggest that whether a woman develops cervical cancer depends on a variety of factors acting together with high-risk HPVs. The factors that may increase the risk of cervical cancer in women with HPV infection include smoking and having many children."

" What tests are used to screen for and diagnose precancerous cervical conditions?

A Pap test is the standard way to check for any cervical cell changes. A Pap test is usually done as part of a gynecologic exam. The U.S. Preventive Services Task Force guidelines recommend that women have a Pap test at least once every 3 years, beginning about 3 years after they begin to have sexual intercourse, but no later than age 21.

Because the HPV test can detect high-risk types of HPV in cervical cells, ***the FDA approved this test as a useful addition to the Pap test*** *to help health care providers decide which women with ASC–US need further testing, such as colposcopy and biopsy of any abnormal areas. (Colposcopy is a procedure in which a lighted magnifying instrument called a colposcope is used to examine the vagina and cervix. Biopsy is the removal of a small piece of tissue for diagnosis.) In addition,* ***the HPV test may be a helpful addition to the Pap test*** *for general screening of women age 30 and over."*

In the above quoted paragraphs, the NCI also emphasizes that the currently FDA- approved HPV assay is an "addition" to the Pap test. Based on the reasons presented above, the petitioner respectfully requests the FDA to rule that the subject device referred to in the K063649 application and in this petition is a moderate risk *in vitro* diagnostic device that should be reclassified as a Class II device. The general characterization of the HPV test as a "helpful addition" to the Pap test is further affirmed by the NCI FactSheet on HPV quoted above in bold-faced Italics in the preceding paragraph.

Appropriateness of Class II classification.

Class II and the 510(k) pathway is an appropriate route to market for a PCR-based HPV DNA detection device, "a laboratory test to detect the presence in women of human papillomavirus (HPV), one of the most common sexually transmitted infections." [1]

PCR-amplification of target viral genomic DNA followed by nucleic acid-based genotyping for HPV is coming into the practice of medicine with or without an FDA-approved PCR test. A cursory browsing on the Web revealed the following links, leading to advertisement of a clinical PCR test with proprietary primers.

West Coast Pathology Labs : DNA Tests Supporting Documentation
The Analyte Specific Reagents (ASRs) were developed by **Access Genetics**, a U.S. Food and ... **HPV** DNA positive **PCR** products were subjected to digestion by the ...
www.wcpl.com/dna_support_doc.asp - 10k - Cached - Similar pages

Physicians Lab
The Four-Step **ACCESS PCR™** Method uses proprietary **HPV** primers that have greater ...
Access Genetics' Four-Step DNA Testing System uses leading-edge testing ...
www.plpath.com/subcategory.cfm?cat_id=16&subcat_id=96 - 28k - Cached - Similar pages

These assays are all in-house, "home-brew", using analyte-specific reagents to perform the tests. The PCR products are usually subjected to various methods of genotyping. There is no information on the number of clinical laboratories using these "home-brew" assays for HPV detection and HPV genotyping in the United States. There is no interlaboratory comparison of results. The PCR primers used in the above-referenced laboratory are "proprietary HPV primers". Quality assurance is not possible without certain established performance standards. PCR methodology varies in sensitivity and specificity. Without a standardized protocol, PCR may be less sensitive than the Digene HC2 assay [67]. Down-classification to Class II of the present Class III status of PCR-based HPV DNA detection devices will authorize the OIVD to take a leadership position in guiding the numerous small innovative manufacturers to introduce their *in vitro* devices for this test through the open 510(k) route in order to bring "safe and effective new technologies to the market quickly" [1], consistent with the least burdensome principles of the FDAMA of 1997.

The present FDA-approved HPV DNA detection kit (the Digene HC2 Hybrid Capture HPV DNA detection test) is an outdated inaccurate technology. It is known to generate numerous false negative and false positive tests [25-28]. HC2 cannot provide genotyping information for following patients with persistent infections and for evaluation of prevention strategy for the individual patients to be immunized with type-specific HPV vaccines. The similarities and dissimilarities between nested PCR and HC2 test are tabulated for comparison on the next page.

SUMMARY OF COMPARISON WITH DIGENE hc2 HPV TEST IN TABLE FORMAT

SIMILARITIES		
Item	**HPV Nest PCR**	**Digene hc2**
Intended Use	Qualitative detection of all HPV types, with end-product suitable for direct DNA sequencing	Qualitative detection of 13 high-risk HPV types, not to be genotyped
Indications for its use	Screen patients with ASCUS Pap smear results Used adjunctively with Pap smear in women 30 years and older	Screen patients with ASCUS Pap smear results Used adjunctively with Pap smear in women 30 years and older
Target molecules for detection	HPV genomic DNA	HPV genomic DNA

DIFFERENCES		
Item	**HPV Nest PCR**	**Digene hc2**
Method of amplification	Nested PCR amplification of target DNA	Signal amplification
Mechanism of amplification	By consensus primer PCR	By reporter molecules
Results detected by	Agarose gel electrophoresis of target DNA PCR products	Chemiluminescence generated by enzyme substrate
Validation of end-product by direct DNA sequencing	Possible and recommended	Not possible
Sensitivity of detection (purified HPV DNA)	1-10 copies of HPV genomic DNA	10^5-10^6 copies of HPV genomic DNA
Sensitivity of detection (clinical specimens)	100%	37.9-100% depending on genotypes
Specificity (clinical specimens)	100% with validation by DNA sequencing for genotyping	74.6% (high-risk HPV) confirmed by DNA sequencing in split parallel clinical specimens
Specimen types	Liquid-based alcohol-preserved cell suspensions	Liquid-based alcohol-preserved cell suspensions and tissue biopsies
Multiple HPV infections	DNA sequencing not possible for specific genotyping	Capable of detecting 13 high-risk HPV types as a group

Special Controls on Safety and Effectiveness
[21 CFR § 860.123(a)(6)]

For a Class II HPV DNA Nested PCR device, there is sufficient information to achieve reasonable assurance of safety and effectiveness by establishing performance standards and providing guidance document and testing guidelines to the professional users of the device. These performance standards, documents and guidelines are presented as materials to be communicated to the users of the device as follows.

1. A Scientific Synopsis of the Device with Petitioner's Comments

Like any virology test, an HPV assay should be as sensitive and specific as possible, to be validated according to principles based on the science of microbiology. HPV testing is an adjunct to, but independent of, the Pap smear cytology, a generally accepted diagnostic tool for precancerous and cancerous conditions. The role of HPV in carcinogenesis is in its ability to initiate and maintain a persistent infection, namely a chronic inflammation. Contrary to acute inflammation which tends to suppress carcinogenesis, chronic inflammation is the tumor promoter in cancer induction [59, 68]. The causative agent initiating an infection that may eventually lead to the formation of cancer does not invariably cause chronic inflammation or lead to cancer development.

A sensitive method for HPV detection is needed because the cells of a high-grade squamous epithelial lesion (HSIL) are usually small [69] and are associated with a lower HPV load than those of a low-grade SIL (LSIL) [24]. At the cytological level, the typical HSIL cells with a high nucleus/cytoplasm ratio are generally smaller in size than the koilocytes of LSIL and the cells with abundant cytoplasm observed in the "atypical squamous cells of uncertain significance" (ASCUS) category. Based on studies of keratinocyte cultures infected by HPV-16, it is estimated that the small cells contain ~100 episomal copies of HPV per cell whereas the large cells contain ~3500 copies per cell [70]. Therefore, to be of practical value for following persistent infections with the potential of progression to precancerous or cancerous conditions, a nucleic acid-based HPV test must be capable of detecting a minute quantity of HPV DNA in the clinical specimens and providing accurate genotyping information when HPV DNA is detected. The viral load per abnormal cell may decrease during cancer development as the cytopathology progresses from metaplasia, to dysplasia and carcinoma.

The methods currently available for HPV genotyping, although not commonly in use, include line probe hybridization assays, restriction fragment length polymorphism analysis and Sanger DNA sequencing, all using PCR amplification of a target DNA for detection and for test material preparation [71]. DNA sequencing is considered the "gold standard" in developing new methods for HPV genotyping [50, 52, 72]. Cycle sequencing has been a standard scientific tool for accurate microbial and viral typing in the past two decades, but only used in research institutes and pharmaceutical

companies. However, with the recent rapid progress in the science of molecular medicine, DNA sequencing technology is becoming more readily available to clinical laboratories, especially in the field of diagnostic virology.

The well characterized MY09/MY11 and GP5+/GP6+ nested PCR is a highly sensitive method for HPV detection and specific for preparation of clinical materials suitable for genotyping with direct DNA sequencing [27, 28, 49].

To convert this research tool into a diagnostic procedure, the petitioner adapted a new low temperature (LoTemp™) ready-to-use PCR polymerase mixture and optimized all the reagents for its routine application in a clinical laboratory. The device uses a combination of two newly patented DNA polymerases that are stable at room temperature for at least six weeks in final working solutions containing proprietary dsDNA-melting agents and dNTP-protectives, and are highly processive in repeated cycle enzymatic nucleotide primer extension. Since the ready-to-use polymerase mixture contains all the required ingredients for PCR, the need for in-house pipetting is minimal. Since the DNA polymerase and other reagent components are stabilized for storage at room temperature, there is no need to keep ice-cold blocks for cooling of reagents while setting up the PCR at the bench. Since this system uses chemical melting agents for dsDNA denaturing and a high-processivity DNA polymerase for nucleotide primer extension under partial isostabilization in the presence of highly effective melting agents, it allows thermocycling at 85°C for denaturing, 40°C for annealing and 65°C for primer extension, respectively. By lowering the cycling temperatures, the rate of heat-induced mutations [73], namely depurination [74] and deamination [75] of the nitrogenous bases, in the DNA molecules during PCR amplification is reduced. As a result, the PCR products are more homogeneous and purification of templates is no longer necessary in the nested PCR protocol.

The LoTemp™ PCR system can amplify 1-10 copies of purified HPV-16, -18 or -6B to generate a corresponding type-specific 150 bp nested PCR product confirmed by DNA sequencing. This sensitivity and specificity are at least comparable to those of the traditional heat-resistant DNA polymerase PCR systems used in nested PCR [27, 28, 49]. The MY09/MY11 and GP5+/GP+6 consensus primers are degenerate PCR primers with numerous mismatched bases to each target HPV DNA molecule. Optimization of the PCR protocol is essential to yield reliable reproducible results. Without optimization, a PCR method may have a lower sensitivity than the Digene HC2 test in detecting HPV DNA in clinical specimens [67].

In a pre-submission study, the petitioner has demonstrated that using an optimized protocol, the nested PCR technology identified 107 HPV-positive cases among 513 samples and the results have been compared with those obtained by the present FDA-approved Digene HC2 test on split samples of each case (Attachment 3, Volume A). All positive results obtained by the subject device have been validated by genotyping with direct DNA sequencing. The computer-generated DNA sequencing data with the corresponding BLAST algorithm analyses on individual positive cases are enclosed (Attachment 3, Volume B).

In this study, twenty-three (23) HPV genotypes have been identified, including 12 of the 13 "high-risk" genotypes, i.e. HPV-16, -18, -31, -33, -35, -39, -45, -52, -56, -58, -59, and -68, which are targeted by the Digene HC2 test. The lack of representation of HPV-51 that is also targeted by the HC 2 test probably reflects a low regional prevalence of this genotype. Using the same primers for nested PCR amplification followed by genotyping with DNA sequencing, HPV-51 was found to be a relatively common genotype in Germany, constituting about 5% of the total HPV isolates detected [28]. However, it was not recorded even once among 894 HPV isolates in Denmark [27].

Among the 107 PCR-positive cases, Digene High-risk HC2 classified 67 as HPV-positive, at a detection rate of 62.6% (*see below*). When the HC2-targeted "high-risk" HPV genotypes are used for comparison, the subject nested PCR device detects 74 HPV-positive cases while HC2 test identifies 50 in this group, a sensitivity of 67.6% (50/74).

HPV genotype prevalence is subject to regional variations. The petitioner found that in the New Haven area the most prevalent is HPV-16, constituting 27.2% of the total isolates *(see below)*. This percentage is similar to those reported by others who used nested PCR/DNA sequencing for their study, e.g. 26% in Denmark [27] and 26.2 % in Germany [28]. Digene HC2 test fails to identify 18 of the 29 HPV-16 positive cases, a failure rate of 62% in this series. This discrepancy is probably in part due to the fact that there are numerous HPV-16 sequence variants [76] that may not be all targeted by the HC2 RNA cocktail probe, but share a highly conserved region of the L1 gene that the MY09/MY11 primers amplify effectively.

HPV-56 is the second most prevalent high-risk genotype detected (8.5%), followed by HPV-31, -18, -54, -58 and -66, sharing about the same rate of prevalence (5.6-6.5%). The combined number of HPV-16 and -18 cases constitutes only 32.8% of the single HPV isolates in this series. This raises the question if a type-specific vaccine targeting HPV-16 and HPV-18 can be as effective in prevention of HPV infection among the women living in the county of New Haven, Connecticut, as in other parts of the world. A similar question has been raised by a group of investigators in Quebec, after reviewing their DNA sequencing data which show that HPV-18 seems to play a relatively minor role among the high-risk HPV infections in Canada [77].

It has been reported by others [78] that the Digene HC2 high-risk test may be able to detect HPV types -53, -54, -62, -66 and –83 and label them as high-risk HPVs although these genotypes are not intentionally targeted in its high-risk cocktail probe. The petitioner's findings confirm these cross reactions (*see below*). Sequence variation within the probe binding sites [79] and non-specific binding between the probe and non-targeted mismatched DNA [80-83] are well recognized sources of error when nucleic acid hybridization is relied upon for accurate microbial and viral genotyping. When the GP5+/GP6+ PCR products with a hypervariable DNA sequence are targeted for developing a multiplex genotyping method [84], the DNA probe designed for HPV-66, a recently recognized high-risk type [85-87], is found to react with HPV-52 and the probe

for HPV-82 with HPV-51 due to cross-hybridization despite the presence of four base mismatches in each pair, a potential pitfall to accurate genotyping associated with all diagnostic methods relying on the nucleic acid hybridization principle.

Fifty percent (50%) of the HPV-54 isolates are identified by HC2 test as high-risk HPV although it is not a target of the cocktail probe. HPV-54 has been classified as a low-risk virus based on studies in other countries [86, 88], but is found to be associated with a 40-fold increase in risk among American Indian women with CIN 2/3. Only HPV-16 has shown a higher risk than HPV-54 among this subpopulation [89]. The latter finding supports the observation that genetic make-up of a patient may have to be considered in using HPV genotyping information for the follow-up of persistent infections [90].

When multiple genotypes are encountered in one specimen, the GP6+ general sequencing primer generates two or more overlapping DNA sequences in the primer extension/termination reaction mixture. These superimposed sequences cannot be resolved by sequence alignments with BLAST algorithm. This limitation of an excellent scientific tool has been cited as a reason for not recommending DNA sequencing as a means for genotyping in clinical practice [91]. Various approaches have been proposed to overcome this technical obstacle in HPV DNA sequencing, including special software computer-analyses of the overlapping sequences [43], hybridization probe assays [91] and the use of type-specific multiple sequencing primers for pyrosequencing [50]. In the petitioner's opinion, none of these alternatives can generate accurate genotyping information comparable to that with classic DNA sequencing especially when the large number of sequence variants of HPV-16 must be taken into consideration. For the mixed infection cases, the petitioner recommends using single primers specific for HPV-6, -11, -16 and -18 [50] to perform individual primer extension/termination reactions to repeat DNA sequencing in order to determine if the mixed infection includes any of these vaccine-relevant HPV types. The rationale for this choice is that the majority of multiple HPV infections are transient [4-11, 20, 21] although the natural course of multiple infections is not clear at the present time and needs to be further investigated when the HPV genotyping test becomes more widely available. The immediate concern to the patient and her health care provider is whether the mixed infection is caused by any of the vaccine-relevant HPV types if the patient is considering vaccine immunization. With this approach, the petitioner found that 2 of the 5 multiple infections contain at least one of these four HPV genotypes, with one sample infected by HPV-16 and the other by both HPV-16 and HPV-18. The remaining 3 samples contain at least two HPV types which are not HPV-6, -11, -16 or -18.

The rate of multiple HPV infections is known to vary among patient populations and is also influenced by the stage of carcinogenesis. Multiple HPV infections were found in less than 5% of the HPV-positive samples from patients with invasive cancer lesions, but over 15% of the positive samples in the control group [49]. Multiple HPV infections tend to evolve into single HPV infections as the infection becomes chronic and persistent while the cervical cytopathology progresses from metaplasia, LSIL, HSIL, carcinoma-in-situ to invasive cancer [92].

2. Guidance Document and Testing Guidelines

Reasonable assurance of Safety and Effectiveness can be provided through Labeling/Promotional Materials as follows.

(1) Proprietary & Established Names

Device Name: **Human Papillomavirus DNA Nested Polymerase Chain Reaction Detection Kit**

Trade Name: HPV DNA Nest PCR Test™

(2) Intended Uses

The HPV DNA Nest PCR Test™ (Nest PCR)* using the nested polymerase chain reaction (PCR) technology is an *in vitro* nucleic acid assay with target DNA amplification in PCR tubes or in microplates for the qualitative detection of human papillomavirus (HPV) DNA in cervical specimens. A positive Nest PCR test is presumptive evidence for the presence of an HPV DNA in the specimen and provides materials suitable for direct automated DNA sequencing for HPV genotyping. All known clinically relevant HPV genotypes can be detected, including HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, the high-risk HPV genotypes recognized by the FDA for a legally marketed *in vitro* diagnostic device.

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

Cervical specimens that may be tested with the Nest PCR Test are alcohol-preserved cells in liquid-based cervicovaginal lavage samples (e.g. Cytyc or Surepath).

The indications for its use are:

1. To screen patients with ASCUS (atypical squamous cells of undermined significance) Pap smear results and to provide materials suitable for HPV genotyping by direct automated DNA sequencing to determine the need for referral to colposcopy. The results of this test are not intended to prevent women from proceeding to colposocpy.

2. In women 30 years and older the HPV DNA Nest PCR Test™ in conjunction with genotyping by direct automated DNA sequencing 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.

HPV DNA Nest PCR Test™ is for professional use within the confines of a licensed high-complexity laboratory, as defined by the Clinical Laboratory Improvement Amendments (CLIA) of 1988.

WARNING

- The Nest PCR 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 (within normal limits) Pap result with concurrent negative HPV Nest PCR test confers low risk similar to consecutive annual, technically adequate WNL Pap results.
- 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 sexually transmitted infections).

The HPV Nest PCR Test™ is designed to augment existing methods for the detection of HPV in cervicovaginal lavage samples and to prepare clinical materials suitable for HPV genotyping. It 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.

HPV Nest PCR Test™ results should not be used as the sole basis for clinical assessment and treatment of patients.

Positive results of HPV Nest PCR Test™ should be confirmed by genotyping with DNA sequencing, or be considered inconclusive if no genotyping results can be obtained.

***A Pap test, HPV genotyping by DNA sequencing and associated testing materials are not included in the test kit and must be obtained separately.**

(3) Summary and Explanation

Human papillomavirus (HPV) testing was introduced to compensate for the poor sensitivity and specificity of the Pap smear cytology often used as a diagnostic tool for borderline precancerous lesions [15]. The Digene Hybrid Capture 2 (HC2) test is commonly used to determine if a cervicovaginal lavage contains "high-risk" oncogenic HPV [16]. The HC2 HPV test cannot provide specific genotyping for the HPV detected and it usually requires 100,000 copies of HPV DNA for a positive reading.

Recently, it has become more widely known that consistent detection of the same high-risk genotype of HPV in a patient on multiple occasions, which may be indicative of a persistent infection, is more clinically significant than finding different high-risk HPV

genotypes over a period of time. Sequential, multiple infections, even caused by different high-risk HPV genotypes, are characteristically not associated with high risk of cervical cancer development. For the development and maintenance of high-grade SIL, the risk is greatest in women positive for the same genotype of HPV on repeated testing [8-10]. High-grade SIL is often associated with a viral DNA load lower than that observed in less severely affected cells [24]. High HPV viral loads and multiple HPV infections are more common in sexually active young women in whom the HPV infection is often transient with normal cervicovaginal cytology [4-7]. In 93% of initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [20]. The median duration of positivity detectable by PCR for a specific HPV type in these young women is 168 days [21].

The recent introduction of type-specific HPV vaccines into the populace may require genotype monitoring of the HPV infection before and after immunization to develop prevention strategy for the individual patients when concurrent infection by a vaccine-relevant HPV is suspected prior to vaccination. According to the VRBPAC Background Document on Gardasil™, the HPV Quadrivalent Vaccine, presented to the FDA by Merck & Co., Inc. at the May 18, 2006 VRBPAC Meeting [14], the vaccine may cause more harm than placebo when it is administered to subjects who have already contracted the infection by HPV-6,-11,-16 or -18. In a subset of clinical trial data, among the 156 subjects who were seropositive and PCR-positive for these so-called vaccine relevant HPV types, 31 subjects developed grade 2/3 or worse CIN lesions after receiving the vaccine while only 19 of the 137 subjects in the same subgroup developed such precancerous lesions after receiving placebo. In other words, the vaccine may increase the risk of developing high-grade dysplasia by 44.6% in a patient if she has concurrent infection by one of the four HPV types contained in the vaccine. In addition, diseases due to other HPV types also have the potential to counter the efficacy results of Gardasil™ for the HPV types contained in the vaccine, according to this document. Therefore, a sensitive, specific and reproducible method for HPV detection and to provide material suitable for genotyping to monitor HPV infection is needed to assist the health care providers in dealing with these new developments in clinical management.

Nested PCR with two sets of consensus primers in two consecutive PCR amplifications in tandem is highly sensitive in detecting a very small quantity of HPV DNA. It can provide nested PCR products suitable for genotyping with direct automated cycle DNA sequencing. A combination of the PCR and DNA sequencing technologies can detect all known HPV types of clinical significance and generate unequivocal genotyping information on the individual HPV isolates [26-29, 40, 42, 48, 49]. Accurate genotyping following PCR detection of HPV has been recognized as the HPV testing of choice to further improve the quality of clinical management. [71, 93]

(4) Test Principle

The HPV Nest PCR Test™ is a nucleic acid assay using PCR amplification to detect target HPV genomic DNA extracted from the alcohol-preserved cells in liquid-based

cervicovaginal lavage specimens. A conserved L1 region of the HPV genome is first amplified with a pair of MY09/MY11 consensus primers to generate a 450 bp PCR product which may or may not be visible on electrophoresis, followed by a nested PCR with a pair of GP5+/GP6+ general primers annealed to two DNA segments located internal to the MY09/MY11 binding sites. The appearance of a band of 150 bp nested PCR products stained with ethidium bromide visualized under UV light after agarose gel electrophoresis is presumptive evidence that HPV DNA is present in the clinical specimen.

The positive nested PCR product should be subjected to confirmatory DNA sequencing for genotyping. The HPV genotype is determined by alignment of a hypervariable segment of the computer-generated DNA sequence in the amplified L1 region of the HPV genome against those known HPV sequences stored in the GenBank database using on-line BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST). DNA sequencing is a well characterized and generally accepted "gold-standard" method for accurate microbial and viral typing, as applied in HPV genotyping.

Reagents and equipment needed for DNA sequencing are not included in this test kit.

(5) Reagents and Materials Provided

There are 96 tests in one HPV Nest PCR Test™ kit. The number of patient results will vary depending on the number of uses per kit because appropriate positive and negative controls must be included in each test run. One HPV Nest PCR Test™ kit includes the following reagents:

36 strips x 8 PCR tubes LoTemp™ HiFi® DNA polymerase ready-to-use mix** for 288 PCRs

1 x 200 µL MY09 primer 5'-CGTCCMARRGGAWACTGATC-3' 10 µmolar

1 x 200 µL MY11 primer 5'-GCMCAGGGWCATAAYAATGG-3' 10 µmolar

1 x 200 µL GP5+ primer 5'-TTTGTTACTGTGGTAGATACYAC-3' 10 µmolar

1 x 200 µL GP6+ primer 5'-GAAAAATAAACTGTAAATCATATTC-3' 10 µmolar

1 x 200 µL ß-globin gene primer F 5'-ACACAACTGTGTTCACTAGC-3' 80 µmolar

1 x 200 µL ß-globin gene primer R 5'- CAACTTCATCCACGTTCACC -3' 80 µmolar

1 x 50 µL Full-length purified HPV-16 genomic DNA 100,000 copies/µL in TE buffer.

1 x 100 mL Cell-washing buffer, pH 8.1

1 x 10 mL Proteinase K solution, 0.1 mg/ml in cell washing buffer.

10 x 4G Agarose gel powder

1 x 50mL 50 X Electrophoresis buffer

1 x 0.5 mL Ethidium bromide, 10mg/mL

1 x 0.5 mL Sample Loading Dye Buffer

1 x 0.5 mL Molecular ruler 100-1000 bp

**LoTemp™ HiFi® DNA polymerase ready-to-use mix contains in each 0.2 ml PCR tube all the components needed for low temperature PCR, including dNTPs, Mg++, buffer, two HiFi® DNA polymerases, proprietary dsDNA melting agents and dNTP preservatives. All components are in 20 µL solution to be used in the final reaction volume of 25µL. The reaction mix remains PCR-active for at least 10 months when stored at 4°C and at least 8 weeks at ambient temperature not to exceed 25°C. Do not freeze.

Materials Required But Not Supplied

General PCR equipment and accessories

PCR Thermocycler (Model TC-412 thermal cycler, Techne Incorporated, Burlington, NJ) or equivalent

Microwave oven

Horizontal Electrophoresis Set

Bio-Rad Sub-Cell Systems with PowerPac Basic Power Supply or Equivalent

UV light source for viewing PCR products, with photographic option.

Micropipettors with disposable tips for 1-10 µL.

Incubator for 45-55°C.

Heating block for 1.5 ml Eppendorf tubes.

Centrifuge for 1.5 ml Eppendorf tubes to be used at room temperature.
Eppendorf 5424 with 5424R rotor or equivalent

Microcentrifuge with adaptors for 0.2 ml PCR tubes in strip.

Vortex mixer.

Clean glass rods of 1.5 mm in diameter with wettable surface.

Molecular biology grade pure water (to be used in all procedures).

Powder-free gloves.

WARNING 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-HIH manual, Biosafety in Microbiological and Biomedical Laboratories, 3rd Edition, pp. 10-13 and National Committee for Clinical Laboratory Standards (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.

6. SPILL: Clean and disinfect all spills of specimens using 0.5% solution hypochlorite. Base-containing spills should be neutralized, wiped dry, and then the spill areas should be wiped with a 0.5% sodium hypochlorite solution.

7. The wiped area should be covered with absorbent material, saturated with a 0.5% sodium hypochlorite solution and allowed to stand for at least 10 minutes. A glass or plastic cover or tray can be used to reduce exposure to fumes.

8. Treat all wiping materials as hazardous waste.

SAFETY AND HEALTH RISK INFORMATION

Ethidium bromide is a very strong mutagen, and may possibly be a carcinogen.

- Gels containing ethidium bromide should be incinerated with biohazard waste
- Aqueous solutions containing <10μg/ml ethidium bromide can be released to the drain.
- Aqueous solutions containing >10μg/ml ethidium bromide should be deactivated before disposal or placed in a container for incineration. Please consult the following website:

  (http://web.princeton.edu/sites/ehs/chemwaste/etbr.html)

(6) Specimen Collection & Preparation

Specimens used for the HPV DNA Nest PCR Test™ are alcohol-fixed liquid-based cell suspensions in the cervicovaginal lavage samples collected in the Cytyc or Surepath vials for cytologic examinations. Specimens stored between 2°C and 30°C up to three months are suitable for this test. About 1 ml of cell suspension containing 10^5 to 10^6 cells is recommended for each test. Measures must be taken for proper labeling and to prevent leakage of contents during specimen transportation. Please refer to *Test Procedure* section for sample processing steps.

(7) Test Procedure

All clinical specimens may contain infectious agents and should be handled accordingly.

Reagent preparation, pre-PCR specimen preparation, post-PCR analysis and preparation for the nested PCR must be performed under a PCR station hood in separate rooms with proper air-flow direction to avoid cross contamination. PCR, especially when nested PCR is used, is an extremely sensitive method to amplify and detect a very minute quantity of target DNA. Cross contamination by a single copy of target DNA molecule can produce false positive results in a PCR laboratory. Diagnostic laboratories using PCR technology must follow strict protocols to avoid cross contamination and aerosol contamination. All molecular diagnostic laboratory personnel must be aware of the serious consequence of amplicon contamination to the entire laboratory operation.

Extraction of DNA from alcohol-fixed cells in liquid-based materials for PCR

1) Centrifuge about 1 ml cell suspension (10^5-10^6 cells) in a 1.5 ml Eppendorf tube at 13,000 rpm for 5 min.

2) Discard supernatant with a micropipette without disturbing the pellet.

3) Pipette 1 ml water into tube to suspend pellet, vortex and centrifuge at 13,000 rpm for 5 min.

4) Discard supernatant with a micropipette.

5) Add 1 ml cell-washing buffer, pH 8.1 and vortex to suspend cells.

4) Centrifuge cell suspension at 13,000 rpm for 5 minutes.

5) Remove all supernatant with a micropipette.

6) Add 100 µL proteinase K solution, 0.1 mg/ml for cell digestion.

7) Vortex and incubate samples at 45-55°C overnight.

8) Heat the tubes in 95°C block for 10 min.

9) Centrifuge the sample for 5 min at 13,000 rpm.

10) Harvest supernatant for PCR or store it at -20°C.

Primary MY09/MY11 PCR (in PCR Room 1)

The primary MY09/MY11 PCR and the ß-globin PCR are set up together to initiate the HPV Nest PCR test

1) Prepare one PCR tube for each specimen extract for MY primer amplification, plus one negative control tube for every 20 specimens and one tube for positive HPV-16 control for the entire batch.

The total number of PCR tubes needed for X specimens is X + (X/20 or 1) negatives + 1 positive =Y. Centrifuge briefly to collect all fluid to bottom of tubes before opening the caps of the tubes. Inspect the fluid level in each tube before use. Every tube should have the same amount of 20 µL fluid.

2) Prepare master MY primer mix (volume in µL) in a tube by adding the following:

Water	2 x (Y+ 1) or 2 x (Y + 0.1 Y) if Y > 10
10 µMolar MY09	Y+1 or Y + 0.1Y
10 µMolar MY11	Y+1 or Y + 0.1Y (or use the chart below)

MY Primer Mix Requirement: Reagent Preparation
(all volumes expressed in µL rounded up to whole number)

Number of Tests	Y+1 (or 0.1Y)	MY09	MY11	Water	Total Volume
1	4	4	4	8	16
2	5	5	5	10	20
3	6	6	6	12	24
4	7	7	7	14	28
5	8	8	8	16	32
6	9	9	9	18	36
10	13	13	13	26	52
11	15	15	15	30	60
20	25	25	25	50	100
30	36	36	36	72	144
40	48	48	48	96	192
50	59	59	59	118	236
60	70	70	70	140	280
70	83	83	83	166	332
80	94	94	94	188	376
90	105	105	105	210	420
100	117	117	117	234	468

3. Vortex master MY primer mix and centrifuge briefly to collect all fluid to bottom of tube.

4. Transfer 4 µL of master MY primer mix into each PCR tube containing 20 µL ready-to-use polymerase mix.

5. Add 1µL of sample extract into each PCR tube to reach a total reaction volume of 25µL.

6. Add 1µL water into a negative control tube for every 20 samples and 1 µL HPV-16 (100,000 copies) to the positive control tube and mix well.

7. Make sure all caps are tightly closed. Then bring all tubes to the thermocycler for 30-cycles LoTemp™ program set for an initial heating at 85° C for 2 min, followed by 30

cycles at 85° C for 30 sec, 40° C for 30 sec, and 65° C for 1 min. The final extension was 65° C for 10 min.

ß-Globin Gene Control PCR (in PCR Room 1)

1) Prepare one PCR tube for each specimen extract for ß-globin primer amplification, plus one negative control tube for every 20 specimens and one tube for positive control (a known specimen with adequate cellularity) for the entire batch.

The total number of PCR tubes needed for X specimens is X + (X/20 or 1) negatives + 1 positive =Y. Centrifuge briefly to collect all fluid to bottom of tubes before opening the caps of the tubes. Inspect the fluid level in each tube before use. Every tube should have the same amount of 20 µL fluid.

2) Prepare master ß-globin primer mix in a tube by adding the following:

Water	2 x (Y+ 1) or 2 x (Y + 0.1 Y) if Y > 10
80 µMolar ß-globin gene primer F	Y+1 or Y + 0.1Y
80 µMolar ß-globin gene primer R	Y+1 or Y + 0.1Y

ß-Globin Primer Mix Requirement: Reagent Preparation
(all volumes expressed in µL rounded up to whole number)

Number of Tests	Y+1 (or 0.1Y)	ß-Globin F	ß-Globin R	Water	Total Volume
1	4	4	4	8	16
2	5	5	5	10	20
3	6	6	6	12	24
4	7	7	7	14	28
5	8	8	8	16	32
6	9	9	9	18	36
10	13	13	13	26	52
11	15	15	15	30	60
20	25	25	25	50	100
30	36	36	36	72	144
40	48	48	48	96	192
50	59	59	59	118	236
60	70	70	70	140	280
70	83	83	83	166	332
80	94	94	94	188	376
90	105	105	105	210	420
100	117	117	117	234	468

3. Vortex master ß-globin primer mix and centrifuge briefly to collect all fluid to bottom of tube.

4. Transfer 4 µL of master ß-globin primer mix into each PCR tube containing 20 µL ready-to-use polymerase mix.

5. Add 1µL of sample extract into each PCR tube to reach a total reaction volume of 25µL.

6. Add 1µL water into a negative control tube for every 20 samples.

7. Make sure all caps are tightly closed. Then bring all tubes to the thermocycler for 30-cycles LoTemp™ program, as described for MY PCR.

Nested GP5+/GP6+ PCR (in PCR Room II)

The number of PCR tubes needed for GP nested PCR amplification is identical to that for MY PCR (=Y).

1) Prepare master GP primer mix in a tube by adding the following:

Water	3x (Y+0.1Y) µL	
10 µMolar GP5+	Y+0.1Y µL	
10 µMolar GP6+	Y+0.1Y µL	(or use the chart below)

GP5+/GP6+ Primer Mix Requirement: Reagent Preparation
(all volumes expressed in µL rounded up to whole number)

Number of Tests	Y+1 (or 0.1Y)	GP5+	GP6+	Water	Total Volume
1	4	4	4	12	20
2	5	5	5	15	25
3	6	6	6	18	30
4	7	7	7	21	35
5	8	8	8	24	40
6	9	9	9	27	45
10	13	13	13	39	65
11	15	15	15	45	75
20	25	25	25	75	125
30	36	36	36	108	180
40	48	48	48	144	240
50	59	59	59	177	295
60	70	70	70	210	350
70	83	83	83	249	415
80	94	94	94	282	470
90	105	105	105	315	525
100	117	117	117	351	585

3. Vortex master GP primer mix and centrifuge briefly to collect all fluid to bottom of tube.

4. Transfer 5 µL of master GP primer mix into each PCR tube containing 20 µL ready-to-use polymerase mix to reach a total reaction volume of 25 µL.

6. Transfer a trace of the MY PCR products by dipping a 1.5 mm diameter clean glass rod into the contents of each MY PCR tube and "inoculating" the MY PCR products into the corresponding nested GP PCR tube prepared above.

7. Make sure all caps are tightly closed. Then bring all tubes to the thermocycler for 30-cycles LoTemp™ program, as described for MY PCR.

Agarose Gel electrophoresis

This is conducted in the Analysis Room which is considered the "most DNA-contaminated" room. One-Way-Out regulations should be instituted to prevent any items of this room from re-entering the other spaces of the laboratory.

Agarose gel preparation

1. Add 2 grams of agarose gel powder and 100 mL of water into a glass bottle.
2. Add 4 µL of ethidium bromide solution.
3. Heat the mixture in a microwave oven to a boil and mix well.
4. Cool the melted agarose gel to about 60°C.
5. Pour the melted agarose gel into the UV-transparent gel tray with a fixed height comb in place.
6. Remove comb carefully after agarose gel solidifies.
7. Transfer the gel tray to the base electrophoresis base.
8. Dilute 4 mL of 50x electrophoresis buffer into a glass bottle with 196 mL of water.
9. Fill the base with buffer to the surface level of the gel.

Specimen loading

1. Transfer 2 µL of Sample Loading Dye Buffer into each of a row of empty small plastic tubes.
2. Pipette 5 µL of the contents from each PCR tube after completion of the thermocycling program and mix it with 2 µL Sample Loading Dye Buffer.
3. Load the 5 µL mixture into one of the wells formed in the agarose gel.
4. Load about 7-10 µL of the molecular ruler into a well for each gel run.

Electrophoresis

1. Connect the electrodes of the cassette to the power supply.
2. Turn on the power and set the voltage to 100 v.
3. Observe the migration of the blue front away from the wells.
4. The electrophoresis process will complete in 30-45 minutes.
5. Remove the gel tray with care and place it under the UV light for observation and for photographic documentation of results.

(8) Results

After electrophoresis, first inspect the lanes for negative controls of the MY09/MY11, GP5+/GP6+, and ß-globin primer PCR. They should show no visible bands under the UV light to confirm that there is no evidence of cross contamination during the test procedure. The MY PCR positive control lane should show a 450 bp PCR product, and the positive GP nested PCR control lane should show a 150 bp band. The positive ß-globin primer lane should show evidence of human genomic DNA amplification in which a 110 bp specific PCR product may or may not be clearly visible.

A sample that does not show a 450 bp band on the MY09/MY11 PCR gel or a 150 bp band on the GP5+/GP6+ PCR gel, but shows evidence of positive β-globin gene amplification is interpreted as **negative** for HPV DNA.

A sample that does not show any PCR products in all three electrophoresis lanes is considered unsatisfactory for evaluation due to low DNA extraction or presence of a PCR inhibitor, and is reported as **inadequate for evaluation**.

A sample that shows a 150 bp nested PCR band on electrophoresis gel is considered to be **probably positive for HPV DNA**, pending confirmation by genotyping with automated direct DNA sequencing.

In certain cases, mutation of the HPV L1 gene at the binding sites or near the binding sites of the nested PCR primers has taken place that the classic GP primer nested PCR can no longer generate a visible 150 bp product band on gel electrophoresis even when there is a 450 bp PCR product clearly visible on the MY09/MY11 primer PCR gel. Under these circumstances, a PCR using the combination of a GP6+/MY11 primer pair, or the combination of GP5+/MY09 primer pair, may generate a semi-nested PCR product suitable for genotyping with DNA sequencing.

(9) Special Comments on

-HPV Genotyping with Automated Cycle DNA Sequencing-

Genotyping with automated cycle DNA sequencing, the generally accepted "gold standard" method for viral typing, is the chosen confirmatory procedure for each HPV nested PCR product and to provide an accurate genotype of the HPV detected for

clinical follow up. However, the automated dye-labeled terminator cycle sequencing technology is owned and strictly controlled by Applied Biosystems, Inc. (ABI, Foster City, CA), the manufacturer of the DNA sequencers and the chemical reagents needed for performing DNA sequencing. The laboratories that perform the HPV Nest PCR test must contact ABI directly to set up the DNA sequencing facilities and follow the users' instructions provided by the manufacturer to generate useful DNA sequencing data for analysis.

Alternatively, the laboratories performing the HPV Nest PCR test may make necessary arrangements with a specialty laboratory that offers DNA sequencing services to molecular diagnostic laboratories.

In general, 1 μL of the nested PCR products, if positive, can be used as template directly to perform the BigDye® enzymatic cycle termination reaction for DNA sequencing.

The sequencing primer is 1 μL of 5 μmolar GP6+ (a 1:1 water dilution of the 10 μmolar GP6+ PCR primer).

The BigDye® Terminator v 1.1/Sequencing Standard Kit is recommended.

The automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis is used according to the protocol supplied by the manufacturer (Applied Biosystems).

Sequence alignments are performed against various standard HPV genotype sequences stored in the GenBank database by BLAST (www.ncbi.nlm.nih.gov/BLAST) algorithm to arrive at specific genotyping. Usually, a 34 bp DNA sequence downstream to the GP5+ binding site is sufficient to identify a genotype of HPV and validate the result of the PCR product [42]. However, HPV-16 is known to have numerous sequence variants [76], some sharing an identical 34 bp sequence in this region with some HPV-33 and HPV-31 strains. Under these circumstances, a 50 bp sequence BLAST algorithm may be needed to establish the final genotyping.

The principle of HPV genotyping with DNA sequencing is briefly summarized as follows. However, the users of DNA sequencing for genotyping must consult the sequencer manufacturer and other scientific and technical sources for proper application of this technology.

HPV-16 genotyping with DNA Sequencing
(for reference only)

A portion of the 7904 bp HPV-16 genomic DNA sequence located in the L1 (5526-7154) region, including a hypervariable segment from base position 6647 to 6681 in bold

Italics downstream to the GP5+ primer binding site, 5' tttgtta ctgttgttga tactac 3' (beginning 23 bp within [ ]), is illustrated below.

MY09/MY11 PCR amplification with GP5+/GP6+ nested PCR generates a 150 bp nested PCR product in this region among all known clinically relevant HPV genotypes. Alignments of a DNA sequence of 28 to 50, usually 34 bp (bold-faced, underlined), in this region against the known sequences stored in the GenBank data base is sufficient to determine a genotype via on-line BLAST alignment algorithm.

```
6481 ctactgcaaa tttagccagt tcaaattatt ttcctacacc tagtggttct atggttacct
6541 ctgatgccca aatattcaat aaaccttatt ggttacaacg agcacagggc cacaataatg
6601 gcatttgttg gggtaaccaa cta[tttgtta ctgttgttga tactacacgc agtacaaata
6661 tgtcattatg tgctgccata tctacttcag aaactacata taaaaatact aa]ctttaagg
6721 agtacctacg acatggggag gaatatgatt tacagtttat ttttcaactg tgcaaaataa
6781 ccttaactgc agacgttatg acatacatac attctatgaa ttccactatt ttggaggact
6841 ggaattttgg tctacaacct cccccaggag gcacactaga agatacttat aggtttgtaa
6901 cccaggcaat tgcttgtcaa aaacatacac ctccagcacc taaagaagat gatcccctta
6961 aaaaatacac tttttgggaa gtaaatttaa aggaaaagtt ttctgcagac ctagatcagt
7021 ttcctttagg acgcaaattt ttactacaag caggattgaa ggccaaacca aaatttacat
7081 taggaaaacg aaaagctaca cccaccacct catctacctc tacaactgct aaacgcaaaa
7141 aacgtaagct gtaa
```

GP5+ primer site is represented by sequence 5'-tttgtta ctgttgttga tactac- 3'

The 34 bp HPV Type-16 hypervariable region [***acgc agtacaaata 6661 tgtcattatg tgctgccata t***] is located downstream to the GP5+ primer site.

On-line BLAST analysis with GP6+ primer sequencing of the last segment (underlined) of the nested PCR product determines the HPV genotyping. In practice, since GP6+ is used as a general sequencing primer for the GP5+/GP6+ nested PCR products, a 34 bp sequence preceding the 3' end of GP5+ primer sequence generated by the sequencer software is excised (see the following **underlined** sequence example where the sequence GTAGTA represents the 3' end of the GP5+ primer) for sequence alignment analysis.

5' ..[TT AGTATTTTTA TATGTAGTTT CTGAAGTAGA TATGGCAGCA CATAATGACA TATTTGTACT GCGTGTAGTA TCAACAAC..].. 3'

Depending on the sources of the clinical specimens, 4 -15% of the HPV infections are caused by more than one genotype. When this happens, DNA sequencing with the GP6+ general primer will generate overlapping peaks in the sequencing tracing, rendering accurate sequence alignment analysis impossible. For these mixed infection cases, a type-specific sequencing primer is needed to target each individual HPV type for DNA sequencing. Since there are more than 100 clinically relevant HPV genotypes, it is not practical to perform more than 100 DNA sequencings for each case of mixed

HPV infection. It is recommended to perform only our specific DNA sequencings to rule out or to confirm a mixed infection caused by at least one of the HPV vaccine-relevant HPV genotypes. This information may be useful for immunization counseling before and after vaccination with Gardasil™. The type-specific sequencing primers for the Gardasil™ vaccine-relevant HPV genotypes are listed as follows: [50]

HPV 16 type-specific sequencing primer 5'-GCTGCCATATCTACTTCAGA-3'
HPV 18 type-specific sequencing primer 5'-GCTTCTACACAGTCTCCTGT-3'
HPV 6 type-specific sequencing primer 5'-GTGCATCCGTAACTACATCTT-3'
HPV 11 type-specific sequencing primer 5'-GTGCATCTGTGTCTAAATCTG-3'

(10) Limitations

A negative HPV DNA Nest PCR Test does not guarantee that there is no HPV in the clinical specimen tested. Failure to detect HPV DNA may be caused by extremely low HPV viral load in the material, or due to existence of a PCR inhibitor in the specimen that cannot be eliminated by washings and dilutions. If the clinical specimen contains less than 1,000 copies of HPV genomic DNA per ml, the Nest PCR test may be negative.

The combination of MY09/MY11 and Gp5+/GP6+ nested PCR is highly sensitive for detection of HPV DNA. However, they may amplify certain HPV DNA genotypes more efficiently than others. It is possible that only one genotype in multiple HPV infections is preferentially amplified and detected.

If a positive GP5+/GP6+ PCR product detected on electrophoresis is validated by DNA sequencing for definitive genotyping, the HPV diagnosis is unequivocal provided no cross contamination has occurred during the test procedure. In case of failure to obtain a readable DNA sequencing for genotyping by BLAST analysis, the result for a positive HPV in the specimen cannot be confirmed. If the failure of genotyping is caused by the presence of overlapping peaks in the DNA sequencing tracing, the result can be reported as "mixed HPV infection". If no sequencing termination is obtained for the GP5+/GP6+ PCR product, there is probably an inhibitor in the specimen that prevents the BigDye® primer extension/termination reaction. Then the HPV DNA Nest PCR Test should be considered "inconclusive for HPV" due to sequencing failure.

There is no simple linear relationship between positive high-risk HPV test and Pap cytology findings. The Nest PCR test results must be interpreted and used by a physician in the context of clinical findings, cytologic findings, biopsy findings and the clinical history of the patient.

(11) Expected Values

The positive rate of HPV DNA Nest PCR Detection test™ is between 11% and 35% in cervicovaginal lavage specimens, subjected to geographic and demographic variations. No quantitative determination of viral load is implied. Most of the positive nested PCR products can be genotyped with direct automated DNA sequencing, using the GP6+ general PCR primer as the sequencing primer. Multiple HPV infections may be encountered in 4-15% of the HPV positive cases. Repeating the test may be indicated for the PCR-positive cases in which DNA sequencing fails to produce a definitive genotyping result through BLAST analysis or multiple HPV infections are detected. The natural history of multiple HPV infections is not known. High-risk persistent HPV infection is usually associated with one single HPV genotype infection.

3. Performance Standards

The performance characteristics of the device were demonstrated as follows.

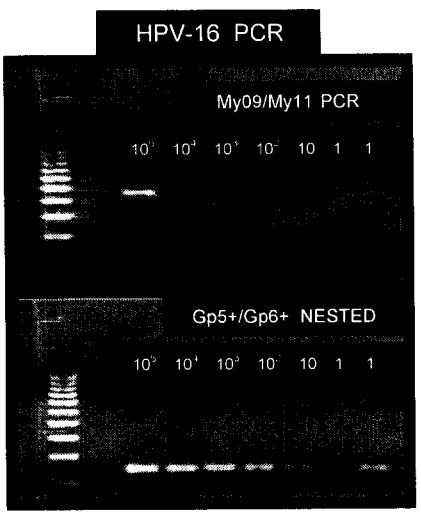
(1) Sensitivity in detection of purified HPV DNA

The standard full-length genomic DNA of HPV type-16, type-18 and type 6B certified by American Type Culture Collection (ATCC) was used to perform the sensitivity study for HPV DNA Nest PCR Detection test™, using the test procedure described above. The stock DNA standard was diluted in TE buffer to various concentrations to contain 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 copies of HPV DNA per µL. One µL was the volume of template used for each primary MY09/MY11 primer PCR amplification. The theoretical number of copies of HPV DNA in the template was calculated according to a generally accepted conversion formula (http://www.uri.edu/research/gsc/resources/cndna.html). A trace of the primary MY PCR products was re-amplified by nested PCR, using the GP5+/GP6+ primer pair. All positive nested PCR products detected by agarose gel electrophoresis were confirmed by DNA sequencing to be the HPV types expected. The limit of detection of these three standard HPV types is found to be 1-10 copies in one µL used as the PCR template (Fig. 1).

Figure 1

Limits of detection for purified HPV genomic DNA by Nest PCR Test

10^5, 10^4, 10^3, 10^2, 10, 1 = copies of HPV DNA per reaction
Sensitivity of detection: MY09/MY11 PCR =10^4-10^5copies of HPV DNA
GP5+/GP6+ nested PCR=1-10 copies of HPV DNA



(2) Sensitivity in detection of HPV DNA in clinical samples, compared to FDA-approved device

To perform the sensitivity study of the subject device on actual clinical specimens, compared to the FDA-approved Digene HC2 kit, 515 alcohol-preserved liquid-based cervicovaginal lavage samples (Cytyc or Surepath) submitted by physicians in the New Haven area as part of routine gynecologic examinations were used. After the material was taken from each sample for routine cytology and HC2 High-Risk HPV DNA Test, about 1 mL of the cell suspension from the residual lavage was placed in a 1.5 mL Eppendorf tube, blind-coded with a case number and transferred to the petitioner's laboratory for HPV Nest PCR detection followed by genotyping by direct DNA sequencing according to the procedure outlined above. Age distribution of the patients and the cervical pathologic conditions were not the subjects of this study. The HC2 High-Risk HPV DNA Tests were performed independently by Quest Diagnostics Laboratory, Wallingford, CT or by Pathology & Laboratory Services, LLC, Woodbridge, CT.

DNA extraction from the alcohol-fixed cells in the lavage was accomplished according to a National Cancer Institute (NCI) protocol [12] with minor modification. Briefly, the cell suspension was first centrifuged in an Eppendorf microcentrifuge (model 5424) equipped with a rotor (model 5424R) for 5 min at 13,000 rpm. The cells in the pellet were washed in 1 mL reagent grade water and then in 1 mL buffer consisting of 50mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The washed cell pellet was re-suspended and digested at 45-55°C overnight in100 µL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) dissolved in the same washing buffer. After denaturing the proteins in the cell digestate in a metal block heated to 95°C for 10 min, the digestate was subjected to a final centrifugation at 13,000 rpm for 5 min. The supernatant was carefully pipetted out and placed in a clean microcentrifuge tube to be used for PCR without further purification or stored at -20°C.

The general methodology of primary PCR amplification of a 450 bp segment of the HPV L1 gene with a pair of consensus MY09/MY11 primers as outlined above was followed, using 1 µL of cell digestate supernatant as the PCR template. After completion of the primary PCR thermocycling, a "trace" of the MY09/MY11 PCR products was transferred by a glass rod with clean wettable surface of about 1.5 mm in diameter to a second PCR tube containing 25 µL of complete nested PCR reaction mixture consisting of 20 µL LoTemp™ HiFi® DNA polymerase ready-to-use mix, 1 µL of 10 µmolar GP5+ primer, 1 µL of 10 µmolar GP6+ primer and 3 µL of water, using the same thermocycling program as described above for a 30-cycle nested PCR amplification.

After completion of the primary and the nested PCR, a 5 µL aliquot of the PCR products was pipetted out from each tube and mixed with 2 µL sample loading dye buffer for electrophoresis in a 2% agarose gel containing ethidium bromide. The gel was

examined under UV light. A 450 bp PCR product band in the MY09/MY11 lane and/or a 150 bp band in the nested PCR lane on the agarose gel provided presumptive evidence of HPV DNA detected in the sample, pending validation by genotyping with direct automated DNA sequencing.

For DNA sequencing, 1 µL of the nested PCR products, if positive, was pipetted out from the nested PCR tube for direct DNA sequencing, using 1 µL of 5 µmolar GP6+ primer as the sequencing primer, the BigDye® Terminator v 1.1/Sequencing Standard Kit for enzymatic termination reaction in a total reaction volume of 20 µL and the automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis, according to the protocol supplied by the manufacturer (Applied Biosystems). Alignment comparison based on BLAST (www.ncbi.nlm.nih.gov/BLAST) analysis of a 34 bp DNA sequence in the hypervariable region of the L1 gene downstream to the GP5+ binding site [42] against known HPV genotype sequences stored in the GenBank database determined the genotype of the HPV detected.

Another 1 µL of each DNA extract was placed in a separate PCR tube with a ß-globin primer pair [94] for human genomic DNA amplification as a control of specimen adequacy. The primers for ß-globin gene amplification were 1 µL of 80 µmolar 5'-ACACAACTGTGTTCACTAGC and 1 µL of 80 µmolar 5'-CAACTTCATCCACGTTCACC in a 20 µL of LoTemp™ HiFi® DNA polymerase ready-to-use mix with 2 µL of water added. The thermocycling program described above was used.

Three PCR tubes per sample were used routinely for the ß-globin gene, the MY09/MY11 primer and the GP5+/GP6+ nested amplification, respectively.

Samples that did not show an MY09/MY11 or a GP5+/GP6+ PCR band, but showed evidence of positive ß-globin gene amplification were interpreted as HPV-negative. Specimens that did not show any PCR products in all three lanes were considered unsatisfactory for evaluation due to low DNA extraction or presence of a PCR inhibitor. There were two unsatisfactory cases among a total of 515 processed. Of the 513 cases accepted as satisfactory for analysis, there were 107 positive nested PCR products, all proven to be those of HPV DNA by direct automated sequencing, using GP6+ as the sequencing primer.

Samples infected with more than one genotype of HPV were indicated by the appearance of numerous ambiguous or overlapping peaks in the DNA sequencing tracings. For each of these mixed infections, the nested PCR products were subjected to additional four individual sequencing reactions each using a single type-specific primer to rule out infection by the Gardasil™ vaccine-relevant HPV types 16, 18, 6 or 11. The type-specific primers used according to Gharizadeh et al. [50] are listed as follows:

HPV 16 type-specific sequencing primer 5'-GCTGCCATATCTACTTCAGA-3'
HPV 18 type-specific sequencing primer 5'-GCTTCTACACAGTCTCCTGT-3'
HPV 6 type-specific sequencing primer 5'-GTGCATCCGTAACTACATCTT-3'
HPV 11 type-specific sequencing primer 5'-GTGCATCTGTGTCTAAATCTG-3'

The validating DNA sequencing data including the DNA sequence tracings and the on-line BLAST genotyping analysis on each positive nested PCR product for the 107 HPV-positive cases are presented in Attachment 3 (Volumes A and B). All 107 positive nested PCR products which provide presumptive evidence for the presence of HPV DNA in the clinical specimens have been proven to be HPV DNA by DNA sequencing, including 102 single HPV genotype infections and 5 mixed HPV infections.

HC2 test returned a positive result for high-risk HPV DNA in 75 cases, a positive rate of 75/513, or 14.6%, including 8 cases in which Nest PCR test failed to detect HPV DNA, compared to a detection rate of 107/513, or 20.9% when HPV Nest PCR test was used as the method of detection (Table 1). This discrepancy in the rates of detection by these two methods is not unexpected since the sensitivity of detection for Nest PCR test is 1-10 copies of HPV genomic DNA per assay and that for HC2 test is about 100,000 copies per assay.

When only the high-risk HPV types were used for the comparison, Nest PCR detected 74 cases which were positive for high-risk HPV genotypes validated by DNA sequencing while HC2 test detected 50 of these 74 PCR-positive cases and classified them as positive for high-risk HPV. All 9 cases of HPV-56, all 6 cases of HPV-18, all 4 cases of HPV-59, both cases of HPV-39, a single case of HPV-33 and a single case of HPV-68 detected in this series were identified by HC2 test as positive for high-risk HPV DNA. However, 18 of 29 HPV-16 cases, 2 of 7 HPV-31 cases, 1 of 6 HPV-58 cases, 1 of 3 HPV-45 cases, 1 of 2 HPV-35 cases, and 1 of 2 HPV-52 cases were not detected by HC2 test (Table 2).

In summary, the sensitivity of HC2 test in detecting the predetermined high-risk HPV genotypes is as follows:

HPV-18	100%
HPC-33	100%
HPV-39	100%
HPV-56	100%
HPV-59	100%
HPV-68	100%
HPV-58	83.3%
HPV-31	71.4%
HPV-45	66.7%
HPV-35	50 %
HPV-52	50 %
HPV-16	37.9%

In comparison, HPV Nest PCR detected all the 74 high-risk HPV isolates validated to be those of high-risk HPV genotypes by DNA sequencing. In conclusion, HPV Nest PCR has been shown to be a sensitive device in the detection of HPV DNA in clinical

specimens with a higher sensitivity than the present FDA-approved HC2 kit.

The unusually high difference in the rate of detection for HPV-16 between these two methods may be due to the fact that there are numerous sequence variants of HPV-16 and some of the HPV-16 DNA variants may not be targeted by the RNA cocktail probe formulated for the Digene HC2 kit. The well characterized MY09/MY11 consensus primers are designed to amplify a highly conserved L1 region of the HPV genome [76], including a hypervariable sequence downstream to the GP5+ binding site.

specimens as the predicate device, with a higher sensitivity than the present FDA-approved HC2 kit.

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TABLE 1

HPV Nest PCR/DNA Sequencing and Digene HC2 Test Results on 107 HPV positive Clinical Specimens in Order of Genotype Frequency (Genotypes underlined are targeted by HC2 High-Risk Probe)

PCR/DNA Sequencing			Test Results by Digene HC2	
Type	Positive Cases	Prevalence (%)	High-risk+	Negative
16	29	(27.2)	11	18
56	9	(8.5)	9	0
31	7	(6.5)	5	2
6	6	(5.6)	2	4
18	6	(5.6)	6	0
54	6	(5.6)	3	3
58	6	(5.6)	5	1
66	6	(5.6)	6	0
59	4	(3.7)	4	0
45	3	(2.8)	2	1
83	3	(2.8)	2	1
32	2	(1.9)	0	2
35	2	(1.9)	1	1
39	2	(1.9)	2	0
40	2	(1.9)	0	2
52	2	(1.9)	1	1
33	1	(0.9)	1	0
53	1	(0.9)	1	0
62	1	(0.9)	1	0
68	1	(0.9)	1	0
70	1	(0.9)	1	0
72	1	(0.9)	0	1
73	1	(0.9)	0	1
M16	1	(0.9)	1	0
M 16, 18	1	(0.9)	1	0
M others	3	(2.9)	1	2
Total+	107	(100)	67	40

HC2 HPV detection rate = 67/107 = 62.6%

Total samples tested = 513
%HPV+ = 107/513 =20.9%

M16= multiple infections with HPV 16 identified by type-specific sequencing primer.
M 16, 18 =multiple infections with HPV 16 and HPV 18 identified by type-specific sequencing primers.
M others= multiple infections by HPV types which cannot be sequenced with type-specific primers for HPV 6, 11, 16 or 18.
Digene High-risk + includes those cases reported as positive for both High-risk and Low-risk HPV types
HPV genotypes targeted by Digene HC2 High-risk cocktail probe are underlined

TABLE 2

Comparison of High-risk HPV Detections by Nest PCR and by Digene HC2 In 74 positive Clinical Specimens confirmed by DNA Sequencing

PCR/DNA Sequencing		Test Results by Digene HC2	
Type	Positive Cases	High-risk+	Detected by HC2
18	6	6	100%
39	2	2	100%
56	9	9	100%
59	4	4	100%
58	6	5	83.3%
31	7	5	71.4%
45	3	2	66.7%
35	2	1	50%
52	2	1	50%
16	29	11	37.9%
33	1	1	
68	1	1	
M16	1	1	
M 16, 18	1	1	
Total+	74	50	
		HC2 HPV detection rate = 50/74 = 67.6%	

M16= mixed infection with HPV 16 identified by type-specific sequencing primer.
M 16, 18 =mixed infection with HPV 16 and HPV 18 identified by type-specific sequencing primers.
Digene High-risk + includes those cases reported as positive for both High-risk and Low-risk HPV types

(3) Specificity in detection of HPV DNA in clinical samples

In a study on 107 positive Nested PCR products isolated from 513 clinical specimens, all final nested PCR products have been validated by DNA sequencing to be those of HPV DNA. One hundred and two (102) of the 107 cases were confirmed to be single HPV genotype infections. Five were mixed or multiple HPV infections. Two of the multiple HPV infections contained at least one HPV-16 type, which was confirmed by DNA sequencing with a type-specific sequencing primer. Since all positive results have been validated by DNA sequencing to represent HPV DNA, either in the form of single

HPV infections or multiple HPV infections, the specificity of the final positive results of the Nest PCR test is 100%.

The sequencing data are summarized in Attachment 3, Volumes A and B.

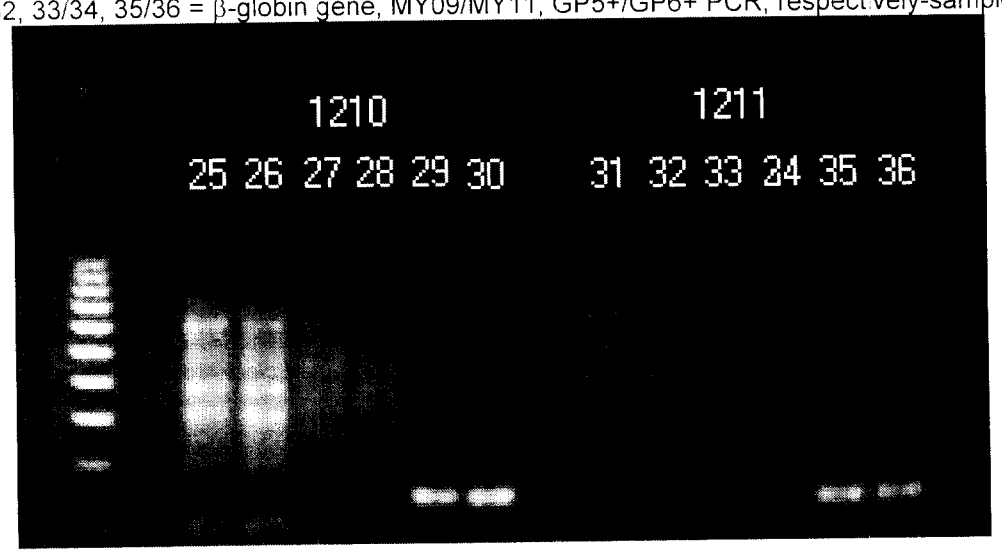
(4) Reproducibility of HPV Nest PCR in clinical specimen testing

The HPV Nest PCR Test in the detection of HPV DNA in clinical specimens is highly reproducible. It has been confirmed by running two parallel sets of PCRs with split pair of single sample digestate as the template, including the ß-globin gene, the MY primer and the GP nested primer amplifications for each set. Pairs of identical results on electrophoresis gel were obtained in all three amplifications (Fig. 2) for the 30 split samples. The nested PCR products obtained on the duplicate sets were confirmed by DNA sequencing to be of the same HPV genotype in the paired sets. Therefore, it is concluded that the reproducibility of HPV Nest PCR in clinical specimen testing is 100%.

Figure 2

Reproducibility of HPV Nest PCR in Clinical Samples

Agarose gel showing PCR products of targeted DNAs extracted from two clinical samples in duplicate. The targeted β-globin DNA amplicon is 110 bp long, as seen clearly in lanes 25 and 26 (#1210), but is hardly visible in lanes 31 and 32 (#1211). Co-amplification of other human genomic DNA fragments and a positive nested PCR amplicon assure specimen adequacy in both samples.
Molecular ruler = 100-1000 bp (far left).
Lanes 25/26, 27/28, 29/30 = β-globin gene, MY09/MY11, GP5+/GP6+ PCR, respectively-sample #1210
Lanes 31/32, 33/34, 35/36 = β-globin gene, MY09/MY11, GP5+/GP6+ PCR, respectively-sample #1211

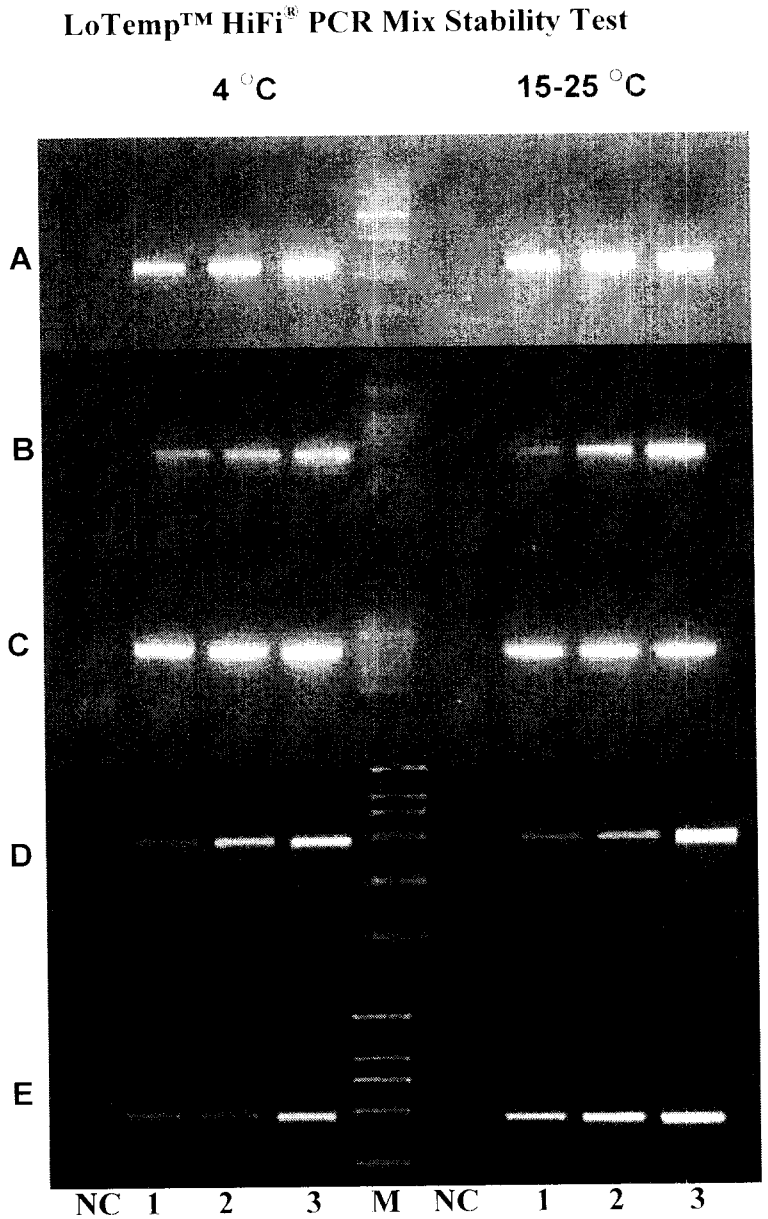


(5) Stability of HPV Nest PCR Reagent

Since successful clinical PCR assays depend on stability of the reagents, especially the DNA polymerases and the dNTPs in the ready-to-use mix, a time course study was performed to confirm that the shelf life of the LoTemp™ HiFi® PCR mixture is adequate to maintain its effectiveness in amplification of HPV DNA during routine clinical laboratory operation.

In this study, a number of 20μL aliquots of LoTemp™ HiFi® PCR ready-to-use mixture in 0.2 mL PCR tubes were stored in a 4°C refrigerator and in a 25°C incubator, respectively. At day 0, day 16, day 31, day 52 and day 65, the reagents stored at 25°C and at the end of each month the reagents stored at 4°C refrigerator were tested for MY09/MY11 PCR amplification with various amounts of HPV-16 plasmid DNA as the template, using the LoTemp™ PCR protocol described above. The results showed that there was no significant decrease in PCR products when 10 – 1,000 pg of plasmid DNA were used for amplification, during the entire course of study. The ready-to-use PCR DNA polymerases and the dNTPs in the reagents were found to remain active for at least 65 days when stored at room temperature not to exceed 25°C (Fig. 3). They were found to remain stable for at least 10 months when stored at 4°C. Therefore, the device is considered to be suitable for application in qualified clinical laboratories at the point of care.

Figure 3



Storage day 0 (A), day 16 (B), day 31 (C), day 52 (D), and day 65 (E).
Lane 1-3: HPV16 plasmid DNA 10, 100 and 1,000 pg amplified with MY09/MY11 primers.
NC: negative control. M: molecular marker.

VIII. Representative data and information known by the petitioner that are unfavorable to the petitioner's position. [21 CFR § 860.123(a)(7)]

The petitioner is unaware of any data and information that is unfavorable to reclassification of HPV DNA PCR detection devices as Class II devices.

IX. If the petition is based upon new information under section 513(e), 514(b), or 515(b) of the act, a summary of the new information. [21 CFR § 860.123(a)(8)]

Although this petition is not based upon new information under section 513(e), 514(b), or 515(b) of the act, a summary of the new information relevant to the review of this petition which has become publicly available since 1988 when the approval order for the VIRAPAP Human Papillomavirus DNA detection Kit dated December 23, 1988 was issued is summarized as follows.

1) HPV test is a laboratory test to detect the presence in women of human papillomavirus (HPV), one of the most common sexually transmitted infections [1]. This is at variance to the 1988 understanding of HPV infection which considered HPV test a device for use in identifying and typing HPV infection to stratifying women at risk for cervical cancer [2].

2) The HPV DNA test does not test for cancer, but for the HPV viruses that can cause cell changes in the cervix. If left untreated, these changes can eventually lead to cancer in some women [1].

3) Most women who become infected with HPV are able to eradicate the virus and suffer no apparent long-term consequences to their health. But a few women develop a persistent infection that can eventually lead to pre-cancerous changes in the cervix [1].

4) Most infections are short-lived and not associated with cervical cancer [1].

5) Persistent HPV infection, not the HPV virus itself, is the pivotal promoter in causing cervical precancerous lesions and cancer [8-11]. Most of HPV infections, even caused by "high-risk" genotypes, are transient with normal Pap cytology in sexually active young women [4-7]. In 93% of initially infected women, the same viral type is not detected upon re-examination four menstrual cycles later [20]. The median duration of positivity detectable by PCR for a specific HPV type in these young women is 168 days [21].

6) For the development and maintenance of a high-grade squamous intraepithelial lesion (SIL), the risk is greatest in women positive for the same genotype of HPV on repeated testing [8-11]. Viral load is not a useful parameter to predict high-grade SIL [23]. High-grade SIL is often associated with a viral DNA load lower than that observed in less severely affected cells [24].

7) The present FDA-approved HPV HC2 test is associated with a significant number of false-negative and false-positive results when compared with other more stringent PCR-based HPV

genotyping assays [26-29]. It is reported to generate 25% false-negative results in cases with biopsy-proven high-grade SIL even when all these biopsies have been proven to contain high-risk HPV DNA by PCR [30].

8) Injection of HPV vaccines into women who have concurrent vaccine-relevant HPV type infections evidenced by sero-positive and PCR-positive tests may increase the risk, by about 44.6%, of developing high-grade precancerous lesions in the cervix [14]. Therefore, it would be prudent to perform a genotype-specific HPV assay if prior HPV infection is suspected.

9) DNA sequencing is the "gold standard" method for accurate genotyping of HPVs detected in clinical specimens [50, 72].

10) Nested PCR with consensus primers is a highly sensitive molecular biology tool in amplifying and detecting HPV DNA [27, 28, 49].

11) Compared to the potential carcinogenic bacterium, Helicobacter pylori, which may initiate chronic inflammation that may lead to stomach cancer with an annual 11,430 deaths [62], HPV is an infective agent of a lower risk, causing 3,700 cervical cancer deaths [66] in the U.S.A.

X. Copies of source documents from which new information used to support the petition has been obtained (attached as appendices to the petition) [21 CFR § 860.123(a)(9)]

Copies of source documents from which new information used to support the petition has been obtained are attached as appendix [21 CFR § 860.123(a)(9)].

XI. Financial certification required by part 54 [21 CFR § 860.123(a)(10)]

Financial certification and disclosure by clinical investigators, consistent with 21 CFR Part 54, are required for clinical studies submitted in support of reclassification petitions for medical devices. Because no clinical studies are included in this request for reclassification, financial certification and disclosure forms are not being submitted.

XII. Conclusions and summary

In conclusion, the petitioner is requesting that HPV DNA PCR detection devices be reclassified from Class III to Class II by FDA. There have been significant advances in the understanding of the natural course of HPV infection and its relationship to the development of squamous intraepithelial lesions, precancerous lesions and cancer in the uterine cervix since 1988 when the FDA assigned the Digene's Virapap HPV assay a class III status. Since the 1990's, medical science has confirmed that most of the HPV infections, even caused by the so-called "high-risk" HPVs, are self-limiting. Only persistent infection caused and maintained by certain genotypes of HPV, often with low intracellular viral load, is truly of "high risk" to the host in causing precancerous lesions of the cervix. There is a close similarity between the infection of

Helicobacter pylori (*H. pylori*) and that of HPV. Both agents can initiate chronic inflammation that may lead to cancer in the stomach and uterine cervix, respectively. The annual deaths due to gastric cancer which is related to the infection caused by *H. pylori* are about 3 times the number of deaths due to cervical cancer related to infections caused by HPV in the United States. Therefore, it is reasonable to conclude that *H. pylori* infection is associated with a higher level of risk than HPV infection. Logically, an *in vitro* diagnostic device for preventing the development of gastric cancer is of **more** substantial importance in preventing impairment of human health than an *in vitro* diagnostic device for preventing the development of cervical cancer if the provision under 21 CFR §860.3 (c)(3) is invoked as the criteria for risk-based classification of the subject device. Since the IVDs for the diagnosis of *H. pylori* infection are customarily classified as class II and class I devices by the FDA, the IVDs for the diagnosis of HPV infection should not be classified as higher than class II under 21 CFR §860.3 (c)(3).

The molecular technology of PCR amplification of HPV DNA followed by nucleic acid-based genotyping for following the patients with persistent HPV infection was developed and introduced into the practice of laboratory medicine after 1988. It is self-evident that the PCR-based detection systems represent a newer medical technology than the Digene's Virapap test approved in1988 by the FDA. The use of an old approval order of 1988 to block the introduction of a more sensitive and more specific, thus safer and more effective 2007 PCR-based technology for HPV DNA testing by imposing unnecessary PMA requirements to the sponsors is not consistent with the "least burdensome" principles in regulating the medical device industry set forth by the FDAMA of 1997. Therefore, reclassification of the HPV DNA PRC detection devices into Class II devices by the FDA is long overdue.

The need for an FDA-approved PCR-based HPV DNA detection device with provision to prepare positive clinical samples for genotyping is more urgent now after the Gardasil™ vaccine is made available to the populace. In a document submitted to the FDA by Merck & Co., Inc., it is recorded that injection of the HPV vaccine, Gardasil™, into women who are sero-positive and PCR-positive for the vaccine-relevant HPV genotypes increases the risk of developing high-grade intraepithelial lesions by 44.6%. As of to-date, there are no FDA-approved PCR-based methods for HPV genotyping on the market in spite of the fact the PCR technology has been available for about 20 years. This petitioner urges the FDA to play a leadership role to guide the device manufacturers to introduce their most sensitive and most specific PCR-based IVDs to assist the sexually active women who are still considering immunization against HPV infections without inadvertently receiving a vaccine that is not only ineffective, but may augment the risk of developing a precancerous lesion in the cervix. The first step that the FDA should take is to down-classify the HPV DNA PCR detection devices to Class II as requested by this petitioner.

Appendix [21 CFR § 860.123(a)(9)]

List of copies of source documents from which new information used to support the petition:

[1] FDA News P03-26, March 31, 2003.

[2] FDA OIVD/CDRH Letter to Ventana Medical Systems, Inc. March 18, 2004.

[3] Hojvat SA, Director, Division of Microbiology Devices. OIVD, FDA Letter dated January 9, 2007 re: K063649.

[4] Jacobs MV, Walboomers JM, Snijders PJ, Voorhorst FJ, Verheijen RH, Fransen-Daalmeijer N, Meijer CJ. Distribution of 37 mucosotropic HPV types in women with cytologically normal cervical smears: the age-related patterns for high-risk and low-risk types. Int J Cancer 2000; 87:221-7.

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[12] Copy of October 30, 2006 letter from Dr. S. H. Lee to Dr. SI Gutman, seeking guidance to submission of a 510(k) for HPV PCR reagent.

[13] Manuscript by SH Lee et al. titled "Human Papillomavirus Genotyping by DNA Sequencing-The Gold Standard HPV Test for Patient Care.

[14] VRBPAC Background Document: **Gardasil™ HPV Quadrivalent Vaccine.** May 18, 2006 VRBPAC Meeting www.fda.gov/ohrms/dockets/ac/06/briefing/2006-4222B3.pdf

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