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# Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections

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**Abstract**

*Background:* It is well established that human papillomavirus (HPV) infection is highly related to the development of precursor lesions of cervical cancer and uterine cancers. However, for a pre-cancerous lesion to develop, a persistent infection with a high-risk type HPV is necessary.

The Digene Hybrid Capture II (hcII) assay is the only FDA approved method used in conjunction with cytology for HPV screening of women older than 30. The hcII has moderate sensitivity (64.7%) and is dependent on the cellular content of samples, rendering occasionally false positive and false negative results.

*Objective:* This study aims to evaluate the performance of a new HPV diagnostic kit (High-Risk HPV detection kit, manufactured by Human Genetic Signatures (HGS), Sydney, Australia).

*Methods:* The method under evaluation was assessed by comparing the results obtained from testing 834 cervical specimens with the HGS method and the Digene hcII method, using genotyping as the reference standard.

*Results:* Results of the study showed that the specificity and positive predictive value of the HGS High-Risk HPV detection test are significantly greater than those of the Digene hcII test. Overall the HGS HPV assay provides a more accurate system for the detection of high-risk HPV strains, with simpler technical use compared with PCR-sequencing methods.



*Keywords:* Human papillomavirus (HPV); Cervical cancer; PCR; Diagnostic methods

## 1. Introduction

Human papillomaviruses (HPVs) are small double-stranded DNA viruses that infect the human epithelium and cause hyper-proliferation (Bosch et al., 1995; Clifford et al., 2003). Certain types of HPV (high-risk types) have been found to be closely associated with the development of greater than 90% of cervical cancers (Chacón et al., 2007; Cuschieri et al., 2004; Del Mistro et al., 2006). The use of combined tests to detect the presence of high-risk HPV DNA together with Pap testing has been shown to greatly improve the ability to detect pre-cancerous states (Wright et al., 2004).

There are a variety of diagnostic methods with differing sensitivity and specificity for the detection of high-risk HPV which have been developed to detect HPV in cervical scrapings and biopsy material (Albrecht et al., 2006; Gheit et al., 2006; Oh et al., 2007; Sotlar et al., 2004; van Doorn et al., 2006). The MY primers are the ideal choice for HPV amplification. They were first described in Manos et al. (1989),

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based on the L1 (Late protein) region of the HPV virus and was one of the first PCR primer sets capable of amplifying all HPV strains. More recently the GP primers sets have been designed using DNA alignments of a greater number of HPV subtypes (Coutlée et al., 2002; Kornegay et al., 2003; Qu et al., 1997; Snliders et al., 1990). The MY and GP primers are the most published primer sets in the literature for the detection of HPV in clinical samples and are considered the "reference standard" (Fontaine et al., 2007).

The hcII HPV DNA test (Digene Corporation, USA), is the only Food and Drug Administration (FDA) approved test for detecting 13 high-risk HPV (Clavel et al., 1998, 2000; Kulmala et al., 2004; Soderlund-Strand et al., 2005). However, the hcII occasionally may produce false negative and false positive results (Poljak et al., 1999).

The reference standard for HPV genotyping is sequencing of PCR amplicons. While this technique gives the most conclusive genotype information, it is also the most labour-intensive (Giovannelli et al., 2004; Gravitt et al., 2000; Nelson et al., 2000).

This study aims to evaluate a novel test (High-Risk HPV detection kit, manufactured by Human Genetic Signatures (HGS), Sydney, Australia) developed to improve the specificity of detection of high-risk HPV DNA, and to allow high throughput testing. This was assessed by comparing the ability of the hcII HPV DNA test versus the HGS High-Risk HPV detection test to detect high-risk HPV DNA in cervical specimens. We examined the utility of the HGS test in a high throughput diagnostic laboratory, and compared it with the existing FDA-licensed platform and then measure sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the HGS and Digene hcII methods using genotyping as reference standard (Leisenring et al., 2000; Moskowitz and Pepe, 2006).

## 2. Materials and methods

### 2.1. Specimens

A total of 834 cervical specimens were submitted for testing to SEALS, Prince of Wales Hospital Sydney, Australia during the period between August 2006 and February 2007. Cervical specimens were obtained randomly following histology and/or cytology, de-identified, and entered into the study sequentially until the target number of specimens was achieved.

### 2.2. Specimen collection

Cervical specimens were obtained using a broom-type collection device (Digene Corp, Gaithersburg, MD 20878, USA) and placed in Cytyc PreservCyt Solution, used in making Cytyc ThinPrep Pap Test slides. At least 12.5 mL of PreservCyt Solution was required for the study. Specimens with insufficient volume were excluded.

### 2.3. Sample preparation

Cytyc PreservCyt Solution specimens were held for up to 3 months at temperatures at 2–30 °C prior to testing (Package insert, Cytyc, Corp, Marlborough, MA 01752, USA). PreservCyt Solution specimens were processed using the Digene Sample Conversion Kit (Digene Corp, Gaithersburg, MD 20878, USA) prior to testing with the hcII HPV DNA Test. The Preservcyt Solution liquid medium specimen (about 6 mL) was divided into three aliquots and tested as follows: 0.3 mL were tested by the HGS High-Risk HPV Detection Kit for high-risk HPV DNA and for subsequent genotyping by the HGS Genotyping kit; 4 mL were tested for high-risk HPV DNA using the hcII HPV DNA test; 0.3 mL were reserved for reference standard PCR testing to detect HPV. If HPV was detected in Aliquot 3, the amplicon was sequenced to identify the type of the most prevalent HPV type. The primer sets used from conserved regions of HPV were MY09/MY11 and GP5+/GP6+.

### 2.4. HPV detection using the HGS High-Risk HPV detection kit

The HGS High-Risk HPV detection kit was manufactured and supplied by Human Genetic Signatures, Sydney, Australia. This test uses the same starting material as the other tests (Pap smear and/or liquid-based cytology (LBC)—sample) and treats the particular sample appropriately to isolate the DNA contained within that sample. The HGS High-Risk HPV detection kit uses genomic simplification to reduce the complexity of the genome from the native four bases to essentially three bases by replacing cytosine with uracil and ultimately with thymine. The kit provides materials to go directly from the LBC to fully simplified DNA. Three hundred microliters of LBC samples are added to each well of the 96-well purification plate and a series of incubations and centrifugations follow as per manufacturer's instructions. The DNA is eluted in 60 μL of elution buffer and 2 μL are added to the High-Risk PCR, followed by detection using agarose gel electrophoresis, stained with ethidium bromide and visualised under ultraviolet light.

### 2.5. Standard high-risk HPV detection using the hcII assay

The Hybrid Capture II (hcII) test is a nucleic acid hybridization assay where specimens containing the target DNA hybridize with a specific HPV RNA probe mixture including probes for 13 High-Risk HPV types. The resultant DNA: RNA hybrids are captured on a microplate coated with antibodies specific for DNA: RNA hybrids. After signal detection with antibodies conjugated with alkaline phosphatase and substrate, the emitted light is measured in a luminometer as relative light units (RLU). Samples are classified as positive for HR HPV if the relative light unit (RLU)

Table 1
Results and testing for HPV using novel HGS and Digene hcII techniques compared with the Reference Standard method (PCR with MY and GP primer sets) for 835 cervical specimens

| | Reference method (PCR) | | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| | + | − | | | | |
| HGS test results | | | | | | |
| + | 197 | 49 | 63.1 | 90.6* | 80.1* | 80.4 |
| − | 115 | 473 | | | | |
| Digene hcII test results | | | | | | |
| + | 202 | 80 | 64.7 | 84.6 | 71.6 | 80.1 |
| − | 110 | 442 | | | | |

Note: (1) (+) positive result; (−) negative result on that test. Number of specimens shown. (2) *$p < 0.001$ for two-tailed student's *t*-test comparing result with HGS and Digene hcII for detection of HPV in cervical specimens.

reading is above 1.0, which (according to the manufacturer) is equivalent to 1 pg HPV DNA/mL.

### 2.6. Reference HPV genotyping

This assay is based on the amplification, using nested PCR, of a conserved region within the HPV matrix protein. The PCR primers sets used for the detection of HPV are the MY09/MY11 and the GP5+/GP6+ (Table 1). The final product is detected by agarose gel electrophoresis, which is stained with ethidium bromide and visualised under ultraviolet light.

### 2.7. Sequencing to determine genotype

Amplicons were purified using the Marligen rapid PCR purification kit according to the manufacturer's instructions (Marligen Biosciences, Inc. Ijamsville, MD, USA). One microliter of a 100 ng/μL solution of the reverse PCR primer was added to 10 μL of the purified DNA and the volume of the reaction made to 15 μL by the addition of molecular grade water. Sequencing reactions and sequence determination was then performed at a commercial DNA sequencing facility (Supamac, Australia). The identity of the HPV strain was determined using BLAST against the NCBI database (http://www.ncbi.nlm.nih.gov/blast).

### 2.8. Statistical analysis

Sensitivity, specificity PPV and NPV were determined using standard methods (Leisenring et al., 2000; Moskowitz and Pepe, 2006).

## 3. Results

The sensitivities of the HGS test and the Digene hcII test were 63.1% and 64.7%, respectively, compared with the reference PCR test. The difference between the HGS and Digene hcII tests was not statistically significant ($p = 0.398$). In turn, the specificities of the HGS test and the Digene test were 90.6% and 84.6%, respectively. The specificity of the HGS test for detection of HPV in cervical Cytic specimens was statistically significantly higher ($p < 0.001$) than the specificity of the Digene hcII, when compared with detection using the reference standard PCR method.

The positive predictive value (PPV) of the HGS test and the Digene test was 80.1% and 71.6%, respectively. The HGS High-risk HPV test had a statistically significant higher PPV than the Digene test ($p < 0.001$). Thus, the rate of false positives for the HGS test is significantly lower than the rate of false positives for the Digene test (19.9% vs. 28.4%; $p < 0.001$).

The negative predictive values (NPV) of the HGS test and the Digene hcII test were 80.4% and 80.1%, respectively. However, there was no statistically significant difference in NPV between the HGS test and Digene hcII test ($p = 0.677$).

## 4. Discussion

This study demonstrated that the HGS High-Risk HPV detection assay was more reliable that the Digene test in detecting high-risk HPV. There was no apparent difference between the two assays in detecting negative results, i.e. the absence of high-risk HPV in the specimens.

The direct comparison of the Digene Hybrid Capture II Assay and the HGS High-Risk HPV detection kit was performed on a set of specimens to evaluate the *specificity* (accurate identification of high-risk HPV genotypes) and *sensitivity* (ability to detect low concentrations of virus) of the assays. The fact that the Digene Hybrid Capture II Assay is approved by the US FDA (Poljak et al., 1999) does not provide any indication of absolute accuracy. Indeed the hcII assay is dependent on the cellular content of samples, rendering occasionally false positive results with low RLU readings. Furthermore, it also yields false negative results and cross contamination between low and high-risk HPV types (Poljak et al., 2002). The test, also cross-reacts with at least 15 non-target genotypes such as a number of the low risk types and unsequenced HPV types (termed HPV X). This potential

weakness of the hcII assay could lead to patient undergoing unnecessary medical interventions such as colposcopy due to incorrect diagnosis. In addition, it has been reported that there is a "grey area" around the Hybrid Capture II cut-off in which samples should be re-tested as results obtained within this area are unreliable (Muldrew et al., 2007). However, a recent report cited no improvement in the overall assay performance even with repeat testing of samples displaying RLU values at or near the cut-off value of the test (Seme et al., 2006). Another draw back of the current Hybrid Capture II method is that although the test can detect the presence of high-risk HPV the technique cannot genotype the strain(s) present in the sample. Notwithstanding that one of the major predisposing factors for the development of cervical disease is the persistent infection of the patient with the same high-risk strain, particularly HPV 16 and 18 (van Doorn et al., 2006).

The HGS High-Risk HPV detection kit uses a method of genomic simplification from the native 4 bases to essentially three bases by replacing cytosine with uracil and ultimately with thymine. Genomic simplification results in genomes that are more similar to each other, enabling the design of primers that contain less mismatches, are more homologous, produce better amplification and importantly have less cross reactivity. These primer sets preferentially amplify high-risk HPV strains over low risk strains. Should the clinician require the particular high-risk strain to be identified, a subsequent reflex PCR test is available to identify the individual strain(s). The subsequent test is undertaken on the material already collected and converted and hence no further sample processing is required, allowing subsequent monitoring of the patient for persistent infection.

The clinical impact of high-risk HPV, particularly HPV-16 and HPV-18 has been evaluated in different studies (Lai et al., 2007; Schwartz et al., 2001; Pilch et al., 2001) and these strains are associated with poor prognosis in cervical cancer patients, poor overall survival and cancer relapse. In one study 20 percent of women with the highest HPV 16 quantities had a 60-fold increased risk of carcinoma in situ of the cervix compared to HPV negative controls (Josefsson et al., 2000). Thus the test under evaluation can provide valuable information to clinicians in terms of early stage cervical cancer at high-risk for disease recurrence. Furthermore, the CDC in its most recent STD treatment guidelines, suggests that HPV testing may be useful for determining optimal follow up of women with atypical squamous cells of undetermined significance (ASCUS) and that HPV testing may have a role in screening programs for women aged >30 years (Workowski and Berman, 2006).

While it may seem advantageous to support the idea that the HGS High-Risk HPV test should be more sensitive, this was not chosen as a primary endpoint due to its debatable clinical utility. A pre-requisite for a pre-cancerous lesion to develop is a persistent infection with the same high-risk HPV type (Cuschieri et al., 2004; An et al., 2003). Indeed most women may carry small amounts of HPV that are readily cleared by the immune system. Thus, an assay that yields most results positive may be useless for clinical purposes. On the contrary, the specificity of the test is critical as high-risk strains are 90% related to cervical cancer. The HGS High-Risk HPV Detection test demonstrated a statistically significant higher positive predictive value, specificity and a statistically significant lower rate of false positives ($p < 0.001$) as compared to the Digene test.

In summary, using a test with better PPV and specificity is ideal because whilst maintaining a similar detection cut-off to that of the Digene test, the HGS test is more capable of identifying high-risk HPV sub-types. Furthermore, from a logistics point of view, the HGS test is simpler to perform, requires less sample volume, it is performed in approximately 1 h and a half less "hands on" time and does not require as much specialized equipment (e.g. Luminometer) making it a viable and more accurate alternative to the Digene Hybrid Capture II Assay.

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