Paper #: C-092

Specific Detection of High-Risk HPV Genotypes Using a Novel DNA Simplification Strategy

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Abstract:

Background: The presence and persistence of high-risk Human Papilloma Virus (HR-HPV) is considered to be the major risk factor in the development of cervical neoplasia. There are over 100 strains of HPV of which only 13 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) are considered as the etiological agents of cervical cancer. Due to the genomic diversity and the large number of strains, it has been technically difficult to design PCR primer sets capable of the specific detection of all high-risk strains. **Methods:** Treatment of DNA with sodium bisulphite chemically converts cytosine residues to uracils, which are subsequently amplified as thymines. This conversion essentially results in the simplification of the conventional 4 base genome to a 3 base genome. This "simplified DNA" increases similarity between members of the HPV family, yet retains sufficient genetic information to allow differentiation between high and low risk strains. To determine if this method could be used to specifically detect HR-HPV in clinical samples we designed a single set of PCR primers based on the E7 gene of HPV and applied these to simplified DNA. **Results:** 266 Liquid based cytology samples of known cytology were tested blind using a commercially available kit for the detection of HR-HPV. The results were compared to those generated by our DNA simplification approach and are summarised in the table below:

Detection of HPV by DNA simplification

Cytology (Number)	Simplified Positive (%)	Hybrid Capture Kit Positive (%)
ASC-US (18)	7 (39%)	7 (39%)
LSIL (59)	53 (90%)	49 (83%)
HSIL (6)	6 (100%)	5 (83%)

183/266 samples were normal by cytology and of these 30 (16.4%) tested positive using the simplification approach compared to 24 (13.1%) using the commercially available kit. All HR-HPV positive normal samples were genotyped using high-risk type specific primers based on simplified HPV sequences to determine if the normal samples did in fact harbour high-risk HPV sequences. The results are summarised in the table below.

Genotyping of Normal Samples

Cytology Normal (183)	Simplified Positive (%)	Hybrid Capture Kit Positive
Positive by Genotyping	23/30 (77%)	12/24 (50%)
Negative by Genotyping	7/30 (23%)	12/24 (50%)
False Positive rate	3.8%	6.6%

Conclusion: DNA simplification is a novel approach for the reliable detection of pathogens consisting of a large number of sub-types such as HPV. In addition the simplified DNA still retains sufficient sequence information for individual genotyping analysis.

Introduction:

Introduction: Sensitive molecular diagnostic assays are of particular importance when the infectious agent is unable to be grown in conventional culture, such as for Human Papilloma Virus (HPV). However, the design of these molecular assays can be technically challenging as many pathogens contain diverse subtypes that can all be involved in diseases, such as in the many genotypes of HPV, influenza and rotavirus.

Human Papilloma Virus is a virus that infects the skin surface of oral, anal and genital cavities. Manifestation of infection is usually transient with patients not expressing symptoms, however persistent infection with certain strains of HPV can lead to more visual and serious manifestations such as genital warts, squamous intraepithelial lesions (SIL), and carcinomas (Cervical Cancer). The American Centre for Public Health Research and Evaluation 2005 report estimates from 14,000 annual cases of cervical cancer in the U.S., 5,000 will die from the disease and worldwide from an estimated 450,000 annual (reported) cases, 200,000 will die as a direct result. To date there are no curative treatments for cervical cancer and HPV infection. Instead research efforts are directed towards alleviating symptoms, vaccination and early stage detection. Based on evidence presented by the Centre of Disease Control and Prevention (United States), it was concluded all cervical cancer tumour masses contain 'high-risk type' HPV DNA, but detection of HPV DNA alone is not a definitive diagnosis for cervical cancer.

The most sensitive HPV detection methodology is PCR, which readily detects a single viral copy in a human genome. The first HPV PCR was directed towards the L1 consensus region with a practical lower detection limit of about 100 viral genomes. Indeed, the L1 consensus primers have become the most widely used in clinical and epidemiological studies, with the MY09/MY11 and the GP5+/GP6+ primers being most frequently used. Although these primer sets have been proven to be very reliable at the detection of HPV, both primer sets react with high risk, low risk and HPV types of unknown origin.

We have developed a broad-range assay that successfully detects all High Risk HPV sub-types (sub-types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Our assay produces a positive result if any of the High Risk HPV subtypes are present. This multi-genotype single assay design is achieved through the conversion of the viral DNA with sodium bisulfite, which converts all unmethylated cytosine bases within a DNA sample to thymine base (via a uracil intermediary) as indicated in Figure 1.

Figure 1: The effect of sodium bisulfite on cytosine

We have called this process DNA simplification. This approach is primarily used to make nucleic acid sequences from different organisms or families more similar to each other enabling the following benefits:

1) The detection of multiple microbial strains in a single reaction without the need for multiplexing, which is complex and difficult to optimise.
2) A single consensus primer, or a small family of primers, can be used to detect the presence of any/all variants of species of interest (e.g. the High Risk HPV subtypes) with a high degree of specificity in a single reaction.
3) Genotyping or specific species detection can still be performed as there is sufficient heterogeneity remaining in the samples post simplification.

An example of how simplification of DNA can be applied to the detection of High Risk HPV is shown in Figure 2. Our assay can effectively reduce the consensus primer heterogeneity from 48 combinations to just 3 combinations and increases the sequence homology from 75% to 95%. This represents a 94% simplification of the original divergent sequences.

Detection of HPV by DNA simplification

	Before Simplification (Wild Type)		After Simplification	
HPV6	GATGG**C**GA**T**A	TGGT**T**GA**C**AC	GATGGTGATA	TGGT**T**GATAT
HPV43	GATGG**T**GA**C**A	TGGT**A**GA**T**AC	GATGGTGATA	TGGT**A**GATAT
HPV44	GATGG**T**GA**T**A	TGGT**G**GA**C**AC	GATGGTGATA	TGGT**G**GATAT
HPV54	GATGG**T**GA**T**A	TGGT**A**GA**T**AT	GATGGTGATA	TGGT**A**GATAT
HPV55	GATGG**T**GA**T**A	TGGT**G**GA**C**AC	GATGGTGATA	TGGT**G**GATAT
HPV30	GATGG**C**GA**C**A	TGGT**T**GA**T**AT	GATGGTGATA	TGGT**T**GATAT
HPV33	GATGG**T**GA**T**A	TGGT**G**GA**C**AC	GATGGTGATA	TGGT**G**GATAT
HPV58	GATGG**T**GA**C**A	TGGT**A**GA**T**AC	GATGGTGATA	TGGT**A**GATAT
HPV18	GATGG**T**GA**T**A	TGGT**A**GA**T**AC	GATGGTGATA	TGGT**A**GATAT
HPV45	GATGG**T**GA**T**A	TGGT**G**GA**T**AC	GATGGTGATA	TGGT**G**GATAT
Consensus	GATGG**Y**GA**Y**A	TGGT**D**GA**Y**AY	GATGGTGATA	TGGT**D**GATAT
	75% homology over 20 bases **48** possible primer combinations		95% homology over 20 bases **3** possible primer combinations	

Figure 2: Consensus sequence of High-Risk HPV virus sequence before and after DNA simplification. The simplification has resulted in an increased homology from 75% to 95%.

To demonstrate the performance of our assay we have recently completed a pilot study comparing 266 samples processed with either our assay or the only FDA approved test for HPV detection, the Digene hc2 HPV DNA test (Gaithersburg, USA). Given the excellent performance of our assay in the pilot study we further commissioned a clinical trial that compared the sensitivity (false negative) and selectivity (false positive) rates of the hc2 HPV DNA test (Digene Corp., USA) against our High Risk HPV DNA Test using PreservCyt Solution (Cytyc Corp., Marlborough USA) specimens obtained from the ThinPrep® Pap Test™

Methods:

We initially analysed 266 Liquid based cytology samples (PreservCyt Solution specimens) of known cytology as a pilot study. These samples were processed using the Human Genetic Signatures High Risk HPV DNA Test (HGS HR-HPV Test) (North Ryde, Australia) according to the manufacturer's instructions. Briefly 300 µL of each PreservCyt Solution specimen was captured within a 96-well purification plate where the cells were lysed and the DNA was denatured. The captured DNA was then treated with sodium bisulfite (to simplify the DNA) and eluted in 60 µL. This eluate was incubated at 95°C to desulfonate the DNA and 2 µL was added to the 1st round PCR reaction. 'Nested' PCR primers were used to ensure good sensitivity and specificity of the High Risk HPV detection, performed in Promega 2x PCR mastermix (Madison, USA) as detailed in Table 1:

Table 1: PCR Condition

Reagent	1x mix
2 x PCR master mix	12.5 µL
Water	8.5 µL
HR-HPV Primer	2.0 µL

The two rounds of PCR were performed according to the following cycling conditions:

95°C/ 3 min	1 cycle
95°C/ 1 min 45°C/ 2 min 65°C/ 2 min	30 cycles
65°C/10 min	1 cycle

Two microlitres of 1st round product was transferred to the 2nd round. Ten microlitres of the 2nd round PCR reaction was electrophoresed on a 2% precast agarose gel (Invitrogen, Carlsbad, USA).

Genotyping was also performed by using the Human Genetic Signatures HPV High Risk Genotyping Kit (North Ryde, Australia) according to the manufacturer's instructions. This genotyping is based on simplified sequences and is therefore compatible with the DNA processed using the HGS HR-HPV DNA Test. Again 2 µL of processed DNA was added to each genotyping PCR, which also contained 10.0 µL Promega 2x PCR mastermix, 5.0 µL H2O and 3 µL of the genotype specific primers. Once again 2 rounds of PCR were performed according to the cycling conditions given above and 2 µL of 1st round product was transferred to the 2nd round. Ten microlitres of the 2nd round PCR reaction was electrophoresed on a 2% precast agarose gel (Invitrogen, USA).

The clinical trial was performed by the South Eastern Area Laboratory Services (SEALS) of the Prince of Wales Hospital, Randwick, NSW, Australia (Principal Investigator: Prof. William Rawlinson). The study design was a double blind, controlled, crossover, comparative study of the sensitivity (false negative) and selectivity (false positive) rates of the hc2 Human Papillomavirus DNA Test (Digene Corporation, USA) versus the HGS HR-HPV DNA Test. These results were compared against the MY09/MY11 and the GP5+/GP6+ L1 PCR based "Gold Standard PCR Test" involving genotyping via sequencing. Both tests were performed exactly as per the recommendations of the manufacturer or according to the literature. The **primary endpoint** of the study was the Positive Predictive Value of each test in determining the presence of High Risk HPV in a sample. Sensitivity, Specificity and Negative Predictive Value were **secondary endpoints**. A total of 834 samples were analysed during the trial.

Results:

An example of the results obtained when using our assay is shown in Figure 3. The HGS HR-HPV test indicated the presence of one or more High Risk HPV types in a sample. Further genotyping was achieved by using the HGS HPV High Risk Genotyping Kit to determine the exact HPV type present in each positive result.

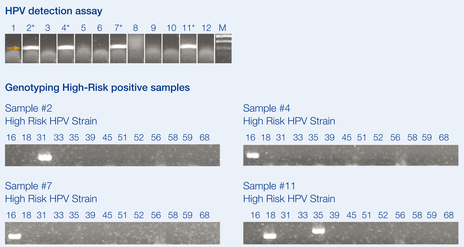

Figure 3. The results of the Human Genetic Signatures High Risk HPV test are shown in the top panel. Samples 2, 4, 7 and 11 are found to contain at least one of the 13 High Risk HPV genotypes. These samples were further genotyped using the Human Genetic Signatures HPV High Risk Genotyping Kit. As shown in the bottom panel sample number 2 harboured HPV type 31, sample numbers 4 and 7 harboured HPV type 16 and sample number 11 harboured a mixed infection of HPV types 18 and 35.

Pilot Study

The pilot study involving 266 samples showed that the HGS test was able to identify High Risk HPV virus in a greater number of LSIL and HSIL graded samples than the Hybrid capture kit (summary table in abstract). In addition, 183 samples were found to be cytologically normal and of these 30 (16.4%) tested positive using our HPV assay as compared to 24 (13.1%) using the Digene hc2 kit. We subsequently genotyped each of the HR-HPV positive cytologically normal samples in order to determine the absolute presence of High Risk HPV in these samples. The results are again summarised in the abstract and show that our assay had a false positive rate of approximately half of the FDA approved hc2 method (Digene Corporation, USA).

Clinical Trial

The SEALS clinical trial compared the sensitivity (false negative) and selectivity (false positive) rates of the hc2 Human Papillomavirus DNA Test (Digene Corporation, USA) versus the HGS HR-HPV DNA Test. A test result was recorded as True Positive or True Negative only if the selected test (hc2 HPV DNA Test or the HGS HR-HPV Test) result agreed with the Gold Standard PCR test according to Table 2. The "Gold Standard PCR Test" comprised PCR amplification of the L1 region using the MY09/MY11 and the GP5+/GP6+ primer sets, and sequencing to confirm the most prevalent genotype.

Table 2: Determination of Sensitivity, Specificity Positive Predictive Value and Negative Predictive Value.

	'Gold Standard' PCR Test result Positive	'Gold Standard' PCR Test result Negative	Total
Criterion Test Positive	a (number true positive)	b (number false positive)	a+ b
Criterion Test Negative	c (number false negative)	d (number true negative)	c + d
Total	a + c	b + d	

Sensitivity = a/a+c; Specificity = d/b+d; PPV = a/a+b; NPV = c/c+d

The trial results demonstrated that our assay **had a statistically significant higher PPV than the Digene test (P < 0.001).** The PPV of the HGS test was 197/246 or 80.1% (95% Confidence Interval (CI) 74.5% to 84.9%) and the PPV of the Digene test was 202/282 or 71.6% (95% CI 66.0% to 76.8%).

Using the same approach as we used to test for differences in PPV between the two tests it can also be inferred that the rate of false positives for the HGS test is lower than the rate of false positives for the Digene test (19.9% vs 28.4%; P < 0.001).

The increased specificity of our assay was achieved at the same level of sensitivity as seen in NPV values. The NPV of the HGS test was 473/588 or 80.4% (95% Confidence Interval (CI) 77.0% to 83.6%) and the NPV of the Digene test was 442/552 or 80.1% (95% CI 66.0% to 76.8%) (Not statistically significant, P=0.677). The sensitivity of the HGS test is 197/312 = 63.1% (57.5 to 68.5%) and the sensitivity of the Digene test is 202/312 = 64.7% (59.2 to 70.0%) but the difference between the two tests is not statistically significant (P = 0.398).

The specificity of the HGS test is 457/522 = 90.6% (87.9 to 93.0%) and the specificity of the Digene test is 442/522 = 84.7% (81.3 to 87.7%) and the difference in specificity between the two tests is statistically significant (P < 0.001), consistent with significant improvements in PPV.

A breakdown of the concordance between the 2 tests is given in Table 3. The overall concordance between the two assays was 86.9%. Further analysis of the discordant samples via genotyping to determine true HPV presence showed that the total HGS HR-HPV test accuracy was 94.7% as compared to the total Digene hc2 test accuracy of 92.2%.

To further understand the prevalence and distribution of the High-Risk HPV subtypes within a normal population we used the HGS HPV High Risk Genotyping Kit to fully characterize each sample. We found that the most prevalent sub-type was HPV type 16, followed by types 31, 51 and 56 (Figure 4). Figure 4 only shows relative distribution of the HPV sub-types as multiple infections of at least two sub-types was observed in 36% of all specimens.

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Table 3a: HGS test results compared with the Digene test result among subjects with the HPV virus.

		Digene test result: Positive	Digene test result: Negative	
HGS test result	Positive	182	15	
HGS test result	Negative	20	95	
				N=312

Table 3b: HGS test results compared with the Digene test result among subjects without the HPV virus.

		Digene test result: Positive	Digene test result: Negative	
HGS test result	Positive	27	22	
HGS test result	Negative	57	420	
				N=522

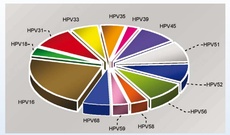

Figure 4. The relative distribution of HPV sub-types in the clinical trial samples.

Conclusion:

We have demonstrated that the simplification of DNA using sodium bisulfite to reduce the 4 base genome to 3 bases is a novel method that enables the detection of multiple species in one reaction without the need for multiplexing. A single consensus primer or small family of primers has been shown to successfully detect the presence of any/all variants of species of interest with high specificity in a single reaction. The simplification of DNA still retains sufficient sequence heterogeneity to allow for genotyping or specific species detection. Our technology is adaptable to any DNA detection methods, including isothermal and real-time detection.

Our High Risk HPV detection assay has demonstrated a statistically significant higher Positive Predictive Value (P<0.001) and a statistically significant lower rate of false positives (P<0.001) as compared to the only FDA approved test for HPV detection (the Digene hc2 test). This was achieved at a similar level of sensitivity as there was no statistically significant difference between the tests in terms of Negative Predictive Value.