Comparison of the Third Wave Invader HPV Assay and the Digene HPV Hybrid Capture 2 Assay for the Detection of High-Risk Human Papillomavirus DNA

C. C. Ginocchio, D. Barth, and F. Zhang

North Shore – Long Island Jewish Health System Laboratories, Department of Molecular Diagnostics, Lake Success, NY

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Corresponding author:
Christine C. Ginocchio
North Shore Long Island Jewish Health System Laboratories
10 Nevada Drive
Lake Success, NY 11042
tel: 516-719-1079
fax: 516-719-1254
email: cginocch@nshs.edu

Debra Barth
North Shore Long Island Jewish Health System Laboratories
10 Nevada Drive
Lake Success, NY 11042

Frank Zhang
North Shore Long Island Jewish Health System Laboratories
10 Nevada Drive
Lake Success, NY 11042
tel: 516-719-1079
fax: 516-719-1254
email: fzhang@nshs.edu
This study compared the clinical performance of the Digene Hybrid Capture 2 (HC2) assay to a prototype Third Wave Invader HPV (IHPV) analyte specific reagent based assay for the detection of HR HPV DNA using liquid based cytology specimens. In total, 821 ThinPrep vials were tested using both assays. In accordance with the type specific probes contained within each test, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the IHPV assay were 95.9%, 97.6%, 97.5% and 96.1%, respectively and for the HC2 assay were 98.1%, 86.2%, 87.1% and 97.9%. Overall, the sensitivity and NPV were comparable between the assays, but the IHPV assay demonstrated a better specificity and PPV since the IHPV assay had less false positive HR HPV results. The incorporation of an internal control to evaluate the cellularity of the test material is an important feature of the IHPV assay and should reduce the risk of false negative results due to insufficient sample collection rather than the lack of HR HPV DNA. An additional benefit of the IHPV assay was the smaller sample volume required (1 mL) versus 4 ml for the HC2 assay.
INTRODUCTION

Cervical cancer is the second most common malignancy of women worldwide, and a leading cause of death for middle-aged women (age 35-55) in low-resource settings (8, 12). In the US, the American Cancer Society estimated that ~10,370 new cases of cervical cancer would be diagnosed in 2005 and that ~3,710 women would die of the disease (1). Cervical cancer is a highly preventable disease when cytologic screening programs are employed that facilitate the detection and treatment of precancerous lesions. When detected early, the 5-year relative survival rate for the earliest stage of invasive cervical cancer is 92% and the overall (all stages combined) 5-year survival rate for cervical cancer is about 73% in the US (1). Beginning in 2002, patient management guidelines have been published by various groups of healthcare professionals that recommend how women should be screened for cervical cancer according to age, other factors, and the presence of cytological abnormalities in a Pap test (6, 14, 20-24). These patient management guidelines all recommend testing for the presence of high-risk types of human papillomavirus (HPV) as an additional diagnostic tool for equivocal or ambiguous cytology results, including ASC-US, ASC-H, and LSIL.

Research worldwide has clearly shown that virtually all cervical cancer is caused by chronic infection with certain carcinogenic types of HPV (2, 3, 9, 10, 11, 18, 19). Over 100 types of HPV have been documented in the literature, approximately 40 of which are known to be sexually transmitted. In the US, genital infection with HPV is the most common sexually transmitted viral infection
with an estimated 6.2 million new infections each year (1). Of the sexually
transmitted types of HPV, approximately 15 types have been classified as
oncogenic or “high-risk” in epidemiological studies (2, 9, 11, 19). Persistent
infection with one or more of these HR types of HPV causes 95-100% of all
cervical cancer (9, 11, 18, 19).

There are thirteen types of high-risk (HR) HPV DNA for which clinical
utility has been established and for which testing is recommended: HPV types
16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (11, 14, 18-22). Recently,
the International Association for Research on Cancer (IARC) re-classified HPV
66 as a high-risk, carcinogenic type of HPV and recommended that it be included
as part of routine screening (3). Under current patient management guidelines,
testing for HR HPV DNA is conducted under one of two settings: as a means to
triage patients with equivocal or ambiguous cytology results (ASC-US) to
determine the need for referral to colposcopy, or, as an adjunct to cervical
cytology analysis in women 30 years of age and older (6, 20-24). In either
setting, the presence or absence of HR HPV DNA is considered in light of the
patient’s cytology history and other risk factors in order to guide the patient’s
management.

Currently, the Digene Hybrid Capture 2 (HC2) assay (Digene,
Gaithersburg, MD) is the only method FDA cleared for the detection of HPV DNA
from both endocervical samples and liquid based cytology specimens (17).
Several other methods, including the PCR based Roche AMPLICOR and Roche
LINEAR ARRAY HPV tests (16), the GenProbe Aptima HPV test for the detection
E6/E7 messenger RNA, and the Third Wave Invader HPV assay (Third Wave Technologies, Madison, WI) (15, 25) are currently under investigation as alternative technologies for HPV detection. This study evaluated the use of the Invader chemistry as an alternative method for detecting HR HPV DNA in samples submitted for routine HPV DNA testing (15, 25). Similar to all Invader assays, the prototype Invader HPV (IHPV) detection method (Research Use Only) uses isothermal signal amplification and requires no specialized instrumentation to perform (4). The prototype IHPV method utilizes sequence specific Invader DNA probes, a structure specific Cleavase enzyme, and a universal fluorescent resonance energy transfer system (FRET) combined with interpretive software and multi-well fluorometer to semi-quantitatively detect fifteen high-risk types of human papillomavirus (HPV) DNA in cervical epithelium cell specimens (4). The reagents are designed to query for the presence of known sequence polymorphisms and to identify specific nucleic acid sequences through the analysis of structure-specific cleavage events driven by the presence of any one of fifteen types of HPV and single copy human DNA sequences, respectively. The HPV types detected by the IHPV prototype assay are the HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and non-oncogenic types 67 and 70. The IHPV reagents were also designed to be able to detect mixed infections of certain combinations of HR HPV. The biplex format of the IHPV reagents enables simultaneous detection of two DNA sequences, HPV DNA if present and a non-varying segment of the human alpha actin 1 (ACTA1) gene in a single well. The detection of the ACTA1 DNA serves as an internal control for
both assay performance and for determining if sufficient cellular material is present in the reaction. IHPV results were compared to those obtained using the current laboratory assay, the Digene HC2 HR HPV test.

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MATERIALS AND METHODS

Sample Acquisition. Endocervical specimens (n=821) of unknown cytological profile were submitted in ThinPrep vials (Cytyc, Marlborough, MA) for routine Pap smear analysis and HR HPV DNA testing using the HC2 test. After completion of the HC2 testing, the residual samples were de-identified and used for IHPV testing under an IRB approved protocol.

Hybrid Capture 2 (HC2) HPV DNA Test. HC2 tests were performed and analyzed as per the manufacturer’s instructions using the HR HPV Probe cocktail (17). The HR Probe cocktail is capable of detecting HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Based upon in-house validation studies, the established laboratory criteria for the interpretation of results were as follows: samples were scored negative for HR HPV DNA when samples yielded RLU values ≤0.8, scored indeterminate for RLU values >0.8 to <5.0 and positive for high risk HPV DNA with RLU values ≥5.0. When sufficient volume was present, samples with values in the indeterminate range were retested with the HC2 assay. If the repeat HC2 test was <1.0 the samples was considered negative.
For a sample to be reported as positive, both RLU values had to be $\geq 2.0$. If only 1 result was $\geq 2.0$ then the result would remain indeterminate. If insufficient sample was available for retesting, the result would remain indeterminate.

**DNA Extraction for Invader HPV Assay.** DNA was extracted from 1 mL of the residual ThinPrep solution using a modified PureGene (Gentra, Minneapolis, MN) protocol. Briefly, cells were lysed using the PureGene Cell Lysis Solution with the assistance of heating to 55°C for 15 minutes. Protein was precipitated using the PureGene Protein Precipitation Solution. Next, 20 mg/ml glycogen was used to create a DNA pellet. Finally, the DNA was washed, dried and rehydrated in 60 µL using nuclease-free water.

**Invader HPV Reactions.** The IHPV reagents were composed of three pools of HR HPV type-specific probes and FRET cassettes, a probe set specific for the ACTA1 gene, Cleavase enzyme and Invader buffer (Third Wave Technologies). IHPV reactions were performed in triplicate using 10 µL of the nucleic acid extract and three separate oligonucleotide pools for each sample. Pool A5/A6 contained the oligonucleotides required for the detection of HPV 51 and 56, pool A7 for the detection of HPV 18, 39, 45, 59, 68, and 70, and pool A9 for the detection of HPV 16, 31, 33, 35, 52, 58, and 67. Reactions were allowed to proceed for four hours at 63°C before signal was read using a multi-well Genios FL fluorometer (Tecan, Durham, NC). HPV signal generated using each of the three oligonucleotide pools produced a FAM signal, while RED signal was produced by the internal control ACTA1 contained within each reaction.
**Invader Data Analysis.** Raw data from the multi-well fluorometer was exported and analyzed using Microsoft Excel 2000. Fold over zero values (FOZ) were calculated for standards and unknown samples by dividing the raw signal obtained for each by the raw signal of the standard no-target control. For HPV qualitative detection, the FOZ values indicative of the presence of HR HPV and for a positive internal control are shown in Table 1. Invader reactions would be repeated when indeterminate results were generated due to either assay failure or if the sample was determined to have an insufficient amount of cellular DNA based upon a RFOZ result of <1.2. If not enough sample remained for repeat testing, the result would be scored as indeterminate.

**Discordant Analysis.** Discrepant results between the HC2 and the IHPV methods were resolved using consensus PCR with sequencing utilizing GP5+/GP6+ and L1C1/L1C2 primer sets as previously described (5, 26). PCR reactions were done in a volume of 25 µL with 1 µL of template DNA. The PCR mixture consisted of 1X PCR buffer without MgCl₂ (Roche Molecular Systems, Pleasanton, CA), 0.4 µL of a 1:1 mixture of Amplitaq polymerase (Roche Molecular Systems) and TaqStart antibody (BD Biosciences, San Jose, CA), 200 uM each dNTP, 4 mM MgCl₂, and 1uM of each of 2 primers (GP5+ and GP6+ or L1C1 and L1C2). Reactions were run in a Hybaid thermocycler (Thermo Fischer Scientific, Inc., Waltham, MA) for 1 cycle of 5 minutes at 95°, then 8 cycles of 94° for 30 seconds, 40-47° for 30 seconds (starting at 40°, then increasing 1° per cycle), and 72° for 1 minute. This was followed by 35 cycles of 94° for 30 seconds, 48° for 30 seconds, and 72° for 1 minute. Total volume of each PCR
product was run on a 2% agarose in 1X TAE gel with ethidium bromide. The approximately 150 bp band from the GP5+/GP6+ amplifications and the approximately 250 bp band(s) from the L1C1/L1C2 amplifications were excised and transferred to individual 1.7 mL tubes. The DNA was extracted with the QiaexII gel extraction system (Qiagen, Valencia, CA) and eluted with 20 µL Tris EDTA buffer. The products were ligated into the pGEM-T Easy vector (Promega, Madison, WI), transformed into chemically competent JM109 cells, then plated on two LB+ampicillin plates containing IPTG and X-gal. After overnight growth, 2 white colonies were picked from each plate (total of 4 per sample) and grown overnight for DNA extraction (using Qiaprep Spin, Qiagen) and sequencing (with the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI 377 Sequencer, Applied Biosystems, Foster City, CA). The primer used for sequencing was J5: 5’- GACGTCGCATGCTCCC-3’. The sequences were trimmed to exclude the amplification primers and vector sequence. The resulting sequences were BLASTed verses Genbank and the top hit for each sequence listed as the genotype.

Statistical analysis. Assay sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulas.

RESULTS

Of the 821 samples tested (Table 2), initial results were concordant for 673 samples (81.97%) and discordant for 148 samples (18.03%). Discordant
results included 16 IHPV positive/HC2 negative samples (1.95%), 73 IHPV negative/HC2 positive samples (8.89%), 20 indeterminate HC2 results (2.44%) and 41 indeterminate IHPV results (4.99%), of which 2 samples were indeterminate by both methods. Twenty six (26) of the 41 IHPV samples were scored as indeterminate due to insufficient cellular material in the samples, as denoted by a RFOZ of < 1.2.

PCR and sequencing were performed on 84/89 positive/negative discordant samples to determine if HR HPV DNA was in fact present in the sample. The remaining 5 samples (IHPV negative/HC2 positive) did not have sufficient quantity remaining for sequencing studies (Table 3). In addition, PCR and sequencing were performed on 1 of the 2 samples indeterminate by both HC2 and IHPV, and 10 HC2 and 11 IHPV indeterminate samples (Table 4). Of the 84 positive/negative discordant samples sequenced, 60 (71.43%) resolved in favor of the IHPV assay (Table 3) when considering the detection probes present within the IHPV assay. The HC2 assay gave false positive results for 38 samples, including 13 samples with no HPV DNA detected by PCR and 25 samples with low risk (LR) HPV types shown in Table 3. The HC2 assay detected 15 additional positive samples than the IHPV assay for HR types listed in Table 3. The HC2 assay also detected 15 samples with HR HPV type 66 although a specific probe for type 66 is not present in their HR HPV probe cocktail. This version of the IHPV assay does not contain a probe for type 66 and therefore, these samples were negative with the IHPV assay. The IHPV assay gave false positive results for 8 samples, including 3 samples with no HPV
DNA detected by PCR and 5 samples with LR HPV LR types (Table 3). The IHPV assay detected HR HPV types in 7 additional samples negative with the HC2 assay.

Repeat testing of all the indeterminate HC2 and IHPV results with the appropriate assay was not performed due to insufficient residual specimen. However, sufficient sample was available to perform PCR and sequencing for 22 indeterminate samples and the results are shown in Table 4. For the HC2 indeterminate/IHPV negative samples, 4 had no HPV DNA detected by PCR, 4 had a LR HPV strain and 2 were positive for HR HPV (types 16 and 31). The two HR positive samples were positive by the IHPV assay. For the samples with IHPV indeterminate_HC2 negative results, 3 had no HPV DNA detected by PCR and 3 had a LR HPV strain. For the IHPV indeterminate_HC2 positive samples, 2 contained a LR HPV strain and 3 HR HPV (types 51 and 66). As noted above the IHPV assay does not contain a probe for type 66. Two of the three samples with HR HPV types would have been reported out as indeterminate due to insufficient cellular material.

For the final overall analysis there were in total 757 samples with valid HC2 and IHPV results, and when indicated for discordant resolution, the comparison to PCR and sequencing analysis (Table 5). Results for each method are presented in two manners: 1) based upon the detection of only the HR HPV types with the type specific probes present in the HR Probe Mixes (Table 5, Rows 2 and 4) and 2) detection of all HR HPV types, including HR type 66 which was not present in either the IHPV or HC2 Probe Mixes (Table 5, rows 3 and 5).
Overall, there were 380 HR HPV positive samples (including 15 type 66) and 377 HR HPV negative samples. Based upon resolution with PCR and sequencing, overall, the HC2 test was slightly more sensitive for the detection of HR HPV DNA than the IHPV assay (98.08%, 98.16% versus 95.89%, 92.11%, respectively), and therefore had a higher negative predictive value (97.94%, 97.4% versus 96.08%, 92.46%, respectively) (Table 5). The increased sensitivity of the HC2 assay was primarily related to the detection of HR HPV type 66, for which a type specific probe is not present in the HC2 probe mix nor in the IHPV probe pools. The HC2 positive results may be due to the presence of other HR HPV types for which probes are present in the HC2 probe mix but were not detected by PCR and sequencing. Alternatively, type 66 may have been detected due to cross reactivity with other HC2 probes present in the probe mix. Analysis of the discordant results demonstrated that the HC2 method, in comparison to the IHPV assay, has a lower specificity (86.41%, 89.87% versus 97.87%, 97.87%, respectively), a lower positive predictive value (86.16%, 89.67% versus 97.61%, 97.41%, respectively) and a higher overall rate of false positives (7.0%, 5.02% versus 1.19%, respectively). This rate of HC2 false positives is a result of samples found to be HPV HR positive, while determined by PCR/sequencing that either no HPV DNA was present, there was a substantial degree of cross-reactivity with HPV types not included in the HC2 HR probe set, or for which the test has label claims of being able to detect with the HC2 HR probe pool. The HC2 method demonstrated cross-reactivity with the low risk HPV types 6, 30, 42, 43, 53, 61, 72, 73, 84, 87, 91, while the IHPV
method demonstrated cross-reactivity with low risk types 6, 73, 84, and 91. In addition to false negatives that were PCR/sequencing positive, the HC2 method does not contain probes capable of detecting “possible HR” HPV type 66, yet demonstrated significant cross-reactivity for this type. However, one must use caution when interpreting these rates of false positives and false negatives. The methods employed here will only detect false positives/negatives that occur in only one method, but not the other, since only discrepant samples were sequenced. To determine the true rates of false-positive and false-negative results for each method, all samples would have to be sequenced. In addition, low levels of HR HPV may have been present in some samples but was not detected by the PCR method used in this study.

In addition to lower rates of false positives, the IHPV method is also capable of detecting the presence of multiple types of HR HPV DNA present within a single sample. The use of three separate oligonucleotide pools enables the IHPV method to detect these mixed infections, something the HC2 method is unable to do. The IHPV method was able to detect at least 47 positive samples that contained mixed HR HPV infections (data not shown).

DISCUSSION

Overall, the HC2 and the IHPV assays demonstrated comparable sensitivity for the detection of HR HPV when considering the type specific probes present in each assay. When taking into account all positive results, the slightly increased sensitivity of the HC2 assay was mainly based on the ability of HC2 to
detect HR HPV type 66 due to potential probe cross reactivity. Recently, the International Association for Research on Cancer (IARC) re-classified HPV 66 as a high-risk, carcinogenic type of HPV and recommended that it be included as part of routine screening (3). While the IHPV probe pools used in this study did not contain probes for type 66 the manufacturer has announced its inclusion in all newer versions of the reagents (25). In this study and in previous studies, the HC2 HR probe pool, which does not contain a type 66 specific probe, has been shown to detect type 66 due to a high degree of cross-reactivity between type 66 and other HR probes present in the HC2 HR probe pool (13). Although the cross reactivity resulted in more HR HPV positive samples, the HC2 product was not FDA-approved with label claims for reliably and reproducibly being able to detect type 66 and its demonstrated cross-reactivity with other non-targeted HPV types. Therefore, the ability of the HC2 assay to detect type 66 should not be relied upon.

When combined with a negative Pap smear, HR HPV DNA testing has been shown to have a negative predictive value of disease progression to cervical cancer of greater than 98% (19, 20). However, other studies have also shown that the significant degree of cross-reactivity exhibited by the HC2 HR HPV probe pool does not have the same effect on the positive predictive value of HPV DNA testing using that assay since the types detected are not always HR HPV. Previous investigation has shown that the HC2 HR probe pool cross reacts with at least 15 types that its probes were not designed to detect, many of them low risk HPV types (13). The IHPV probes in the three probe pools used in this
study were designed to identify the presence of type-specific single nucleotide polymorphisms (SNPs) and by taking this approach were able to significantly decrease the cross reactivity with low risk HPV types and the potential for false-positive results being generated. This factor accounts for the better specificity, and PPV of the IHPV assay as compared to the HC2. Recent studies by Schutzbank et al. that compared the IHPV assay and the HC2 assay, also demonstrated a higher specificity of the IHPV assay than the HC2 assay, due to cross reactivity of the HC2 assay with HPV LR types (14). A high specificity and PPV is desired to reduce the number of unnecessary colposcopies that might be conducted based upon a false positive HR HPV result. One limitation of this study was that only those samples with discordant results were sequenced, thereby not identifying all samples within the total data set that may have been called HR HPV positive but contained only LR types. In addition, although 4 clones per sample were sequenced, a low level HR HPV type may have actually been present in the sample but not detected. Conversely, samples negative by both assays may have contained HR HPV. PCR and sequence analysis of all 821 samples was not feasible.

The manufacturer of the IHPV reagents has also improved the designs of several of the probes in the A5/A6 and A9 probe pools to further enhance the ability of laboratories to detect specific HPV types, and has removed the probes specific for the non-oncogenic HPV types 67 and 70. Studies by Wong et al. using this second generation IHPV assay demonstrated good concordance with the HC2 assay (86.6%) and an overall sensitivity and specificity of 96% (25).
addition, since both the IHPV testing and HC2 utilize signal amplification instead of target amplification, both tests allow for the detection of clinically significant levels of HPV DNA, avoiding issues associated with target amplification technologies that often detect low levels of HPV DNA that may lack clinical significance in the disease progression to cervical cancer.

Another feature incorporated into the IHPV assay was the use of ACTA1 probes as an internal control for the degree of cellularity in the ThinPrep samples tested. Since the ACTA1 gene is found in two copies per human cell, a positive ACTA1 signal assures that the sample contains sufficient DNA to allow for the detection of HR HPV DNA should it be present in the sample. The lack of such an internal control in the HC2 test results in the possibility that a negative HR HPV result may actually be due to insufficient cellularity of the sample instead of the actual lack of HR HPV DNA. Low cellularity was found in 26/41 (63.4%) of the indeterminate IHPV samples, which included 2 samples that contained HR HPV types 51 or 66. Without the internal control the 26 samples would have been reported out as potential false negative for HR HPV DNA. Low cellularity in a ThinPrep sample may also result in an insufficient volume of residual sample being available after cytology testing for HPV testing using HC2 since HC2 requires 4 mL of sample. The requirement of less than 2 mL of sample for the Invader based test minimizes the occurrence of samples with insufficient volume for HPV DNA testing.

Recent studies have suggested that the risk of progression towards cervical cancer can be as high for simultaneous infections with multiple HPV
types as it is for infection with just one of the highest risk HPV types such as type 16 (19). This study has demonstrated that the IHPV testing is capable of identifying mixed infections, with multiple HR HPV types in 47 of 821 samples (5.7%) identified in this sample population alone. The use of three separate probe pools, one each for phylogenetically related HR HPV types, allows the IHPV test to identify samples that contain such mixed HPV infections, something unable to be done using the HC2 test.

The results of this study demonstrated the improved specificity of the IHPV reagents, that the incorporation of an internal control was important to assure that negative results are due to the absence of HR HPV DNA instead of a lack of sample cellularity, and the assay’s ability to detect mixed HR HPV infection within a single sample. Due to the manual extraction process, the IHPV assay required approximately 1 hr more hands on time than did the HC2 assay. However, studies have shown that the extraction process can be adapted to automated platforms such as the Qiagen BioRobot M48 (Qiagen, Inc., Valencia, CA) and the bioMérieux NucliSENS easyMAG (bioMérieux, Durham, NC) (15, 25). Overall, these findings suggest that the IHPV method for the detection of HR HPV DNA is an easy to use and robust technology that may offer several advantages over current methods for the detection of HPV DNA.

ACKNOWLEDGEMENTS

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their technical support. This study was funded in part by the Jane and Dayton Brown and Dayton Brown Jr. Molecular Diagnostics Laboratory Research Fund. Invader HPV test reagents were kindly provided by Third Wave Technologies.

REFERENCES:


TABLE 1. User-defined criteria for Invader® data analysis using Excel.

<table>
<thead>
<tr>
<th>Description</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>Positive FFOZ* Ratio ≥</td>
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<tr>
<td>Negative FFOZ* Ratio &lt;</td>
<td>1.500</td>
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<tr>
<td>Valid Average RFOZ* ≥</td>
<td>1.30</td>
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<tr>
<td>Valid % CV (RFOZ*) &lt;</td>
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</tr>
<tr>
<td>HPV FFOZ* Flag (A5/A6 Pool) ≥</td>
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<tr>
<td>HPV FFOZ* Flag (A7 Pool) ≥</td>
<td>3.00</td>
</tr>
<tr>
<td>HPV FFOZ* Flag (A9 Pool) ≥</td>
<td>3.00</td>
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*FFOZ indicates FAM fold-over-zero, RFOZ indicates RED fold-over-zero.
Table 2: Summary of initial test results comparing HC2 and Invader HPV Assays

<table>
<thead>
<tr>
<th></th>
<th>Indeterminate</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
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<tr>
<td>HC2 Assay</td>
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<td></td>
</tr>
<tr>
<td>Indeterminate</td>
<td>2</td>
<td>15</td>
<td>3</td>
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</tr>
<tr>
<td>Negative</td>
<td>21</td>
<td>330</td>
<td>16</td>
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<tr>
<td>Positive</td>
<td>18</td>
<td>73</td>
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<td>434</td>
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<tr>
<td>Total</td>
<td>41</td>
<td>418</td>
<td>362</td>
<td>821</td>
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</table>
Table 3: Resolution by PCR/sequencing for discordant positive/negative samples

<table>
<thead>
<tr>
<th></th>
<th>True HR HPV Positive</th>
<th>False HR HPV Positive</th>
<th>HPV Negative Results</th>
</tr>
</thead>
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<tr>
<td></td>
<td>HR DNA positive</td>
<td>Type 66 Cross reactivity with HR probes</td>
<td>HPV DNA negative</td>
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<tr>
<td>Invader HPV Assay</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 (Types 16, 45, 56, 59, 66)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>HC2 Assay</td>
<td>15 (Types 16, 31, 35, 52, 53, 56, 58, 59, 68)</td>
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<td>13</td>
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Table 4: Resolution of indeterminate HC2 and IHPV results

<table>
<thead>
<tr>
<th>Results</th>
<th>PCR/sequencing Results&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>No HPV DNA detected</td>
</tr>
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<td>HC2 Ind&lt;sup&gt;e&lt;/sup&gt; Invader Neg&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4</td>
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<td>(Types 6, 41, 42, 81, 90)</td>
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<tr>
<td>HC2 Ind Invader Pos&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>(Types 16, 31)</td>
<td></td>
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<tr>
<td>HC2 Ind Invader Ind</td>
<td>0</td>
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<tr>
<td>(Type 67)</td>
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</tr>
<tr>
<td>HC2 Neg Invader Ind</td>
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<tr>
<td>(Types 6, 91)</td>
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<tr>
<td>HC2 Pos Invader Ind</td>
<td>0</td>
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<tr>
<td>(Types 53, 90) (Type 51) (Type 66)</td>
<td></td>
</tr>
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</table>

<sup>a</sup> Number of samples per category based on PCR and sequencing results

<sup>b</sup> LR = low risk

<sup>c</sup> HR = high risk

<sup>d</sup> HR HPV detected due to cross reactivity with probes in HC2 HR probe mix

<sup>e</sup> Ind = indeterminate

<sup>f</sup> Neg = negative

<sup>g</sup> Pos = positive
Table 5: Summary of results after discordant analysis

<table>
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<tr>
<th></th>
<th>Total Pos/Neg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>True Pos&lt;sup&gt;a&lt;/sup&gt;</th>
<th>False Pos&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>NPV&lt;sup&gt;f&lt;/sup&gt;</th>
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<td>IHPV (w/o 66)</td>
<td>365/392</td>
<td>350</td>
<td>9</td>
<td>368</td>
<td>15</td>
<td>95.89</td>
<td>97.61</td>
<td>97.49</td>
<td>96.08</td>
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<tr>
<td>IHPV With 66</td>
<td>380/377</td>
<td>350</td>
<td>9</td>
<td>338</td>
<td>30</td>
<td>92.11</td>
<td>97.41</td>
<td>97.49</td>
<td>91.85</td>
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<tr>
<td>HC2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>365/392</td>
<td>358</td>
<td>53</td>
<td>330</td>
<td>7</td>
<td>98.08</td>
<td>86.16</td>
<td>87.10</td>
<td>97.94</td>
</tr>
<tr>
<td>w/o 66</td>
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<tr>
<td>HC2&lt;sup&gt;h&lt;/sup&gt;</td>
<td>380/377</td>
<td>373</td>
<td>38</td>
<td>330</td>
<td>7</td>
<td>98.16</td>
<td>89.67</td>
<td>90.75</td>
<td>97.94</td>
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<tr>
<td>With 66</td>
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</tbody>
</table>

Rows 2 and 4: w/o = without including data for type 66. Data is presented based upon detection of HR HPV types in accordance with type specific probes present in each assay.

Rows 3 and 5: with type 66: Data is presented including detection of HR HPV type 66 due to HC2 probe cross reactivity, since samples were scored as positive with HC2 assay and confirmed by PCR/sequencing to contain type 66.

<sup>a</sup> Total true positive and true negative samples based upon concordant HC2/IHPV testing and discordant analysis.

<sup>b</sup> Samples were scored as false positive or false negative if not concordant between IHPV, HC2 and sequencing.
c. Sens = sensitivity

d. Spec = specificity

e. PPV = positive predictive value

f. NPV = negative predictive value

g. Results based upon correlation with HR HPV probe types present in HC2 assay

h. Results based upon the inclusion of the detection of type 66 due to HC2 probe cross reactivity