Human Papillomavirus in Atypical Squamous Cervical Cytology: The Invader HPV Test as a New Screening Assay

Running Title: The Invader HPV test for screening cervical cytology

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ABSTRACT

In surveillance for cervical neoplasia, cytologically atypical cells of undetermined significance (ASCUS) present a significant clinical issue, often dependent on high risk (HR) human papilloma virus (HPV) testing for triage of patients. HPV type 16 now appears as a critical concern in the follow-up of ASCUS patients.

The Invader HPV test (Inv2) by Third Wave Technologies, Inc. is a recently developed analyte specific reagents (ASRs) assay employing probe sets for detection of 14 HR HPV subtypes, and grouped as probe sets A5/A6 (HPV 51,56,66), A7 (HPV 18,39,45,59,68) and A9 (HPV 16,31,33,35,52,58). This report describes performance characteristics of the Inv2 test in screening ASCUS cervical cytology specimens with correlation to the results of the Hybrid Capture II HPV test (HC2) by Digene. The Linear Array HPV Genotyping Test (PCR-LA) (Roche Molecular Systems) was employed as a reference method for discordant results.

Ninety-four Pap smear samples with cytologic diagnosis of ASCUS, and 39 samples with a negative diagnosis were tested. The Inv2 HPV test demonstrated good (86.6%) concordance when compared to the HC2 HPV test with an overall sensitivity and specificity of 96%. Additionally, the Inv2 assay with high throughput, semi-automated DNA extraction offers subgrouping of HPV types by differential probe sets, and could provide a useful test for HPV screening with potential for improved risk stratification and selection of patients for further HPV subtyping.
INTRODUCTION

The human papillomavirus (HPV) is responsible for 95-100% of cervical cancers—worldwide the second most prevalent malignant neoplasm among women (15, 18, 25). Approximately 50 million females undergo the Papanicolaou (Pap) test, annually, with 7% of individuals diagnosed with a pathological abnormality (10, 11). Atypical squamous cells of undetermined significance (ASCUS) remains a common cytological diagnosis comprising 4% of clinical cases. The poor cytotologic reproducibility of this diagnosis remains a problem since 5-17% of ASCUS cases are subsequently diagnosed as cervical intra-epithelial neoplasia (CIN) 2 or 3 on cervical biopsy (4-6, 13, 16, 20-24, 27), a significant clinical management issue.

HPV typing and colposcopy are ancillary tests for patients with abnormal cervical cytology while cervical biopsy demonstrating CIN3 remains the only diagnostic test for cervical neoplasia. Colposcopy is invasive, expensive, and can miss one third of CIN3 lesions (8). Although cervical cancer is HPV induced, HPV types are not equally carcinogenic. Fifteen oncogenic or high risk (HR) strains are implicated in cervical carcinogenesis. Of these HPV types 16 and 18 (HPV 16/18) are etiologically associated with CIN3 and invasive cervical carcinomas ($\geq$ CIN3) in 60% and 10-20% of specimens, respectively (17). The ASCUS LSIL Triage Study (ALTS) Group reported that the 2-year cumulative absolute risk for $\geq$ CIN3 is 32.5% for HPV 16 positive ASCUS specimens (2), which raises a clinical urgency. Moreover, the persistence of HPV infection is most often associated with HPV 16/18 (9, 14, 24, 26), suggesting that identification of these two HPV types may play an important role in risk stratification of ASCUS patients for appropriate monitoring and possibly heightened follow-up.
Approximately 50% of ASCUS specimens demonstrate HR HPV infections (7). Algorithmically, clinical follow-up for ASCUS is considerably intensified by a positive screen for HR HPV. For most contemporary HPV testing, positive assays do not yet specifically identify HPV types 16/18, nor do they indicate the level of inherent patient risk for positive assays. As one approach to the recently recognized need for a level of specificity for HPV 16/18 in cervical cytology specimens (2, 12), we examined a new and yet unreported HPV screening assay, a second generation Invader HPV Assay (Inv2) (Third Wave Technologies, Inc., Madison, WI). Inv2 is a screening assay for HPV DNA, which subgroups HR HPV types according to three HR separate probe sets.

The Inv2 assay is available as analyte specific reagents (ASRs) and is a potentially semi-automated, high-throughput system for detection of 14 HPV HR types. According to the manufacturer of the Inv2 ASRs, Inv2 is similar to the first generation Invader HPV HR assay, with the addition of the HR HPV type 66 (Table 1). Inv2 HR probe sets include: A5/A6 (HPV types 51,56,66), A7 (HPV 18,39,45,59,68), and A9 (HPV 16,31,33,35,52,58). Probe positive A9 and A7 specimens are suggestive of the presence of HPV 16 and 18 respectively (Table 1). The Inv2 assay features an internal positive control through detection of the human histone-2, or H2be gene, ensuring quality DNA and informative results for negative assays.

We describe the performance characteristics of the Inv2 assay with particular attention to HR HPV screening and subgrouping of ASCUS cytology specimens. We compared the Inv2 assay to a commonly employed HPV HR screening test, the Hybrid Capture II HPV Test (HC2) (Digene Corporation, Gaithersburg, MD). The HC2 assay (HC2 Probe B) screens for thirteen HR HPV types associated with cervical carcinoma.
(HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). This evaluation of the Inv2 assay and HR grouping is undertaken in the context of an increasing clinical focus on HPV16/18 and the need for refining risk stratification for ASCUS cervical cytology results.

METHODS

Cytology and Hybrid Capture II HPV testing. Cervical cytology specimens were submitted in SurePath, an ethanol based transport medium, from December 2006-2007, which for this study included 39 negative cytology and 94 ASCUS cytology specimens. SurePath is a liquid medium employed for routine Pap smears, a fixative providing excellent preservation of squamous cells for accurate morphologic interpretations (Figure 1), and extraction of high quality DNA for concurrent HPV testing.

SurePath specimens were vortexed for 20 seconds at 3000 rpm, subsequently enriched by dispensing approximately 8mL of specimen over 4mL of PrepMate Density Reagent, and then centrifuged at 800 x g for 10 minutes. The supernatant was decanted and the pellet vortexed. Digene Hybrid Capture 2 Probe B (Digene, Gaithersburg, MD) was employed on the post-enrichment sample, with residual specimen stored at 4 °C for a period up to 3 months prior to Invader 2 and amplification assays.

Nuclisens easyMAG total nucleic acid extraction. Total nucleic acids were extracted from SurePath specimens using the NucliSens easyMAG platform, a second-generation system for automated isolation of nucleic acid from clinical samples based on
silica extraction technology (bioMérieux, Durham, NC). One to two mL of the SurePath sample was centrifuged at 1150 x g for 15 min. The supernatant was removed and the cell pellet was resuspended in 400 uL of Agencourt Genfind Lysis Buffer (Agencourt Bioscience Corporation, Beverly, MA). This mixture was incubated at 99˚C for 10 minutes and then cooled to ambient (room) temperature. Nine uL of Proteinase K (Agencourt Bioscience Corporation, Beverly, MA) was added to each tube containing the reaction mixture and incubated at 37 °C for 30 min on a thermomixer. Following incubation, DNA was extracted by Nuclisens easyMAG and eluted to a final volume of 110 uL.

Invader HPV typing. Extracted DNAs were tested for the presence of HR HPV DNAs in a laboratory-developed HPV assay employing the Invader ASRs, including HPV Oligos V2.0 (Third Wave Technologies, Inc., Madison, WI). Three Inv2 HPV reaction mixtures representing Invader HPV Oligos A5/A6, A7 and A9 (Probe sets) were prepared containing specific oligonucleotides, the enzyme cleavase, MgCl₂, and oligonucleotides specific for the human H2be gene.

Ten µL of each sample or control was added to 3 separate wells, representing the target for the 3 HPV probe sets, in a 96-well reaction plate. Twenty µL of Chill-Out Liquid Wax (Bio-Rad, Hercules, CA) was added to each well. After incubation at 95 °C for 5 minutes and cooling to 63 °C, 10 µL of each Inv2 reaction mix was added to appropriate specimens and controls. The reaction plate was incubated at 63 °C for 4 hours and then read in a Tecan GENios Reader (Tecan Systems, Inc., San Jose, CA) with the following settings: FAM, excitation 485nm/emission 535nm; Red, excitation...
560nm/emission 612nm. Data analysis and HR HPV detection were performed using a Microsoft Excel-based HPV 2.0 Invader Data Analysis Worksheet (Third Wave Technologies, Inc.).

Analytic criteria for Invader 2 results and cut-off values. All specimens’ fluorescent signals were expressed as FAM (HPV specific) and Red (H2be specific) signals. Red-Fold Over Zero (Red FOZ) and FAM-Fold Over Zero (FAM FOZ) were calculated for each of the three probe sets as follows:

\[
\text{FAM FOZ} = \frac{\text{FAM Signal of Sample}}{\text{FAM Signal of No DNA Target Solution}}
\]

\[
\text{Red FOZ} = \frac{\text{Red Signal of Sample}}{\text{Red Signal of No DNA Target Solution}}
\]

The FAM FOZ ratio was subsequently calculated using the FAM FOZ values from each of the three probe sets for a given sample (see below). When the lowest FAM FOZ value was < 1, the value was set to 1.0 for the calculation of the FAM FOZ ratio:

\[
\text{FAM FOZ ratio} = \frac{\text{Highest FAM FOZ}}{\text{Lowest FAM FOZ}}
\]

\[
\text{FAM FOZ ratio} = \frac{\text{Middle FAM FOZ}}{\text{Lowest FAM FOZ}}
\]

Specimens with a FAM FOZ ratio \( \geq 1.4 \) were considered positive for HR HPV. Where the FAM FOZ ratio was < 1.4, but the specimen demonstrated FAM FOZ > 1.5 in
all three HPV probe sets, the specimen was called multiple positive, or ‘Three Fluorescence Signal Presence’ (3-FS).

An average Red FOZ was calculated for each specimen and served as an indicator for genomic DNA levels. A negative HPV reaction obtained from specimens with average Red FOZ < 1.5 was considered insufficient for resulting.

Linear Array HPV Genotyping Test. The linear array HPV genotyping assay (PCR-LA) (Roche Molecular Systems, Inc., Branchburg, NJ) employs amplification of target DNA by PCR and nucleic acid hybridization, and has been designed to detect 37 cervical HPV DNA genotypes, including 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The PCR-LA is a research use test, only (Figure 1).

The HPV PCR-LA was performed using extracted DNAs according to the manufacturer’s instructions. Briefly, DNAs were amplified in a final reaction volume of 100 µl with working master mix containing Tris buffer, KCl, dATP, dCTP, dUTP, dGTP, dTTP, AmpliTaq Gold DNA polymerase, uracil-N-glycosylase, MgCl2, biotinylated HPV specific and β-globin primers. The mixture was incubated in a GeneAmp PCR System 9700 for 2 minutes at 50 °C and 9 minutes at 95 °C, followed by 40 cycles of 30s at 95 °C, 1 minute at 55 °C, and 1 minute at 72 °C, with a final extension at 72 °C for 5 minutes. Positive and negative controls were included in all runs to assess the performance of the assay. Following amplification, HPV and human β-globin biotinylated amplicons were denatured by adding 100 µl of denaturation solution to each PCR tube. The denatured amplicons were hybridized to an immobilized probe array strip. Detection and HPV genotyping were achieved following the protocol provided by the manufacturer.
Hybridization strips were manually interpreted using the Linear Array HPV reference guide.

RESULTS

Inv2 semi-automated extraction of SurePath cytology specimens. High quality DNA was successfully extracted by semi-automated techniques from all 133 specimens as demonstrated by a Red FOZ > 1.5 (see Methods).

For determination of reproducibility of the Inv2 assay, the coefficients of variation (cv’s) were established through repeat testing for high to low viral ‘loads’ in dilution assays, through replicate testing in separate runs, on different days. CaSki and Hela cell DNA, source materials for HPV 16 and HPV 18, respectively, were employed for known viral ‘copy’ numbers (Advanced Biotechnology, Inc., Columbia, Maryland). Reactions contained HPV 16 (1.2x10^3 - 10x10^3 copies in 4 dilutions) or HPV 18 (5x10^3 - 10x10^3 copies in two dilutions). For HPV types 16 and 18, cv’s for FAM FOZ ratios ranged from 6.63 - 2.53% and 9.33 - 4.72%, respectively (range from low to high viral loads). FAM FOZ ratios were reproduced with good precision.

All specimens were screened for the presence of HPV by Inv2 and HC2 and the results considered within the context of ASCUS or Negative cytology studies (Tables 2-3).

ASCUS cytology specimens and HPV typing. A total of 94 extracted specimens previously identified cytologically as ASCUS were screened for HPV by Inv2 and HC2 and stratified into four groups (Table 2A): 40 Inv2 negative/HC2 negative; 39 Inv2
positive/HC2 positive; 7 Inv2 negative/HC2 positive; and 5 Inv2 positive/HC2 negative.

Three Inv2 samples demonstrated multiple fluorescence signals (3FS) by parameters outlined in the Methods section. These specimens were all re-tested, with the same results (3FS). Of these specimens, two were negative and one was positive for HPV 59, by PCR-LA. Accordingly, 3FS results were considered diagnostically uninformative, or indeterminate (Table 2A). Otherwise, samples positive by both HPV typing methodologies (Inv2 and HC2) variously included 7 samples positive for Inv2 probe set A5/6, 7 samples for probe set A7, 18 samples for probe set A9, 2 samples mixed positive for A5/6 and A7, 2 samples for A5/6 and A9, and 3 samples for A7 and A9. Five samples of Inv2 positive/HC2 negative were HPV subtyped into 2 specimens of A5/6, 1 specimen of A7, and 2 specimen mixed positive for A5/6 and A9. Inv2 probe set A9 and A7 were positive in 38.5% (35/91) of ASCUS specimens, representative of the possible presence of HPV subtypes 16 and/or 18.

There were 12 discordant ASCUS specimens comprised of 7 Inv2 negative/HC2 positive specimens and 5 Inv2 positive/HC2 negative specimens, all of which underwent PCR-LA testing (Table 3A-B). Of the 7 Inv2 negative/HC2 positive samples, 2 specimens of high risk HPV were detected, which included a sample with HPV types 35, 56 and a second sample typed as HPV 16, 39. The 5 specimens of Inv2 positive/HC2 negative showed three samples with high risk HPV, including a specimen with both HPV 16 and 51, a specimen with HPV 39, and a specimen with HPV 66. Of all the discordant specimens, PCR-LA detected HPV 16 in 2 of these 12 samples: 1 specimen of Inv2 positive/HC2 negative and 1 specimen of Inv2 negative/HC2 positive, with Inv2 and HC2 each failing to detect a sample with HPV type 16 by PCR-LA. For ASCUS cytology
the concordance between Inv2 and HC2 testing was 86.8% (79/91).

Negative cytology specimens and HPV typing. 39 negative cytology specimens underwent HPV testing (Table 2B). Specimens were grouped into four categories: Inv2 positive/HC2 positive (1 specimen); Inv2 positive/HC2 negative (4 specimens); Inv2 negative/HC2 positive (1 specimen); Inv2 negative/HC2 negative (30 specimens). Three specimens were identified (including repeat assays) as 3FS, were considered indeterminate and excluded from analysis. A total of 5 specimens demonstrated HPV by Inv2 with one specimen positive for probe set A5/6, one for A7, and three specimens for A9. Inv2 detected the potential presence of HPV type 16 and/or 18 (probe sets A9 and A7) in 11.0% (4/36) of negative cytology specimens.

For the negative cytology specimens, five discordant specimens were identified in 36 informative specimens (3 of 39 specimens were 3FS, and indeterminate). These included 4 specimens positive by Inv2 and 1 specimen negative by Inv2 but positive by HC2 for HPV (Tables 3A-B). PCR-LA was performed on 3 of the 5 discordant cytology negative specimens, with detection of HR HPV type 56 in a single specimen which was Inv2 positive (Table 3B). The concordance rate between Inv2 and HC2 in negative cytology specimens was 86.1% (31/36).

Overall the Inv2 demonstrated a concordance of 86.6% when compared to HC2. With PCR-LA as reference for discordant specimens, Inv2 had an overall sensitivity of 95.6% and specificity of 96.1%, a positive predictive value of 93.6% and a negative predictive value of 97.4%.
All of the ASCUS and negative cytology specimens were re-evaluated in the context of the Inv2 results. No cyto-morphologic differences were seen among specimens, whether positive, negative, or indeterminate for HPV (Figures 2, 3).

**DISCUSSION**

The use of the Papanicolaou test in the prevention of invasive cervical epithelial disease represents a medical triumph (7). There is now an increasingly important role for the clinical characterization of the etiologic agents in this disease, HR HPV. In a recent study Castle et al (2) were able to stratify absolute risk for progression to ≥CIN3 in women with ASCUS cytology, based upon the type of HR HPV detected at baseline screening. Women with ASCUS cytology positive for HPV 16 had the highest risk for progression, fivefold greater than the collective risk attributable to other HR HPV type infections. In the future, tests for HPV must anticipate such priorities—the clinical implications of HPV 16/18—augmenting screening methods commensurate with the urgency of these two HR HPV subtypes.

This study describes the performance characteristics of a new HPV test, a second generation Invader HPV assay (Inv2). Clinically, the Inv2 test detects 14 oncogenic HPV types and segregates the viruses into three groups according to separate probe sets, thus allowing selection of women for further subtyping as necessary. In this study, cervical samples were submitted via SurePath ethanol-based transport medium, with DNA extractions for Inv2 testing obtained by semi-automated methods. SurePath has been increasingly popular as a liquid fixative for gynecologic cytology; moreover, HPV
screening from liquid fixative is rapidly growing as an important modality in cancer prevention. For the Inv2 assay, \textit{H2be} serves as an internal control ensuring the presence of informative specimen extracted DNA. Inv2 extractions in this study were ‘clean’, that is of low extraneous background in the semi-automated extraction of quality specimen DNA.

In examining the performance of Inv2 for ASCUS cytology specimens, the results of Inv2 were compared to those obtained by the HC2 assay. Discordances between the two tests were examined by a third method, PCR-LA. For ASCUS specimens, Inv2 demonstrated good correlation (86.8\%) with HC2 screening. The Inv2 test demonstrated 48.4\% (44/91) of ASCUS specimens as positive for HR HPV with 38.4\% (35/91) representing potential HPV types 16/18. This HR incidence correlates with previously published reports of 45.5 - 59\% of females with HR HPV in ASCUS cytology (7).

Otherwise, twelve ASCUS specimens were discordant for HPV typing by Inv2 and HC2. With the PCR-LA test as reference, for ASCUS cytology specimens, false positives for HR HPV were Inv2 4.6\% (2/44) and HC2 10.9 \% (5/46). False negatives were Inv2 4.3\% (2/47) and HC2 6.7\% (3/45). Inv2 was falsely negative for HR HPV types 16, 35, 39, and 56 in 2 specimens, HC2 for HPV types 16, 39, 51, and 66 in 3 specimens. There were two ASCUS cases that screened negative for HPV, one each for Inv2 and HC2, notable since each was positive for HPV 16 by PCR-LA.

HPV typing of 39 negative cytology specimens demonstrated HR HPV by Inv2 in 13.9\% (5/36) of specimens, 4 (11.1\%) of which involved potential HPV16 and/or 18 (A9 or A7 probe sets). The findings correlate with the published 17\% of patients with HR HPV in negative cytology (7). Negative cytology specimens screened as discordant for
HPV by Inv2 and HC2 were also examined by PCR-LA. Of the three specimens tested, PCR-LA detected HR HPV (type 56) in a single specimen that was Inv2 positive.

Overall the Inv2 demonstrated a concordance of 86.6% when compared to HC2. With PCR-LA as reference for discordant specimens, Inv2 had an overall sensitivity and specificity of 96%, a positive predictive value of 94% and a negative predictive value of 97%.

There were 6 specimens (3 ASCUS and 3 negative cytology specimens) demonstrating 3FS (three fluorescence signal) present, classified as indeterminate in this study. HC2 was negative in all of these. All specimens had a FAM FOZ $\leq$ 4.0. PCR-LA performed on the 3 ASCUS 3FS specimens demonstrated HPV 59 in one. The significance of 3FS results is not clear at the present time. Additional specimens with 3FS results will need to be studied. It is possible that the cutoff values for the Inv2 test will need to be further optimized in the future. Alternatively, for the SurePath fixative, specimen DNA concentration and/or duration of fixation may require standardization for 3FS results. Importantly, no differences were seen in cytologic morphology among specimens, positive, negative or indeterminate for HPV.

Specificity for HPV subtypes is now a major concern, namely that patients may be subjected to unnecessary stress and procedures secondary to false positives for high risk types. The HC2 HPV screening assay detects at least 15 HPV genotypes not included in its high risk probe set (19). It may be argued that it is important to detect HPV with sensitivity, irrespective of specificity; however, this may not attend caveats of risk and economic cost associated with false positive testing. Specificity for HR HPV and the associated clinical implication—the need for close follow-up including colposcopy—are...
relevant issues which suggest high sensitivity and specificity are paramount to clinical acumen. The Inv2 and HC2 tests appear to demonstrate similar sensitivity, although the Inv2 test may offer an improvement in specificity (19). From the present results, Inv2 testing offers reliable grouping of HR types, potentially of advantage in triaging of patients for follow-up of possible HPV 16/18 infections. We have semi-automated the extraction phase for Inv2 testing for high throughput. Otherwise, the Linear Array HPV Genotyping Test includes specific recognition of individual types within both low and high risk groups, although the test remains unavailable for clinical use at this time. In its present format, PCR-LA would be problematic in application of the test (strip hybridization) for high throughput.

In characterizing Inv2, the assay appears simple to perform, is amenable to use with SurePath fixative, may be semi-automated for DNA extraction, and shows good sensitivity and specificity for ASCUS cytology cases. For routine HPV surveillance, the Inv2 test appears as potentially useful as a first line HPV screening test. Inv2 demonstrates few false positives without compromising the sensitivity for critical HPV types, including HPV type 16/18. Patients with Inv2 test positivity for A9 and/or A7 probe sets may warrant further close follow-up and/or testing for possible HPV 16/18.

In the future, new modalities for HPV testing should address HPV types 16/18. However, based on even limited association with invasive cervical disease (28), because of the obvious clinical stakes it will remain prudent to continue to screen for multiple HR HPV types, such as for ASCUS cytology cases, as well as for additional triage of HPV types 16/18.
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TABLE 1. HPV types detected by Invader 2 (Inv2) and Hybrid Capture 2 (HC2) Probes

<table>
<thead>
<tr>
<th>Inv2 HPV Oligos V2.0</th>
<th>HC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5/6 A7 A9</td>
<td>Probe A</td>
</tr>
<tr>
<td>51 18 16</td>
<td>6</td>
</tr>
<tr>
<td>56 39 31</td>
<td>11</td>
</tr>
<tr>
<td>66 45 33</td>
<td>42</td>
</tr>
<tr>
<td>59 35</td>
<td>43</td>
</tr>
<tr>
<td>68 52 58</td>
<td>44 35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Probe B</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>45</td>
</tr>
<tr>
<td>52</td>
<td>51</td>
</tr>
<tr>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>58</td>
<td>56</td>
</tr>
<tr>
<td>59</td>
<td>58</td>
</tr>
<tr>
<td>68</td>
<td>59</td>
</tr>
</tbody>
</table>
**TABLE 2A.** Correlation of HPV Results obtained by the Invader 2 (Inv2) and Hybrid Capture 2 (HC2) high risk probe assays on specimens diagnosed cytologically as ASCUS

<table>
<thead>
<tr>
<th>HC2 (n)</th>
<th>Inv 2 results</th>
<th></th>
<th></th>
<th></th>
<th>Indeterminate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A5/6</td>
<td>A7</td>
<td>A9</td>
<td>Mixed</td>
</tr>
<tr>
<td>Negative (48)</td>
<td>40</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2$^b$</td>
</tr>
<tr>
<td>Positive (46)</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>18</td>
<td>7$^c$</td>
</tr>
<tr>
<td>Total (94)</td>
<td>47</td>
<td>9</td>
<td>8</td>
<td>18</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$Indeterminate: Specimens showing the presence of 3 fluorescence signals (3FS) and diagnostically indeterminate

$^b$ Mixed positive probes: 2 specimens positive for probe A5/6 and A9

$^c$ Mixed positive probes: 2 specimens positive for probes A5/6 and A7; 3 positive for probes A7 and A9; and 2 positive for A5/6 and A9 (total of 7 specimens)
TABLE 2B. Correlation of HPV Results obtained by the Invader 2 (Inv2) and Hybrid Capture 2 (HC2) HR-HPV probe assays on specimens diagnosed cytologically as Negative

<table>
<thead>
<tr>
<th>HC2 results</th>
<th>Inv 2 results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>A5/6</td>
</tr>
<tr>
<td>Negative</td>
<td>(n)</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
</tr>
<tr>
<td>Positive</td>
<td>(2)</td>
</tr>
<tr>
<td>Total</td>
<td>(39)</td>
</tr>
</tbody>
</table>

^a Specimens showing the presence of 3 fluorescence signals (3FS) and diagnostically indeterminate (not included in the analysis)

^b Specimens positive for 2 probe sets
### TABLE 3A. Summary of discordant specimens

<table>
<thead>
<tr>
<th>Cytologic Diagnosis</th>
<th>HPV results&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total Specimens</th>
<th>No. Positive for HR-HPV by PCR-LA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inv2</td>
<td>HC2</td>
<td></td>
</tr>
<tr>
<td>ASCUS</td>
<td>Pos</td>
<td>Neg</td>
<td>5</td>
</tr>
<tr>
<td>ASCUS</td>
<td>Neg</td>
<td>Pos</td>
<td>7</td>
</tr>
<tr>
<td>Negative</td>
<td>Pos</td>
<td>Neg</td>
<td>4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative</td>
<td>Neg</td>
<td>Pos</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inv2, Invader 2; HC2, Hybrid Capture 2 Probe B; Pos, positive for HR-HPV; Neg, negative for HR-HPV

<sup>b</sup> PCR-LA, Linear Array HPV Genotyping Test

<sup>c</sup> Only two of four samples could be tested by PCR-LA

<sup>d</sup> nd, not done
### TABLE 3B. Roche PCR Linear Array (PCR-LA) and HPV types in discordant specimens (HC2 and Inv2)

<table>
<thead>
<tr>
<th>SID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cytology Result</th>
<th>HC2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inv2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PCR-LA Types&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Sp61</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>54,61,GTIS39</td>
</tr>
<tr>
<td>2 Sp67</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>61</td>
</tr>
<tr>
<td>3 Sp84</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>33,35,52,56,58</td>
</tr>
<tr>
<td>4 Sp108</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>82</td>
</tr>
<tr>
<td>5 Sp124</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>53,CP6108</td>
</tr>
<tr>
<td>6 Sp133&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>16,39</td>
</tr>
<tr>
<td>7 Sp134</td>
<td>ASCUS</td>
<td>+</td>
<td>-</td>
<td>53,82</td>
</tr>
<tr>
<td>8 Sp78</td>
<td>ASCUS</td>
<td>-</td>
<td>+</td>
<td>84</td>
</tr>
<tr>
<td>9 Sp79&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ASCUS</td>
<td>-</td>
<td>+</td>
<td>16,51,64,67,84</td>
</tr>
<tr>
<td>10 Sp81</td>
<td>ASCUS</td>
<td>-</td>
<td>+</td>
<td>39</td>
</tr>
<tr>
<td>11 Sp122</td>
<td>ASCUS</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12 Sp142</td>
<td>ASCUS</td>
<td>-</td>
<td>+</td>
<td>66,84</td>
</tr>
<tr>
<td>13 N56</td>
<td>Negative</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14 N167</td>
<td>Negative</td>
<td>-</td>
<td>+</td>
<td>53,56,CP6108</td>
</tr>
<tr>
<td>15 N178</td>
<td>Negative</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>SID - specimen identification numbers of 12 discordant ASCUS, , and 3 discordant cytologically negative specimens

<sup>b</sup>HC2B, Hybrid Capture 2 Probe B; +, positive for HR-HPV; - , negative for HR-HPV

<sup>c</sup>Inv2, Invader 2; +, positive for high risk HPV; - , negative for HR-HPV

<sup>d</sup>Linear Array Genotyping Test, PCR-LA with HR-HPV types in **bold**

<sup>e</sup>Specimen with HPV 16 not detected by Inv2

<sup>f</sup>Specimen with HPV 16 not detected by HC2
**FIGURE LEGENDS**

**Figure 1.** Examples of the Linear Array HPV Genotyping Test (PCR-LA) Results. The HPV Genotyping result of each sample was determined by comparing the band pattern to the PCR-LA Reference Guide: (Lane 1) The PCR-LA HPV negative control with no visible bands; (Lane 2) The PCR-LA HPV positive control with HPV 16 band; (Lane 3) Hela cell DNA positive for HPV 18; (Lane 4) Specimen SP133 positive for HPV 16 and 39; (Lane 5) Specimen N167 positive for HPV 53, 56 and CP6108; (Lane 6) Specimen SP79 positive for HPV 16, 51, 64, 67 and 84. In addition, except for the negative control, all specimens were positive for $\beta$-Globin high and $\beta$-Globin low (see methods).

**Figure 2.** Examples of specimens cytologically diagnosed as ASCUS. (a) Negative for HPV by both Inv2 and HC2; (b) Positive for HPV by both Inv2 and HC2; (c) Negative for HPV by Inv2 but positive by HC2; (d) Positive for HPV by Inv2 but negative by HC2; (e) Indeterminate for analysis as sample demonstrated multiple fluorescence signals (3FS).

**Figure 3.** Examples of specimens cytologically diagnosed as negative. (a) Positive for HPV by both Inv2 and HC2; (b) Negative for HPV by both Inv2 and HC2.
Figure: 1