DETECTION OF HIGH RISK PAPILLOMAVIRUS DNA WITH COMMERCIAL INVADER® TECHNOLOGY-BASED ANALYTE SPECIFIC REAGENTS FOLLOWING AUTOMATED EXTRACTION OF DNA FROM CERVICAL BRUSHINGS IN THINPREP™ MEDIA

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Abstract

We compared the performance of the Third Wave Technology Invader method, and the Digene Hybrid Capture 2 assay to detect high risk HumanPapilloma Viruses in 87 cervical brushing specimens submitted in Cytyc ThinPrep media. Two different methods for extraction of DNA from squamous epithelial cells were also evaluated.
The Human Papilloma virus (HPV) family consists of more than 100 types. Those shown to be causative agents of cervical cancer are referred to as “High Risk” strains. Current guidelines recommend testing all women with a cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS) for infection by high-risk types of HPV (17, 24). HPV testing has been found to have higher sensitivity than standard or liquid-based PAP testing for detecting high-grade cervical intraepithelial neoplasia (1, 18-21). Only the Digene Hybrid Capture-2 assay (HC2, DIGENE, Gaithersburg, MD) has been approved by the FDA for the detection of high-risk strains of HPV as an ASCUS triage for referral to colposcopy, and in conjunction with cervical cytology as a primary screen for cervical cancer. The purpose of this study was to compare a laboratory-developed test, utilizing analyte specific reagents (ASR’s) developed by Third Wave Technologies Inc. (Madison Wisconsin) (TWT), with the Digene HC2 assay, for the detection of high risk HPV DNA in cervical brushings. The TWT method used in this study, based on proprietary Invader technology, employs isothermal signal amplification to detect 13 HR HPV types utilizing 3 probe pools (A5/A6 [types 51 and 56], A7 [types 18, 39, 45, 59, and 68] and A9 pool [types 16, 31, 33, 35, 52, and 58]), based on phylogenetic relatedness. The assay also incorporated an internal control for human alpha-actin to control for DNA quality and quantity in each reaction.

The 87 specimens used in this study were cervical brushings collected in ThinPrep fluid (Cytyc Corp., Marlborough, MA) submitted to the Cytology laboratory at CompuNet Clinical Laboratories for routine PAP testing. Those samples that received a cytological diagnosis of ASCUS were tested for high-risk HPV using the Digene HC2 High–Risk
HPV test according to the manufacturer’s instructions. Cells were collected from 2 mL of ThinPrep fluid by centrifugation at 14,000 x g, for 10 minutes, and suspended in 0.4 mL of phosphate buffered saline (PBS). DNA was extracted using the Qiagen M48 Biorobot using the MagAttract Virus DNA Minikit (Qiagen Inc. Valencia CA), and eluted into 100 µL of RNase-free distilled water. Duplicate samples were prepared from 2 mL of each cytology sample using Gentra Systems PureGene spin columns (Qiagen Inc. Valencia CA), following the manufacturer’s instructions. All extracted samples were stored at –20°C until tested. The extracted DNA’s were tested for the presence of high risk HPV by a laboratory-developed assay utilizing Invader HPV ASR’s. The TWT ASR reagents consisted of 3 separate oligonucleotide pools with FAM labeled probes, one RED-labeled oligonucleotide set for the internal control, Cleavase X enzyme, and MgCl₂ solution. Each of 3, 10 µL aliquots from each sample was added to separate wells of a 96-well reaction plate, and layered with 20 µL of mineral oil. The plates were incubated at 95°C for 5 minutes in a MJ Research PTC-100 Peltier Thermal Cycler (MJ Research, Waltham MA), and cooled to 63°C. 10 µL of master mix containing one of the three oligonucleotide sets, plus the internal control oligo pool, were added to each of the triplicate aliquots. Each master mix consisted of 4 µL of MgCl₂, 4 µL of one FAM oligo mix, 1 µL of RED oligo mix, and 1 µL of Cleavase X enzyme, per reaction. The plates were incubated at 63°C for an additional 4 hours, and then cooled to ambient temperature for 5 minutes. Readings were taken immediately after cooling using a Tecan GENios microplate fluorometer (Tecan Inc. Durham, NC). Excitation/Emission settings were 485nm/535nm and 560nm/612nm for FAM and RED respectively. Data collected from
the fluorometer was imported into a generic Microsoft Excel spreadsheet programmed with user defined cut-offs to determine the positivity/negativity of the reactions.

For the manually extracted samples, 50 tested negative, and the remaining 37 were positive for HPV DNA. Results using the automated extractor were nearly identical, with 49 and 38 samples testing negative and positive respectively for HPV DNA. The one discordant positive specimen was determined to be HPV type 51 by PCR amplification and sequencing analysis. The Invader internal controls for all DNA extracts, irrespective of extraction methodologies, were positive, indicating that sufficient DNA was extracted from all of the specimens tested. It is not clear as to why the one sample that tested positive with the M48 extracted DNA gave a negative HPV result when extracted with the manual PureGene method. The major advantage of the M48 Biorobot was that it totally automated the extraction process, freeing laboratory personnel to perform other tasks. One technician could comfortably extract 12 ThinPrep specimens per hour using the PureGene method. Total hands on time using the M48 Biorobot was also approximately 1 hour, including instrument setup and the centrifugation steps, however the Qiagen instrument has the capability of extracting 48 specimens per run.

A comparison of the results obtained by Digene HC2 testing versus TWT Invader is shown in Table 1. The agreement between the Digene HC2 and TWT Invader tests was 81.6%. Of the 16 discordant samples, 15 were positive by Digene HC2 and negative by the TWT Invader method, while one was negative by the former and positive by the latter. Therefore, prior to resolution of the discordant results, the sensitivity and
specificity of the TWT Invader method compared to Digene were 77.6% and 97.22% respectively. Of the 16 discordant specimens, 12 were analyzed by PCR using 2 different sets of HPV consensus primers (9, 26). The remaining 4 discordant specimens did not have sufficient ThinPrep material for further testing. Amplified DNA resulting from each positive PCR reaction was cloned into the pGEM-T Easy vector. Two clones from each PCR reaction were sequenced, for a total of 4 sequencing results being obtained for each discordant sample (two for each of the 2 PCR primer sets). Results of the PCR and sequencing analysis are shown in Table 2. In each case with negative TWT Invader/positive Digene HC2 results, there was either no HPV DNA detected by PCR, (4/11) or the HPV sequence was determined to belong to a low-risk genotype (7/11). Sequence analysis of the sole TWT Invader positive/Digene HC2 negative sample was determined to be a high-risk, type 51. As stated above, the Invader internal control demonstrated that sufficient DNA had been extracted for this method. There is no internal control in the Digene assay; therefore it is not possible to determine if results called as negative by the Digene assay resulted from insufficient DNA after the specimen processing procedure used by this method. The Digene method detected one type 53 HPV; the TWT assay is not designed to detect this genotype. HPV type 53 was classified by the International Agency for Research on Cancer (IARC) as a probable high-risk type, but IARC has not recommended its inclusion into high-risk HPV diagnostics (3, 12).

In their review of the clinical relevance of HPV testing, Snijders et al discuss the relationship between analytical and clinical sensitivity and specificity (18). Analytical
sensitivity refers to the number of HPV infected women who are positive for HPV by a
given method, while analytical specificity is the number of HPV negative women
correctly identified by that test. On the other hand, clinical sensitivity refers to the
proportion of women who are determined to be infected with HPV that also demonstrate
cytological findings of high-grade cervical intraepithelial neoplasia (lesions ≥ CIN 3).
The findings of several studies have clearly demonstrated that HPV testing is more
sensitive for detecting high grade CIN than pap smears alone (2, 5, 6-8, 11,13-15,23).
Based on these studies, it has been proposed that HPV testing be performed in addition to
PAP testing on all women over the age of 30, (8). Colposcopic examination is the
standard follow-up for women with abnormal cytological findings, to include ASCUS.
Based on the results from the Digene HC2 testing, colposcopy would have been
performed inappropriately on 11 women. Similar results were obtained in a recent study
comparing the Amplicor HPV PCR assay (Roche Molecular Systems, Branchburg, NJ) to
the Digene HC2 method (10). The PCR assay also demonstrated considerable cross
reactivity with low risk types of HPV since the consensus primers were designed to
amplify all HPV types, not just high-risk (22). With any screening assay, specificity is
typically sacrificed to ensure a very high degree of sensitivity. However, with most
screening assays, (e.g., HIV and HCV EIA testing), positive results are confirmed with a
secondary method with high specificity. Unfortunately no such confirmatory assays are
currently available for HPV testing. Therefore, if HPV DNA testing is to be performed
as an adjunct to PAP testing, the analytical specificity of the HPV test becomes of critical
importance to ensure that women are not subjected to a needless invasive procedure. Our
results clearly demonstrate the TWT Invader HPV ASR methodology is suitable for use
as a companion to PAP testing. It should be pointed out that this study is based on a relatively small number of samples. However the disparity between the performance of the Invader and Digene HPV assays, especially regarding the cross reactivity of the latter with low risk HPV types is very apparent. It is expected that these results will be confirmed using a larger data set; such studies are now in progress. In the limited number of patients tested utilizing the Invader assay, there were no false positive results, and possibly only one false negative (if HPV type 53 is to be placed in the high risk category).
References:


Table 1. Comparison of TWT versus Digene HPV testing results

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Table 2. Resolution of TWT and HPV Discordant Results

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