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Development and Evaluation of a PCR and Mass Spectroscopy-based (PCR-MS) Method for Quantitative, Type-specific Detection of Human Papillomavirus

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Abstract

Knowledge of the central role of high-risk human papillomavirus (HPV) in cervical carcinogenesis, coupled with an emerging need to monitor the efficacy of newly introduced HPV vaccines, warrant development and evaluation of type-specific, quantitative HPV detection methods. In the present study, a prototype PCR and mass spectroscopy (PCR-MS)-based method to detect and quantitate 13 high-risk HPV types is compared to the Hybrid Capture 2 High Risk HPV DNA test (HC2; Digene Corp., Gaithersburg, MD) in 199 cervical scraping samples and to DNA sequencing in 77 cervical tumor samples. High-risk HPV types were detected in 76/77 (98.7%) cervical tumor samples by PCR-MS. Degenerate and type-specific sequencing confirmed the types detected by PCR-MS. In 199 cervical scraping samples, all 13 HPV types were detected by PCR-MS. Eighteen (14.5%) of 124 cervical scraping samples that were positive for high-risk HPV by HC2 were negative by PCR-MS. In all these cases, degenerate DNA sequencing failed to detect any of the 13 high-risk HPV types. Nearly half (46.7%) of the 75 cervical scraping samples

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¹Deceased

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that were negative for high-risk HPV by the HC2 assay were positive by PCR-MS. Type-specific sequencing in a subset of these samples confirmed the HPV type detected by PCR-MS. Quantitative PCR-MS results demonstrated that 11/75 (14.7%) samples contained as much HPV copies/cell as HC2-positive samples. These findings suggest that this prototype PCR-MS assay performs at least as well as HC2 for HPV detection, while offering the additional, unique advantages of type-specific identification and quantitation. Further validation work is underway to define clinically meaningful HPV detection thresholds and to evaluate the potential clinical application of future generations of the PCR-MS assay.

Keywords

Human papillomavirus; cervical tumor; cervical scrapings; quantitative polymerase chain reaction; mass spectroscopy

1. Introduction

Cervical cancer is the second most common cancer among women worldwide, and ranks first in many developing countries. Worldwide, approximately 493,000 new cases of cervical cancer and 274,000 associated deaths occurred in 2002 (Parkin et al., 2005). Persistent infection with 13-15 high-risk types of human papillomavirus (HPV) has been established as a necessary, but insufficient, factor in the development of high-grade cervical dysplasia and cervical carcinoma (Bosch et al., 1995; Munoz et al., 2003; Munoz et al., 2004). HPV DNA has been detected in up to 99.7% of all cervical cancers, and infection with one of four HPV types (16, 18, 31 or 45) is present in nearly 75% of all cervical cancers diagnosed annually in the U.S. (Goldie et al., 2004). Increased risk of viral persistence and development of high-grade dysplasia and/or invasive cancer has been associated with certain HPV types (Bosch et al., 1995; Castle et al., 2005) and high viral load (Cheung et al., 2009; Josefsson et al., 2000; Saunier et al., 2008; Schlecht et al., 2003; Swan et al., 1999; Ylitalo et al., 2000). Recent prospective studies have found elevated risk of cervical disease development in women with HPV 16 (Castle et al., 2005; Khan et al., 2005) and HPV 18 (Khan et al., 2005).

The causal role of HPV in cervical carcinogenesis has established an important role for high-risk HPV testing in cervical cancer screening. Current patient management guidelines recommend testing for the presence of high-risk types of HPV for the triage of women with equivocal or ambiguous cytology results and for use as an adjunct to the Papanicolaou (Pap) test in women over age 30 (Obiso and Lorincz, 2004). The Hybrid Capture 2 High Risk HPV DNA test (HC2; Digene Corp., Gaithersburg, MD) is currently the most widely used HPV test approved for clinical use by the U.S. Food and Drug Administration. The HC2 test is affordable and widely used in clinical practice; however, it does not identify specific HPV types or quantitate the amount of HPV present. In light of our evolving understanding of the role of HPV type and quantity in cervical carcinogenesis, recently heightened by a need to monitor the efficacy of prophylactic HPV vaccines, quantitative, type-specific HPV data has the potential to inform research and clinical practice. This study extends previous work using a PCR and mass spectroscopy (PCR-MS)-based method for detecting HPV 16 and 18 (Yang et al., 2005) to describe a probe set that allows simultaneous analysis of 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) by multiplex. In the present study, PCR-MS is evaluated as an alternative method for detecting high-risk HPV DNA using cervical tumor-derived cell lines, routine cervical cancer screening samples, and cervical tumors.

2. Materials and Methods

2.1 Human subjects

A waiver was obtained from the Institutional Review Board of the University of Michigan Medical School approving this research on residual specimens unlinked to personal identifiers.

2.2 Study samples

Cervical tumor-derived cell lines CaSki, SiHa, and HeLa were purchased from American Type Culture Collection (ATCC, Manassas, VA). De-identified DNA from 77 frozen cervical tumors was provided by K. Cho (University of Michigan). All tumors represented a mixture of invasive squamous cell carcinomas, adenocarcinomas, and adenosquamous cell carcinomas. One hundred ninety-nine cervical scraping samples collected in ThinPrep vials (Cytoc, Marlborough, MA) for routine cervical cancer screening and HPV testing using the HC2 test were provided by D. Newton. After completion of HC2 testing, residual samples were de-identified for analysis using the PCR-MS assay.

2.3 DNA isolation and quantitation

Cell lines were cultured as recommended by ATCC. Tumors were microdissected manually prior to DNA isolation. Cell line and tumor DNAs were isolated by SDS/proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Cervical scraping DNAs were isolated from 200 μ l of ThinPrep solution using spin columns (QIAmp DNA Mini kit, Qiagen, Inc., Valencia, CA) or 3.5 ml of ThinPrep solution using silicon beads (ZR Serum DNA kit, Zymo Research Corp., Orange, CA) per manufacturers' instructions. DNA concentrations were calculated by real-time fluorescent PCR against a standard curve of human genomic DNA (5 pg/ μ l – 20 ng/ μ l) using the β -globin primers forward 5'-TGAGTCCAAGCTAGGCCCTTT-3', reverse 5'-ACCAGCCACCACTTTCTGAT-3', and probe 5'-FAM-CTTATCTTCTCCACAGCTCCTGGGCAACGTGC-Black Hole Quencher_1-3'. Reactions were performed in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5.5 mM MgCl₂ with 200 μ M each dNTP, 200 pM each primer and probe, and 0.025 U/ μ l Platinum Taq enzyme (Invitrogen Corp., Carlsbad, CA). Cycling conditions were 95°C $\times$ 15 sec, 60°C $\times$ 60 sec for 50 cycles. Water was included in the reactions as a negative control.

2.4 High-risk HPV PCR-MS assay

PCR primer pairs were designed within the E6 genes of the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and MassARRAY assay designer software v3.0 [Sequenom, Inc., San Diego, CA (Demeter et al., 1988; Ding and Cantor, 2003; Scheffner et al., 1990)] and are listed in Table 1. The E6 gene was chosen because interaction of the E6 protein with p53 is required to initiate pathogenic cellular proliferation (Guerin-Reverchon et al., 1989). The 10 nt sequence ACGTTGGATG was added to the 5' end of each primer. This inert sequence increased each primer by over 3000 daltons to ensure that unincorporated primers from the initial PCR would not be detected within the experimental window during the mass spectrometry analysis. PCR amplification was performed in the HotStar buffer system (Qiagen, Inc., Valencia, CA) with 2.5 mM MgCl₂, 200 μ M each dNTP, 200 pM each primer, and 0.02 U/ μ l HotStar Taq enzyme. To quantitate HPV DNA load in a given sample, an oligomer was added to the PCR reactions in a final concentration ranging from 1 aM – 100 fM (1×10^{-18} M – 1×10^{-13} M). This oligo "competitor" was identical in sequence to the sample-amplified 53 – 97 bp product except for the substitution of one centrally located C or G nucleotide with its complementary base. Twenty-five μ l PCR reactions were assembled in a 96-well plate, and reactions were

expanded to 4 replicate wells in a 384-well plate containing 5 μ l each using a liquid dispenser robot. Thermocycling was performed at 94°C $\times$ 20 sec, 52°C $\times$ 30 sec, 72°C $\times$ 60 sec for 45 cycles to co-amplify identical length products from the HPV-infected genomic DNA sample and the competitor. Water was included as a negative control.

Amplification products were treated with shrimp alkaline phosphatase (0.04 U/ μ l) at 37°C for 30 min to dephosphorylate residual unincorporated dNTPs. Reactions were then subjected to a second amplification step using a single internal extension primer. Individual extension primers were designed so that the primer's last 3' nucleotide was one base prior to the wobble G/C nucleotide that differentiated the sample/competitor amplified products. In addition, base content and primer length were adjusted to ensure that at least 20 daltons separated the masses of the HPV type-specific extension primers to avoid interference between adjacent peaks during mass spectroscopy analysis. This round of linear amplification was performed in the EXTEND buffer system (Sequenom, Inc., San Diego, CA) with 600 pM each extension primer and 0.064 U/ μ l MassEXTEND ThermoSequenase at 94°C $\times$ 5 sec, 52°C $\times$ 5 sec, 72°C $\times$ 5 sec for 99 cycles. Reaction buffer contained ddGTP and ddCTP and terminated extension at the first nucleotide 3' of the extension primer which was the C/G wobble nucleotide. Reactions were desalted with cation exchange resin, stamped to a 384 bioarray SpectroCHIP™ silicon dioxide chip (Sequenom, Inc., San Diego, CA) using a 24-pin robot, and analyzed by mass spectroscopy.

Mass spectrometry output for a sample analyzed for a single HPV type showed three peaks representing the type-specific extension primer, sample extended product, and competitor extended product. In the triplet, the peak of lowest mass represented the extension primer. The middle peak was 273 daltons greater than the extension peak, representing the extension primer capped with a ddCTP. The heaviest peak was 40 daltons greater than the middle peak and 313 daltons greater than the extension primer, representing the extension peak capped with ddGTP. In negative control reactions with competitor but no DNA template, two peaks representing the extension primer and competitor peak were observed. In negative control reactions with no competitor or DNA template, one peak representing the extension primer was observed.

After optimization of individual HPV type-specific assays, primers for the 13 high-risk HPV assays were combined and run in a single reaction using the same reagent concentrations and incubation conditions. To detect and quantitate type-specific HPV content, DNA samples were initially screened by 13-plex PCR-MS with no competitor and 1 aM competitors. For those HPV types detected, samples were then reanalyzed to quantitate viral load. PCR-MS reactions were run in the presence of individual HPV type primers with competitor concentrations of 1 aM, 100 aM, and 10 fM. Frequencies for the area under the sample-specific peak and the competitor-specific peak were calculated using MassARRAY Typer version 3.4 software and applied to compute sample HPV concentration. If the sample:competitor peak ratio was $\geq$ 95:5 at 10 fM, the sample was diluted and reanalyzed. HPV copies/cell was calculated based on 6.5 pg genomic DNA/human diploid cell.

2.5 Other HPV detection methods

Prior to PCR-MS testing, DNAs isolated from cervical scraping samples were analyzed for HPV by the HC2 assay with the high-risk HPV probe mix according to manufacturer's instructions. This probe mix included RNA probes complementary to the DNA of each of 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Hybridization between HPV DNA and any of the complementary RNA probes was detected using capture antibodies, which targeted RNA:DNA hybrids (Obiso and Lorincz, 2004). Manufacturer guidelines were followed for relative light unit (RLU) cutoff ratios to define HPV positive

and negative samples. Ambiguous specimens that did not repeat as positive were not included in this study.

To determine HPV type by degenerate sequencing, DNA was amplified using the following degenerate primers from the L1 region (de Roda Husman et al., 1995): GP5+, a mixture of primers 5'-GCACAGGGACATAATAAT-3', 5'-GCACAGGGTCATAATAAT-3', 5'-GCCCAGGGACATAAT-3 and 5'-GCCCAGGGTCATAAT-3' and GP6+ primer 5'-GAATATGATTTACAGTTTATTTTC-3'. Reactions were run at 95°C × 15 sec, 52°C × 60 sec for 40 cycles. For type-specific sequencing, DNA was amplified with the PCR-MS type-specific forward and reverse primers using the conditions described above. Products were visualized on an ethidium bromide stained 2% agarose gel in 1x TBE, purified using QIAquick spin columns (Qiagen, Inc., Valencia, CA), and sequenced by the University of Michigan sequencing core using the GP6+ primer or PCR-MS forward primer. Sequences were analyzed by the BLAST algorithm and ClustalW alignment to identify HPV type.

3. Results

3.1 Design and validation of PCR-MS HPV assay

To develop a PCR-MS assay that detected and quantitated each of the 13 high-risk HPV types, we initially optimized individual type-specific assays and then combined all 13 into one reaction. Peak intensity remained robust for almost all primer sets when incorporated into the multiplex assay, and new sequences were designed to improve those few suppressed by the multiplex milieu. Each HPV type yielded an extension product with a unique molecular mass based on the nucleotide length and nucleotide composition of the extension primer, and amplification of the expected type-specific product at its unique molecular weight in the presence of 10 aM of the given competitor sequence but not with the other competitors confirmed the specificity of individual types in the multiplex. Figure 1 depicts a representative profile of the 13-plex PCR-MS reaction.

In the cervical cancer cell lines, viral load values of 1.5 copies/cell HPV 16 in SiHa, 548 copies/cell HPV 16 in CaSki, and 8 copies/cell HPV 18 in HeLa were obtained which were in agreement with published results obtained using other methodologies (Ho et al., 1995; Lizard et al., 1993; Syrjanen et al., 1991). Linear results were observed in both the uniplex and multiplex platforms on serial 10-fold dilutions of HeLa DNA (1.37 pg/μl - 13.7 ng/μl), with calculated HPV 18 concentrations decreasing proportionally as input sample DNA concentrations decreased.

3.2 HPV detection in cervical tumors

All but one cervical tumor (76/77; 98.7%) contained at least one of the high-risk HPV types by PCR-MS. Table 2 and Figure 2A show the distribution of HPV types detected. Most tumors (84.4%) contained only a single high-risk HPV type, with HPV 16 (62.3%) and HPV 18 (18.2%) detected most frequently. HPV total viral load ranged from less than one to almost 6,000 copies/cell (Figure 2B). To validate these results, degenerate DNA sequencing of L1 consensus PCR amplicons was performed. HPV type by degenerate DNA sequencing agreed with HPV type by PCR-MS in 60 (78.9%) samples (Table 3). No product was amplified to sequence for the one tumor negative by PCR-MS, and no sequencing results were obtained for 13 tumors despite the visualization of an amplicon on an agarose gel. For these 13 discordant samples, HPV type determined by type-specific amplification and sequencing matched at least one of the HPV type originally identified by PCR-MS in 11 tumors with sufficient DNA. No sequencing results were obtained with one other tumor with sufficient DNA, and the remaining discordant sample had inadequate DNA for type-specific amplification and sequencing. Degenerate sequencing results for the three remaining tumor

samples with single HPV type infections did not match the HPV type detected by PCR-MS. For two of these, alternative high-risk HPV types present in the 13-plex PCR-MS assay were identified by degenerate sequencing. For the third tumor sample, sequence for the intermediate-risk HPV 73 was obtained. An HPV 73 PCR-MS assay was designed and confirmed this HPV type in this tumor.

3.3 HPV detection in cervical scrapings

Of 199 cervical scraping samples analyzed, 124 (62.3%) were positive for high-risk HPV by HC2 (HC2+), and the remaining 75 (37.7%) were negative for HPV by HC2 (HC2-). A single high-risk HPV type was detected by PCR-MS in 71 (57.3%) HC2+ samples and in 20 (26.7%) HC2- samples (Table 2). No high-risk HPV was detected in 18 (14.5%) HC2+ samples and 40 (53.3%) HC2- samples. All 13 HPV types were detected in at least one HC2+ sample. HPV 16 (29.0%) was the type most commonly detected in HC2+ samples and was second highest in HC2- samples (13.3%), after HPV 33 and HPV 39 (16.0% each; Figure 2A). In HC2+ samples, total HPV copies/cell ranged from 0.06 to over 300,000 (Figure 2B). In HC2- samples, total HPV copies/cell ranged from 0.003 to 4.8. While HPV copies/cell were significantly less in the HC2- samples compared to HC2+ samples, the top 11 HC2- samples with high-risk HPV detected by PCR-MS overlapped with the 20 HC2+ samples with the lowest viral loads in the range of 0.06 to 4.9 copies/cell.

Comparison of high-risk HPV detection by PCR-MS and HC2 assays is shown in Table 4. The two methods agreed for nearly three-quarters (146; 73.4%) of samples as either positive or negative for high-risk HPV types. Sequencing with degenerate primers of the 18 HC2+ samples negative by PCR-MS detected a non-high-risk HPV type in 11 samples, and no sequence similarity to HPV in the remaining 7 samples. For the 35 HC2- samples positive by PCR-MS, PCR amplification was performed with type-specific primers in 28 (80%) samples. PCR products of expected size were visualized on agarose gels from 52/55 (94.5%) reactions representing 25/28 (89.3%) samples containing single and multiple HPV types. Thirty-nine amplicons (75%) were sequenced and matched the type originally detected by PCR-MS in all cases except for three in which no interpretable sequence was obtained.

4. Discussion

This study describes a prototype assay in the initial “proof-of-principle” stages of development and evaluation, and demonstrates that this prototype PCR-MS assay can detect and quantitate individual high-risk HPV types in cervical tumor-derived cell lines, cervical scrapings, and cervical tumors. All 13 types were detected in at least one sample, supporting the functionality of each individual amplification within the multiplex assay. In addition, the most commonly detected high-risk HPV types in both cervical tumors and scraping samples were consistent with the most common types observed in pooled case-control data from nine countries (Munoz et al., 2003).

As the PCR-MS assay generates information not previously available in a single assay (i.e., both type-specific and quantitative HPV characterization), no current clinical assay exists for direct comparison. Therefore, we compared our assay to the only FDA-approved HPV detection assay available at the time for use in the clinical setting (HC2). As HC2 does not provide quantitation, we also applied PCR-MS to analyze DNA from cervical cell lines for which HPV type and quantity have been established in the literature. Analytic validation studies showed that calculated HPV 18 viral loads in HeLa cells were proportional to the amount of sample DNA analyzed over a 5 log range. Further, HPV 16 and HPV 18 copies/cell in the cervical cancer cell lines CaSki, HeLa, and SiHa generally agreed with copy numbers derived by alternative methodologies (Lizard et al., 1993; Syrjanen et al., 1991). Similarly, the range of copies/cell (0.003 to over 300,000) that we obtained in our cervical

scraping samples is consistent with previous reports using real-time PCR (Kulmala et al., 2007) and densitometric scanning of autoradiographs (Wickenden et al., 1987). We acknowledge that 300,000 copies/cell is an apparent outlier. This high value could be explained by multiple reasons beyond issues of the assay itself, including cervical sampling technique and underlying stage of the viral infection cycle. The concentration observed during papillomaviral DNA replication ranges from 50-100 copies per cell during initial amplification in the nucleus of the infected host cell, to thousands of copies per cell during productive replication of the viral genome in differentiated cells of the outer layer of the epidermis (Flint et al., 2004). Information on competing clinical co-morbidities (e.g., HIV status, autoimmune disease, immunosuppressive drugs) in the patients from which the samples were collected is not available, which could also explain the observed high viral load. Finally, we analyzed HPV type and quantity in cervical tumors. Because HPV DNA is detected in virtually all cervical tumors (Bosch et al., 1995), there has been little clinical utility thus far in assaying tumors for the presence of HPV. Only a few, small studies have quantitated viral load in cervical tumor tissue (Chan et al., 2005; Lillo et al., 2008). However, in the context of other HPV-associated cancer sites, there is emerging evidence that HPV type and viral load measured in tumors could have potential clinical utility for guiding treatment (Worden et al., 2008) or monitoring recurrence (Cohen et al., 2008; Li et al., 2003). The PCR-MS assay was recently evaluated in oropharyngeal tumor DNA, and the authors found that response to chemotherapy was affected by HPV positivity (Worden et al., 2008). PCR-MS is an evolving tool with exciting potential to inform the biology, epidemiology, and clinical management of HPV-associated cancers.

While PCR-MS fully agreed with degenerate sequencing in identifying the same HPV types in 59 tumor samples, type-specific amplification and sequencing was needed to confirm PCR-MS types in 11 other tumors for which degenerate HPV primer amplification and sequencing did not identify any HPV. Thus, although type-specific sequencing is impractical for HPV screening, use of the PCR-MS assay enhances specificity in tumors when considering both type-specific and degenerate sequencing results. Furthermore, inherent problems in degenerate sequencing, due in part to reduced hybridization and amplification efficiencies using consensus primers, are avoided in the PCR-MS assays since primer sequences are type-specific. Indeed, the presence of samples that did not produce a sequence underscores the limit of this technique which is not quantitative, not as sensitive as PCR-MS, only specifies the most abundant HPV type, and fails to generate a unique DNA sequence when there are multiple HPV types present in similar concentrations. In this study, HPV identification by degenerate sequencing used primers from the L1 region of the HPV genome, while PCR-MS uses primers from the E6 region for HPV detection. The independence of the methods is a strength of the study design, since errors in detection are not potentially being replicated. Still, while these results are suggestive of improved accuracy of HPV detection using PCR-MS, there is a need for cross-validation with other HPV genotyping tests. Forthcoming work from this research group (manuscript in preparation) compares PCR-MS with reverse line probe PCR in women with mild to moderate cervical intraepithelial neoplasia.

Further evaluation of analytical and clinical performance would be a necessary prerequisite before PCR-MS can be used for cervical cancer screening and for the monitoring of the efficacy of HPV vaccines. Still, these results add to the literature suggesting the potential clinical advantages of alternative HPV DNA detection methods (Ginocchio et al., 2008). Interestingly, PCR-MS did not detect the presence of a high-risk HPV type in 18 (14.5%) of HC2+ cervical scraping samples. From a clinical standpoint, these results could reflect false positive results, which have been shown to occur in about 10% of HC2 tests due to non-specific cross-reactivity of assay reagents (Jastania et al., 2006; Seme et al., 2006). Assays that reduce, or even eliminate, such false positive results could have potential clinical utility

in reducing colposcopy referral rates for women with equivocal cervical cytology as well as over treatment of women with mild cytological abnormalities.

PCR-MS analysis also identified potential false negative results of the HC2 test. Nearly half (35; 46.7%) of the 75 HC2- cervical scraping samples had discrete amounts of HPV that were too low to be detected by HC2 but were detected by PCR-MS. Eleven of these 35 HC2-/PCR-MS+ samples contained HPV copies/cell higher than the minimum copies/cell quantitated by PCR-MS in HC2+ samples, and thus could potentially be considered HC2 false negative results. The remaining 24 HC2-/PCR-MS+ samples contained HPV copies/cell lower than the minimum copies/cell recorded by PCR-MS in HC2+ samples. Clearly, the low levels of HPV DNA detected may lack clinical significance in disease progression to cervical cancer, requiring longitudinal analyses to investigate changes in viral load over time and the prognostic significance for cervical disease progression (Bigas and de Marval, 2005; Moberg et al., 2004). Notably, studies that normalized the results based on copy number of HPV DNA per cell were more likely to demonstrate a relation between viral load and clinical disease severity (Josefsson et al., 2000; Moberg et al., 2004; Ylitalo et al., 2000). In response to these findings, primers from an intron of the β -globin gene have been added to the next generation multiplex PCR-MS assay, allowing cell number determination on the same sample as HPV analysis and eliminating the need for real-time PCR.

5. Conclusions

These results demonstrate that this prototype PCR-MS assay performs at least as well as the HC2 assay, while offering the additional, unique advantages of type-specific identification and quantitation. Further validation work is underway to define clinically meaningful HPV detection thresholds and to evaluate the potential clinical application of future generations of the PCR-MS assay based on well-characterized study populations and clinical endpoints.

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Figure 1.

Representative profile of the 13-plex PCR-MS reaction.

In a control PCR-MS reaction containing extension primers and water as template but no competitor primers, 13 peaks representing the extension primer for each high-risk HPV type distinctly resolve in the multiplex assay. In order of increasing mass are extension peaks for HPV 35, 18, 31, 52, 33, 16, 39, 58, 59, 51, 68, 56 and 45. Paired vertical lines matching the color of the line at each extension primer peak are 273 daltons and 313 daltons heavier than the extension primer peak and mark the location of the ddCTP and ddGTP capped extension primer, respectively. X axis indicates mass in daltons; Y axis indicates intensity of peak in relative units.

Figure 2. Distribution of HPV type and quantity in cervical samples. (A) PCR-MS detected all 13 high-risk HPV types in the cervical tumor and scraping samples. (B) Total HPV DNA detected by PCR-MS ranged from 5 aM - 650 fM in tumor samples, 5 aM - 4 pM in HC2-positive cervical scraping samples, and 6.5 aM - 500 aM in HC2-negative cervical scrapings samples.

Table 1

13-plex HPV PCR-MS primer sequences

	Forward Primer ^{a, b} / Competitor ^{a, d}	Reverse Primer ^{a, b}	Extension Primer ^d	nt ^c / daltons ^e
HPV 16	<u>ACGTTGGATGAACAGTTACTGCGACGTGAG</u> AGCATATGGATTCCCATCTCTATATACTATGCATAAATCCCGAAAGCAAAGTCATATACCTCACGTGCGAGTAAC TGTT	<u>ACGTTGGATGATGATGAGCATATGAGCATATG</u> TGTTCTCTGCGTCTGGAGTCTCTGTCGTCTCGTTGCGAGCACGAATGGCACTGGCCTCTATAGTGCCTCCAGCTAT	<u>TTGCTTTTCGGGATTTATG</u> GCCAATTCGTGCTGCAAC	73 5831 83 5146
HPV 31	<u>ACGTTGGATGCGTGTCTGAGGTTTCACAAAATACTATGTGCTTTATATACCAACCGTTTTCGGTTCACCA</u>	<u>ACGTTGGATGCGTGTCTGAGGTTTCACAAAATACTATGTGCTTTATATACCAACCGTTTTCGGTTCACCA</u>	<u>ATGGCGTCTGTAGGTTT</u>	78 5247
HPV 33	<u>ACGTTGGATGCGATTTTCATGTTTCGGG</u> TCACATGCGAGTTTCTACGTGCGGACCTCCAAACACGCGCGCACAGCGCCCTCCCGAACGCCGAAATATATGAAATCG	<u>ACGTTGGATGCGATGTCAGTGTCTCTACG</u> ACGTTGGATGCGTGTCTGAGGTTTCACAAAATACTATGTGCTTTATATACCAACCGTTTTCGGTTCACCA	<u>ACACGCCGACAGCGCCCT</u>	81 5704
HPV 35	<u>ACGTTGGATGCGTGTCTGAGTTGAAAAG</u> TTCCACAGGACATACACCGACCTGTCCACCGTCCACGGATGTTATGGAATCGTTTTTCTCTCTAAATGTCCTTTTCACTGGACACAGCG	<u>ACGTTGGATGTTGCAAAAAG</u> ACGTTGGATGCGTGTCTGAGGTTTCACAAAATACTATGTGCTTTATATACCAACCGTTTTCGGTTCACCA	<u>ACCTGTCCACCGTCCAC</u>	97 5051
HPV 39	<u>ACGTTGGATGGCATACAAATTGCCAGACC</u> TCGCTGCAATAGACACAGGCTATTTGTAATGTCCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC</u>	<u>TTGCCAGACCTGTGCAAC</u>	82 6062
HPV 45	<u>ACGTTGGATGAAGTGCATTACAGGATGGCG</u> CTGTGCACAAATCTGGTAGTTGTAGGTCGTTCTCTTGGATCGTCAAGCGCGCCATCTGTAATGCACCTT	<u>ACGTTGGATGCTGTGCAAAATCTGGTAGC</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>AAATCTGGTAGCTGTAGGGTCGTT</u>	72 7743
HPV 51	<u>ACGTTGGATGGTTATACCGAAAACGGTG</u> GTCTTCCCTCTGTCTCGAAACATGGTGTCTCTATCTATCTTAAAGACCTGCTGTAATGCACCTT	<u>ACGTTGGATGCTGTCTTCCCTCTGTCTTCG</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>CGGTGCATATAAAAGTGCAGTG</u>	83 6824
HPV 52	<u>ACGTTGGATGATTGTGTGAGGTCGTGGAAG</u> ACCTCTCTGCTGTAGCTCTTTTTTGCACTGCGACACACTGCAAGCCTTATTCATCCACCGATTCTTCCAGCACCTCACACAAT	<u>ACGTTGGATGACCTCTCTGCTGTGAGCTC</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>GTGCTGGAAGAAATCGGTG</u>	84 5620
HPV 56	<u>ACGTTGGATGTTAACTCCGGAGAAAAGC</u> CAACATGACCCCGTCCAACCATGTGCTATAGATGAAATGGTCTTTTTCTGTGCACAAATGCAATTGCTTTCTCGGAGTTAAC	<u>ACGTTGGATGCAAAACATGACCCGGTCCAAC</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>CAACCATGTGCTATTAGATGAAAT</u>	85 7360
HPV 58	<u>ACGTTGGATGTGATTGTGTGAGCGTTGG</u> TACCTCAGATCGCTGCAAAATCTTTTGCATTCAACGCATTTCGAATTCGATTTTCATGCACATGCTCTCCAAACGCTGACAAATCA	<u>ACGTTGGATGTACCTCAGATCGCTGCAAAAG</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>GTGTCAGGCGTTGGAGACAT</u>	88 6213
HPV 59	<u>ACGTTGGATGCTGCCTGATTGTGAGCAAC</u> GCAGTTCCTCTTTCGCAAAACACACAAATTGATGGGAATATCATGTCAGAGGAATAATTCGAATGTGTGCTCAAAATCAGGCAG	<u>ACGTTGGATGCGATTTCCCTTTTGC AAAAC</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>TTGCAAAAACACACAATTTGATG</u>	79 6422
HPV 68	<u>ACGTTGGATGTCAGAAAGCAACTACAACG</u> ACCCGTCCTCTATATACTACATTTAAGTCAAGCAAGGCAAAATTCATATACCTCTGTCCGTTGTAGTTGCCTTCTGCA	<u>ACGTTGGATGACCCCGTCCCTATATACTAC</u> ACGTTGGATGCGTGTCTGCAAGTGGTGTCCAGGTTGTGCGACAGGTCGTGCAATTTGTATGGC	<u>CCCTATATACTACATTTAAGTCA</u>	77 6942

^a All primers are listed 5' - 3'.

^b Underlined sequences in forward and reverse primer sequences are inert non-HPV nucleotides, increasing the mass of primers to ensure that they are heavier than any of the extension product masses

^c Length of HPV type-specific PCR products amplified with forward and reverse primers

^d Enlarged bold C or G indicates "wobble," base of competitor sequence that is complement to the wild-type HPV type-specific sequence. Underlined nucleotides indicate locations of the extension primer sequences (in the reverse complement) within the competitor primers.

^e Mass in daltons of extension primers

Table 2

Distribution of high-risk HPV types detected by PCR-MS assay in cervical samples

Number of high-risk HPV types by PCR-MS	Tumors (n=77) n (%)	Cervical scrapings (n=199) n (%)	
		HC2+ (n=124)	HC2- (n=75)
0	1 (1.3)	18 (14.5)	40 (53.3)
1	65 (84.4)	71 (57.3)	20 (26.7)
2	10 (13.0)	30 (24.2)	8 (10.7)
3	1 (1.3)	3 (2.4)	2 (2.7)
4	0 (0)	2 (1.6)	4 (5.3)
5	0 (0)	0 (0)	1 (1.3)

Table 3

Comparison of high-risk HPV detection by PCR-MS assay and degenerate DNA sequencing in 77 cervical tumors

PCR-MS Assay	Degenerate DNA Sequencing	
	High-risk HPV positive	High-risk HPV negative
High-risk HPV positive	60	16
High-risk HPV negative	0	1

Table 4

Comparison of high-risk HPV detection by PCR-MS and HC2 assays in 199 cervical scraping samples

PCR-MS Assay	HC2 Assay	
	Positive (n=124)	Negative (n=75)
High-risk HPV positive	106	35
High-risk HPV negative	18	40