Facile, Comprehensive, High-Throughput Genotyping of Human Genital Papillomaviruses Using Spectrally Addressable Liquid Bead Microarrays

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Human papillomavirus (HPV) is the worldwide cause of carcinoma of the uterine cervix, a cancer that is the second most common neoplasm in women, resulting in nearly 250,000 deaths a year. The magnitude of the risk of cancer after HPV infection, however, is virus type-specific. Over 40 HPV types can infect the genital tract. Comprehensive, high-throughput typing assays for HPV, however, are not currently available. Blending multiplex PCR and multiplex hybridization using spectrally addressable liquid bead microarrays we have developed a high-throughput, fast, single-tube-typing assay capable of simultaneously typing 45 HPV. The overall incidence of HPV in 429 women tested using this new assay was 72.2% for those with squamous intraepithelial lesions, 51.5% for those with atypical squamous cells of undetermined significance and 15.4% for women with normal cytology, respectively. This compared well with the incidence of HPV detected by a parallel non-typing generic high-risk assay. The new assay detected a wide spectrum of HPV types and a high incidence of mixed infections. We believe our assay may find widespread applications in areas requiring virus type-specific information, such as in epidemiological studies, cancer screening programs, monitoring therapeutic interventions, and evaluating the efficacy of HPV vaccine trials. (*J Mol Diagn* 2005, 7:72–80)

Human papillomavirus (HPV) is the main cause of carcinoma of the uterine cervix. Significant progress has been made in the identification and taxonomic classification of these viruses using polymerase chain reaction (PCR)-based assays. Nonetheless, no currently available assay can type all HPVs that infect the human genital tract, be

fully automated, or be easily deployed on a high throughput platform. There are good reasons to develop such a test. First, clinical, epidemiological, and molecular data indicates that HPV is the etiological agent of nearly all carcinomas of the uterine cervix.^{1–4} Second, the risk of cancer development after HPV infection is type-specific and there are at least 45 HPV types capable of infecting the human genital tract.^{5–8} Moreover, among cancer-associated HPVs the contributions of their intrinsic protumorigenic properties versus their relative prevalence within the “at risk” population are not fully defined. Nor is the interplay between virus and host factors including ethnicity and other genetic factors, which have been suggested by numerous epidemiological studies^{9–11} been explained. The simultaneous analysis of viral type and host genetic factors such as HLA type, and genetic loci conferring susceptibility and/or resistance to HPV infections or progression to cancer, which may be uncovered using genome-wide SNPs screens, may shed further light on HPV cancer susceptibility and needs to be carried out. Epidemiological studies using viral typing assays have begun to answer some of these important questions and assays that improve the quality of typing or sample throughput may contribute further in this area.^{4,6,11–16} In addition, there also are practical reasons to strive for facile and cost-effective genotyping of HPV. Taking in consideration results of the ALTS study¹⁷ the 2001 American Society for Colposcopy and Cervical Pathology (ASCCP)-sponsored consensus conference recommended HPV testing as one of the three appropriate management approaches to patients with equivocal results (abnormal squamous cells of undetermined significance, ASCUS) on their cervicovaginal cytology assays.¹⁸ It further recommended that patients found harboring intermediate and high-risk HPV types undergo more extensive and invasive procedures to rule out intra-

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epithelial carcinoma (carcinoma *in situ*), while those negative for HPV or carrying low-grade HPV types are spared from such procedures and followed with yearly conventional cervical cytology studies. Moreover, because the population at greatest risk for HPV-induced carcinoma of the cervix are women that fail to clear HPV and remain persistently infected^{19–21} some have recommended HPV testing plus cytological examination (pap smear) in women 30 years or older. This recommendation is based on studies indicating that the predictive value for significant uterine cervix lesions of combined HPV typing and cytologic examination in this age group is superior to that of either test alone.²² Lastly, there are two additional areas where comprehensive assays may contribute to patient management or HPV control programs. One of these is in the follow up of patients with definitive therapies such as conization or fulguration of uterine cervix lesions. In these patients a negative HPV test at 6- or 12-month follow up is highly predictive of eradication of the lesion whereas a positive result is associated with persistence of cervical lesion.^{23–27} Secondly, as an increasing number of monovalent or oligovalent vaccine programs get underway,^{28–30} comprehensive HPV typing assays will be essential to assess their efficacy in eradicating HPV in the previously infected host or preventing infection in the HPV-naïve host. This is particularly relevant to HPV vaccines since cross-immunity to viral-like particle vaccines is not extensive and is relatively type-specific.

There are a number of published HPV typing assays (reviewed in references 25, 31, and 32) based on PCR amplification. Most of these assays use post-PCR hybridization approaches to typing, such as Southern blot plus RFLP analysis,³³ dot blot,^{34–37} enzyme linked immunosorbent assay (ELISA)^{38–41} line-blot assay,^{42–45} solid phase optical microarrays^{46,47} or conductivity microarrays.⁴⁸ Although these assays can genotype a relatively large spectrum of HPV types, none types them all or can be automated or deployed on a high-throughput platform, features which are essential for an assay intended for a large volume of patients. We have hypothesized that an assay with optimal performance characteristics for genital HPV genotyping can be developed by combining multiplex PCR amplification with multiplex hybridization to liquid bead microarrays (LBMA). Guided by this principle we have developed a low cost, single-tube high-throughput assay capable of unambiguously typing most, if not all, genital HPVs. We used PGMY09/11 primers because of their well-established performance, but primers in other suitable areas of the genome, particularly those that make it possible to generate smaller amplicons (50 to 100 bp) may offer significant advantages⁴⁹ and are being actively explored in our laboratory. Our assay can type HPV in hundreds of patient samples a day in a nearly automatic fashion. This assay, which we have dubbed BARCODE-HPV for *Bead Array Coded Detection* of HPV types, may facilitate conducting the large-scale studies needed for more precisely defining the interplay between the virus and the host and for assessing the effectiveness of HPV vaccination programs.

Materials and Methods

Source of Clinical Specimens and Genital HPV Type Controls

Clinical specimens were obtained from patients undergoing routine liquid (Cytyc Corporation, Boxborough, MA) cervicovaginal cytology examinations (pap test) at UMass Memorial Health Care (Worcester, MA). All studies were conducted with previous approval by the research in human subjects committee of the internal review board of the University of Massachusetts Medical School. The study population comprised 429 patients for which residual PreserveCyt fluid (Cytyc) was available and included patients with normal cytology and patients with a diagnosis of ASCUS or squamous intraepithelial neoplasia (SIL).

DNA Extraction from PreserveCyt Fluid

One ml of PreserveCyt fluid per sample was aliquoted into a 1.5-ml vial and 0.1 ml of buffer converting fluid (Digene Corporation, Gaithersburg, MD) was added. Samples were then centrifuged at $3000 \times g$ for 5 minutes and supernatants discarded. Samples were resuspended in 100 μ l of digestion buffer containing 0.8X SSC, 200 mmol/L NaCl, 0.5% sodium dodecyl sulfate (SDS), 1 mmol/L 1,4-dithio-DL-threitol (DTT), 2 mg/ml proteinase K, and digested for 30 minutes. Purification of the lysate was done using standard phenol and chloroform procedure. DNA was precipitated with ethanol, and resuspended in 20 μ l of Tris-EDTA (TE). Samples extracted in this manner yielded between 2 and 10 μ g of total DNA as assessed by absorbance measurements at 260 nm.

HPV type standards were a kind gift of Dr. Ethel-Michele de Villiers and included Supergroup A subtypes 6, 11, 16, 18, 45, and 51. Plasmids were electroporated into DH10B *E. coli*, plated on selective media and DNA minipreps (Qiagen, Valencia, CA) prepared from saturated cultures of individual bacterial colonies. Care was exercised not to cross-contaminate the HPV stocks. Dilutions of the stock plasmids were used as templates for multiplexed PCR reactions.

Amplification of L1 Sequences of Genital HPVs

Since our stated objective was to determine the feasibility of using liquid bead microarrays for efficient typing of HPVs, rather than the sensitivity or specificity of HPV DNA amplification, we used one of the most type-comprehensive and well-characterized multiplex PCR assays. The assay uses a family of primers designated PGMY09/11 that improve over the capabilities of the widely used MY09/11 primers and are capable of amplifying at least 37⁴¹ of the 45 genital HPV types included in our LBMA (high-risk HPVs 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 55, 56, 58, 59, 66, 68, 73, 82, 83 and low-risk types 6, 7, 11, 13, 30, 32, 34, 40, 42, 43, 44, 54, 61, 62, 64, 67, 69, 70, 71, 72, 74, 81, 84, 86, 87).^{1,4,5} PCR conditions were as in the published PGMY09/11 version.⁴³ Oligonucleotides that primed the strand complementary to the se-

Table 1. Probe Target Sequence* (L1 gene, + strand)

45 sequences aligned		
HPV6L1p	1	AAAGCCCACTCCTGAAAA 18
HPV7L1p	1	GCGTGATGCACCCCAAAA 18
HPV11L1p	1	GAAACCCACACCTGAAAA 18
HPV13L1p	1	AAAGCCTACACCTGATAA 18
HPV16L1p	1	AAAAATACACCTCCAGC 18
HPV18L1p	1	AAAGGATGCTGCACCGGC 18
HPV26L1p	1	GCGTAACGCCCCCTCCTGT 18
HPV30L1p	1	AAAGGATCAGCCTCCTGC 18
HPV31L1p	1	AAAAACTGCCCCCAAAA 18
HPV32L1p	1	AGCTAAGGTAACAGCACC 18
HPV33L1p	1	AAAAACAGTACCTCCAAA 18
HPV34L1p	1	GCGTCCGCAACCTCCTAA 18
HPV35L1p	1	AAAAACAGTGCACCAAAA 18
HPV39L1p	1	AAAGGATGCTCCAGCACC 18
HPV40L1p	1	GCGCGATGCGCCCCCAAA 18
HPV42L1p	1	GCGTAAGGTAACAACGCG 18
HPV43L1p	1	AAAAAATGCTCCCCCAAAA 18
HPV44L1p	1	AAAGCCACCCCTGAAAA 18
HPV45L1p	1	AAAGGATAGTACACCTCC 18
HPV51L1p	1	AAAGGATACCCCTCCACA 18
HPV52L1p	1	GAAAAACACACCACCTAA 18
HPV53L1p	1	AAAGGATCAGCCCCCTCC 18
HPV54L1p	1	GAATAATGCCCTGCAAG 18
HPV55L1p	1	AAAGCCTCCCCCTGAAAA 18
HPV56L1p	1	ACGGGAACAGCCACCAAC 18
HPV58L1p	1	AAAAACAGCACCCCTTAA 18
HPV59L1p	1	AAAGGACACCGCACCGCC 18
HPV61L1p	1	GGGTGCTGCTGCCCCGTC 18
HPV62L1p	1	GGGGGCTGCCTACCCGTC 18
HPV64L1p	1	GCGTCCGCAACCTCCTAA 18
HPV66L1p	1	GAGGGAACAGCCCCCTGC 18
HPV67L1p	1	AAAAACATCCCCCTCCAA 18
HPV68L1p	1	GAAAGACGCCCCCTGCACC 18
HPV69L1p	1	ACGCGATGCCCTGCACA 18
HPV70L1p	1	AAAGGATGCTCCTACACC 18
HPV71L1p	1	AAACAGTCCTCCTCCTGC 18
HPV72L1p	1	GGGGGCTGCCACCCCTCC 18
HPV73L1p	1	ACGTCTCAACCTCCTAA 18
HPV74L1p	1	AAAACCTACGCCTGATAA 18
HPV81L1p	1	GGGTGCTGCTGCCCCCTGC 18
HPV82L1p	1	CAAGGACAGTCTCCACA 18
HPV83L1p	1	GGGTCTTCCGCCCTGC 18
HPV84L1p	1	GGGGGCCGCGCCGCCCAA 18
HPV86L1p	1	AGGGGCTACTGCACCACA 18
HPV87L1p	1	GGGGTCTGCAGCCACAAA 18

*Probes are reverse and complement of the displayed sequence.

quence of the capture probe attached to beads carried a biotin group in their 5' ends serving as a reporter for hybridizations (see below).

Selection of Probes, Manufacture of a Master 45 Plex-HPV Liquid Bead Microarray (HPV-LBMA), Determination of the Efficiency of Probe-Bead Conjugation and the Analytical Specificity of the Microarray

Probes consisted of 18 mer oligonucleotides targeting a divergent sequence segment within the PGMY09/11 amplicon (Table 1). Probes were selected for most of the HPVs reported to infect the genital tract regardless of risk class. Recursive Clustal-W alignment algorithm (MacVector 7.0, Oxford Molecular Group, Cambridge, UK) was used to select oligonucleotides with the lowest cross-

homology and the greatest discriminating specificity. Forty-five type-specific probes were attached by the end user to their respective bead sets (XMAP beads from Luminex Corporation, Austin, TX) using a heterobifunctional cross-linking reagent (1-ethyl-3-[3-dimethylamino-propyl]-carbodiimide, Pierce, Rockford, IL). Each bead set carries a distinctive spectral signature that is unambiguously recognized by the Luminex 100 instrument when presented with a multibead array such as our 45-plex array. Spectral signatures are encoded in each bead by the manufacturer (Luminex). All conjugated beads were then combined to formulate a master 45-plex HPV-LBMA. Efficiency of conjugation and quality control of each bead/probe combinations was determined empirically by hybridizing each bead set to its cognate target in the form of a biotinylated oligonucleotide perfectly complementary to the probe attached to the bead. Beads with signals above 2000 RLU, when exposed to a 2 nmol/L solution of cognate target were selected for further work. Rarely, however, a conjugation had to be discarded because beads failed to fluoresce above 2000 RLU. As formulated, one master array was sufficient to perform 500 assays (patients). The analytical specificity of the HPV-LBMA *per se* was determined by exposing the whole array to relevant amounts of all targets in consecutive wells. A highly specific array is expected to generate a perfect diagonal on a bivariate plot of probe-bead versus target (see below and Figure 2).

Hybridization of PCR Products to the HPV-LBMA and Analysis in the Luminex 100 Instrument

Amplified targets were typed by hybridization with the HPV-LBMA. Multiplexed hybridization was carried out in a solution containing TMAC (tetramethyl ammonium chloride, Sigma, St. Louis, MO).⁵⁰ Ten μ l of PCR product and 7 μ l H₂O were added to single wells (one well per patient) of a 96-multiplate (MJ Research, Waltham, MA) containing 33 μ l of the HPV-LBMA per well suspended in 1.5X TMAC (1X = 2 mol/L TMAC, 0.1% sarcosyl, 50 mmol/L Tris, 4 mmol/L EDTA). The multiplate was then ramped to 99°C for 5 minutes, to co-denature PCR products with array probes, and then ramped down and kept at 55°C for 30 minutes. Just before completion of hybridization, a multiscreen vacuum plate with manifold (Millipore, Bedford, MA) was prepared by rinsing twice with ice-cold 1X TMAC. Hybridization reactions were terminated by transferring the entire reaction to the multiscreen plate containing ice-cold 1X TMAC buffer and washed twice in the same buffer with intervening vacuum filtration. Each well of the microplate then received 2 μ l of a stock solution of phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) diluted 1:100 in 1X TMAC. The entire plate was allowed to reach room temperature (~30 minutes), and was then incubated at 55°C for 30 minutes before two additional washes with TMAC buffer. The 96-well microtiter plate was then transferred to the XY platform of the Luminex 100 and samples automatically injected into the analyzer. One full 96-well plate was read by the Luminex 100 in less than 1 hour. Numerical results

Figure 1. Schematic representation of the BARCODE-HPV assay. After multiplex PCR amplification of 45 possible HPV sequences with the PGMY09/11 primer set on a single well of a PCR multiplate (A), PCR products are transferred to a sister multiplate containing in each well the 45-plex HPV-LBMA and hybridized for 30 minutes (B and C). Hybridized beads are then injected in the Luminex 100 instrument which, using the bead-encoding infrared spectral signatures, classify each bead into discrete populations (D, bottom) and simultaneously measures their analyte-associated (hybridization signal) fluorescence (D, top). Results are displayed graphically using a routine operator embedded on an Excel file (E).

were expressed as light units normalized for background fluorescence and the varying hybridization efficiencies of the target/probe combinations. Thresholds were set at 10% of the average positive control plasmid signals. A Microsoft Excel algorithm transformed raw numerical instrument output data into graphic display for a large number of patient samples (up to 90 per run) simultaneously (~30 seconds). Statistical analysis was performed using Fisher's exact test for association between two independent variables and kappa statistics to assess the strength of statistically significant associations.

Results

The assay is described schematically in Figure 1 (Figure 1). An essential component of BARCODE-HPV is an infrared fluorophore-encoded liquid bead microarray to which oligonucleotide probes can be conjugated. After PGMY09/11 multiplex PCR amplification on a 96-well plate format (one well per patient) with appropriate negative- and positive-control wells, PCR products are transferred into a sister plate (Figure 1B) containing the custom-built HPV-LBMA. The mix is co-denatured and allowed to hybridize for 30 minutes (see Materials and Methods). The array is then exposed to the reporter fluorophore, washed, and injected into the Luminex 100 instrument using an automated XY platform (Figure 1D).

Data collection proceeds automatically and can be transferred onto an Excel file containing a routine operator to subtract background from the negative control and normalize signal intensity using average signal from the positive controls. Data are displayed as tri-dimensional bar graphs (Figure 1E) or, for ease of interpretation, on a conditionally formatted Excel file in the form of a two-dimensional grid (Table 2).

We first determined the quality of the HPV-LBMA by hybridizing the array to each individual target in the form of a biotinylated oligonucleotide (Figure 2). Analysis of these data indicated that our 45 plex LBMA perfectly discriminated all 18 high-risk HPV targets and 23 of the 27 low-risk HPV targets. Two pairs, HPV34/64 and HPV61/81, could not be fully discriminated. HPV34 and 64 targets exhibited 100% homology in the selected area and generated identical signals. HPV61 and 81 targets on the other hand, despite being only 88% homologous, generated sufficient cross-hybridization signal to make discrimination less than straightforward. Failure to discriminate between HPV34/64 and 61/81 is of no consequence, however, since the two pairs of viruses belong in the low-risk group (Figure 2).

To determine the ability of the HPV-LBMA to specifically detect different HPV types we hybridized it with PGMY09/11 amplicons generated from HPV plasmids or mixture (not shown) of plasmids. Analysis of these data showed that our LBMA perfectly discriminated all targets, regardless of number of viral types preset in the mixture (Figure 3). The data in Figure 3 is presented without subtraction of background to allow the reader to better appreciate the signal-to-noise ratio. Of note, no significant cross-hybridization signals were generated by the individual viruses or by PCR reactions derived from HPV-negative genomic human DNA (Figure 3 and not shown). Only a limited number of viruses were available as plasmids to be used as controls. Nevertheless, the absence of significance cross-hybridization signals in these experiments indicates that good discrimination is possible even using the relatively long (~450 bp) PCR product generated by PGMY09/11 primers.

We next hybridized PGMY amplicons derived from 429 patients undergoing thin-prep pap smear at Umass Memorial Health Care. One hundred and ten patients had been diagnosed with SIL lesions, 202 with ASCUS, and 117 had normal cytology on their current thin-prep pap smears. Amplified samples were analyzed by both agarose gel electrophoresis and HPV-LBMA. Analysis of these data revealed that there was a nearly complete correspondence between gel detection of amplified L1 gene targets and detection using the HPV-LBMA. BARCODE-HPV failed to detect 4 of 206 samples positive by gel electrophoresis (for a presumed false-negative rate of 1.9%) and was positive in four cases that had not clearly visible band of appropriate size on the agarose gel (presumed false-positive rate of 1.8%) (see Tables 2 and 3). These results indicate that BARCODE-HPV can detect nearly all amplicons generated by PGMY09/11 in this study. The overall incidence of HPV in our samples was 47.1% for the entire group and 72.7, 51.5 and 15.4% for SIL, ASCUS, and normal cytology specimens, respec-

Table 2. Two-Dimensional Grid Displaying Results of BARCODE-HPV and HCII Assays for 30 patients with SIL, ASCUS and Normal (NRML) Thin-Prep Pap Cytology

[illegible]

Each HPV type is represented by a column, each sample by a row. Darkened boxes correspond to types identified in each sample. Only the first thirty samples in each series of SIL, ASCUS, and normal cytology specimens is shown. The three broader columns on the right correspond to results of HC-II (1st column), BARCODE-HPV high-risk (2nd column), and BARCODE-HPV high plus low-risk HPVVs (3rd column), and allow a quick visual check on the correlation between HC-II and BARCODE-HPV.

Figure 2. Building an HPV-LBMA. Forty-five HPV type-specific probes were attached to 45 XMAP bead sets and combined into one master HPB-LBMA (bead/probe in figure). The array was hybridized to each individual target (labeled targets in figure). Only bead sets that generated a signal of >2000 relative light units (RLU) when hybridized to target at 2 nmol/L were included in the array. Observe the excellent signal-to-noise ratio of the array and the absence of cross-hybridization signals.

tively (Table 3). Infections with more than one virus were common and accounted for 41.2, 35.6 and 5.5% of SIL, ASCUS and normal cytology specimens, respectively (Table 3). Notably, examination of the whole data set did not reveal patterns that would suggest cross-hybridization or spurious signals (Table 2). In general, the results of BARCODE-HPV and the Hybrid Capture II (HC-II, Digene) assay were highly correlated as shown by the Fisher's exact test and kappa statistics (Tables 3 and 4). The HC-II assay, however, did detect a higher rate of high-risk type HPV infection in SIL lesions (81.8% versus 60.9%) but not in ASCUS (52.4 versus 41.5%) or women with normal cytology (8.5 versus 9.4%). Moreover, in comparisons between the frequency of HPV detection by HC-II and all HPVs included in the BARCODE-HPV assay, the differences in SIL specimens narrowed significantly and were 81.8% versus 72.7%, 52.4% versus 50.4%, and 8.5% versus 15.4% for SIL, ASCUS and normal specimens, respectively (Table 4). The rate of infection detection by BARCODE-HPV and Hybrid Capture II for the entire cohort of patients was nearly identical (47 versus 48, respectively) (Table 3). As in many other studies, the most prevalent HPV across all patient groups studied was HPV16 (Figure 4A). HPV18, however, was only the twelfth, fifth, and third most prevalent high-risk virus among patients with SIL, ASCUS, or normal cervicovaginal cytology, respectively (Figure 4, F, G, and H). Other common high-risk viruses were frequent as well, such as HPVs 39 and 59. Interestingly HPV45, a high-risk virus frequently reported in the literature, was not detected in our study (Figure 4). This was not due to microarray failure to detect this viral type since both synthetic target

(Figure 2) and target generated from a control HPV45 plasmid (Figure 3) using PGMY09/11 primers could be easily detected. However, since PGMY09/11 primers do not amplify HPV45 efficiently, false-negative results for this viral type with our assay cannot be ruled out. HPV31 and 33, two common high-risk viruses in the literature were found at a lower frequency than HPV39, 59, 73, 58 and 35 in SIL lesions (Figure 4F). Interestingly, HPV26 was the seventh, thirteenth, and second most prevalent HPV in SIL, ASCUS and normal cervicovaginal cytology specimens, respectively. Four HPV types not currently included in HC-II (HPV26, 53, 66 and 73) were respectively the seventh, tenth, sixth, and fourth most prevalent high-risk HPVs in SIL samples in our study (Figure 4F). These apparent differences may be due to differences in the local prevalence of different HPV types, different ethnic or racial susceptibilities to different HPV types, or founder effects due to temporal differences in the introduction of different viral types within the at risk population.

Discussion

In this report we demonstrate the feasibility of typing genital HPVs by multiplex PCR using liquid bead microarrays as a readout platform. BARCODE-HPV itself is a high-throughput assay that offers significant comparative advantage over other currently available HPV typing assays. The assay can potentially be fully automated, drastically decreasing the personnel cost component of the assay. Our assay can type nearly all genital HPVs amplified by the PYGM primer set, it can detect infections with

Figure 3. Specificity of BARCODE-HPV on PCR amplicons generated from HPV control plasmids. Ten nanograms of each plasmid was used to perform PGMY09/11-primed PCR reactions identical to those used for clinical samples. At the completion of PCR, amplicons were hybridized to the HPV-LBMA and analyzed in the Luminex 100. Results are normalized for positive-control signals but are displayed without subtraction of average background RLU generated by HPV-negative DNA samples.

Table 3. Number of HPV Types Detected with BARCODE-HPV among Patients with Different Thin-Prep Pap Cytology Smear Results and Correlation with Gel-Detectable Amplification Products

	Type-specific HPV incidence in PreserveCyt fluid from women undergoing cervicovaginal cytology								PGMY 09/11	
	SIL (n = 110)		ASCUS (n = 202)		Normal (n = 117)		Total (n = 429)		Total (n = 429)	
	n	%	n	%	n	%	n	%	n	%
1	47	58.8	67	64.4	17	94.5	131	64.9	—	—
2	21	26.3	27	26.0	1	5.5	49	24.3	—	—
3	11	13.8	9	8.7	0	0.0	20	9.9	—	—
>3	1	1.3	1	1.0	0	0.0	2	1.0	—	—
HPV+	80	72.7	104	51.5	18	15.4	202	47.0	206	48.0
HPV−	30	27.3	98	48.5	99	84.6	227	52.9	223	52.0

PGMY 09/11 represents gel detectable PCR products of size consistent with L1 PGMY 09/11 amplicons.

single as well as multiple HPVs, and can type many more samples than previously available typing assays. We are currently working with commercial vendors to develop a fully automated DNA extraction procedure for liquid cytology specimens. Our assay compares favorably with well-established generic high-risk assays such as HC-II. The differences in sensitivity noted between BARCODE-HPV and HC-II can in fact not be easily attributed to either method alone. It is well established that HC-II under typical operating conditions has a false-positive rates as high as 17% (HCII test package insert), which in part may account for the apparent lower sensitivity of BARCODE-HPV. In addition, the high-risk probes in HC-II may cross-hybridize with some low-risk viral types leading to erroneous assignment of risk when these types are present. This latter consideration may explain the tighter correlation between HC-II and BARCODE-HPV when both high and low-risk viruses in the latter are considered. Conversely, the PGMY09/11 primer set, though purportedly amplifying most of the 45 HPV types included in this study⁴¹ may either not amplify a significant fraction of them or not amplify some types with sufficient sensitivity (for example HPV45) leading to possible false negatives. The relative contributions of HCII false positives and PGMY primer-driven BARCODE-HPV false negatives need to be studied further. It is possible that primer sets

with greater HPV type range could be designed to diminish false negatives in our assay. The final answer to these issues awaits studies with large number of samples comparing BARCODE-HPV and HC-II with single-target type-specific "gold standard" PCR assays that include all genital HPVs. Indeed, the design of new primer sets targeting smaller (100 to 150 bp) amplicons is an ongoing effort in our laboratory but was not an aim of this study.

There are good reasons to believe that BARCODE-HPV or optimized versions of it can find great utility in several areas of HPV research. Because of its favorable cost/benefit ratio and high sample throughput, it can be used in further defining the validity of epidemiological and phylogenetic risk-classification schemes in specific geographical and/or ethnic populations.⁵ Though such studies have been conducted in the past, they have been limited in scope and number of patients and have led to some uncertainty about the attributed risk to some specific viral types. Analyzing the incidence ratio of specific HPV types in women with normal cytology versus those with SIL or invasive cancers may provide a more comprehensive view of the true type-specific cancer risk and clearly separate the role of type-specific prevalence versus that of type-specific intrinsic carcinogenicity.

BARCODE-HPV can also be a valuable tool in the evaluation of women enrolled in HPV vaccine clinical

Table 4. Correlation between HCII (High-Risk Probe Set) and BARCODE-HPV Assays

HCII/High-risk probe set only	BARCODE-HPV					
	High-risk only			High + Low-risk		
	+	−	Total	+	−	Total
Squamous intraepithelial lesion						
+	64 (58.1)	26 (23.6)	90 (81.8)	73 (66.3)	17 (15.4)	90 (81.8)
−	3 (2.7)	17 (15.4)	20 (18.2)	7 (6.3)	13 (11.8)	20 (18.2)
Total	67 (60.9)	43 (39.1)	110 (100)	80 (72.7)	30 (27)	110 (100)
Abnormal squamous cells of uncertain significance						
+	75 (37.1)	31 (15.3)	106 (52.4)	87 (43.5)	18 (8.9)	105 (52.4)
−	9 (4.4)	87 (43.0)	96 (47.6)	15 (6.9)	82 (40.6)	97 (47.5)
Total	84 (41.5)	118 (58.5)	202 (100)	102 (50.4)	100 (50)	202 (100)
Normal cytology						
+	7 (6.0)	3 (2.5)	10 (9)	7 (6.0)	3 (2.5)	10 (8.5)
−	4 (3.4)	103 (88.0)	107 (91)	11 (9.4)	96 (82.1)	107 (91.5)
Total	11 (9.4)	106 (90.5)	117 (100)	18 (15.4)	99 (84.6)	117 (100)

Statistically significant (Fisher's test <0.0001) correlations between BARCODE-HPV and HC-2 occurred throughout. The overall agreement between BARCODE-HPV and HC-2 was high, with kappa values of 0.681 ± 0.034 for all samples and 0.682 ± 0.051 for ASCUS samples, while it was lower for normal and SIL samples with respective kappa values of 0.561 ± 0.138 and 0.394 ± 0.083 .

Figure 4. HPV detection and typing by BARCODE-HPV in 429 women undergoing cervicovaginal liquid cytology studies (thin-prep pap smears). Type-specific incidence of all or high-risk only genital HPVs in women with cervicovaginal cytology studies resulted as SIL, ASCUS, or Normal are shown in **A** and **E**, respectively. The incidence rank order for all HPVs or high-risk HPV only are plotted for SIL (**B** and **F**), ASCUS (**C** and **G**), and Normal cytology samples (**D** and **H**), respectively.

trials as it can help in defining issues of specificity of the immune protective responses, cross-immunity, durability of responses, etc. Undoubtedly viral type-specific assays will be critical in the coming years to assess the effectiveness of HPV vaccine programs and BARCODE-HPV represents a significant step in this direction.

Our findings indicating that the prevalence of certain HPV types in our study population differ from that extracted from large studies performed in other demographic settings appears to be sufficient argument to exercise caution in extrapolating results from one study population to another. It might well be that the dynamics of HPV infection is influenced by numerous local ethnic, socioeconomic, and cultural factors that may lead to significant differences in type-specific prevalence and/or cancer risk. BARCODE-HPV could also easily be configured to subtype the most prevalent high-risk HPVs. Variants of HPV16 in fact may be associated with different risk of carcinoma of the cervix^{51,52} and may be discriminated by single oligonucleotide probes.

Though HPV-typing assays based on solid-phase microarrays have been published^{46–48} and others are being developed, liquid bead microarrays exhibit some comparative advantages over solid phase microarrays that are worth pointing out. Hybridization kinetics with liquid bead arrays closely approximates the kinetics of solution-phase hybridization. This results in short hybridization times and faster turn-around times than possible with solid phase microarrays. In addition, liquid bead

microarrays are more easily quality controlled than solid-phase microarrays since with the former it is possible to test the array's performance by sampling the master array, something that is not possible with solid-phase arrays. Finally, though possible, it is unlikely that the levels of sample throughput and analysis achievable with liquid bead arrays can be equated with the currently available solid-phase arrays platforms as LBMA use low-volume hybridization, fast instrument read-out (30 seconds) and rapid and automated analysis (30 seconds for a set of 90 samples). These properties combined make LBMA highly practical for the detection and typing of genital HPVs and possibly the typing and subtyping of other microorganism for diagnostic, prognostic, or therapeutic purposes.

Finally, based on published cost analysis of bead microarray-driven assays,⁵³ and a Luminex Corporation freely available estimate, the cost per well (per patient) in reagents and consumables (DNA isolation, PCR, array beads, plasticware, etc) is approximately three dollars and seventy-five cents. This compares favorably with other available commercial HPV assays.

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