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3 **Universal Human Papillomavirus Typing Assay: Whole Genome Sequencing**  
4 **Following Target Enrichment**

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27 **Running title: Universal HPV typing by whole genome sequencing**

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29 Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent  
30 the official position of the Centers for Disease Control and Prevention.

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37 **ABSTRACT**

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41 We designed a universal HPV typing assay based on target enrichment and whole genome sequencing (eWGS).

42 The RNA bait included 23,941 probes targeting 191 HPV types and 12 targeting beta-globin as control. We used

43 Agilent SureSelect XT2 protocol for library preparation, Illumina HiSeq 2500 for sequencing and CLC genomics

44 workbench for sequence analysis. Mapping stringency for type assignment was determined based on 8 (6 HPV-

45 positive and 2 HPV negative) control samples. Using the optimal mapping conditions, types were assigned to 24

46 blinded samples. eWGS results were 100% concordant with Linear Array (LA) genotyping results for 9 plasmid

47 samples and fully or partially concordant for 9 of the 15 cervical-vaginal samples, with 95.83% overall-type

48 specific concordance for LA genotyping. eWGS identified 7 HPV types not included in the LA genotyping. Since

49 this method does not involve degenerate primers targeting HPV genomic regions, PCR bias in genotype detection

50 is minimized. With further refinements aimed at reducing cost and increasing throughput, this first application of

51 eWGS for universal HPV typing could be a useful method to elucidate HPV epidemiology.

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53 **Key words:** HPV typing, broad-spectrum assay, whole genome sequencing, target enrichment

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76 **INTRODUCTION**

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79 Human Papillomaviruses (HPV) are double-stranded DNA viruses in the family *Papillomaviridae*. More  
80 than 200 genotypes are recognized based on the sequence of the approximately 8 (k)basepairs [(k)bp] circular  
81 genome, with variants in each genotype based on sequence relatedness (1,2). Detection and typing of HPV has  
82 clinical and public health significance because of the association of some genotypes in the alpha-papillomavirus  
83 genus with anogenital and oropharyngeal cancers. As a result, most assays used in HPV epidemiology and  
84 natural history studies are directed to these genotypes, and many PCR-based assays use degenerate primers  
85 directed to the L1 region of the HPV genome (3,4). Studies examining disease associations of an increasingly  
86 broad spectrum of HPV genotypes have been hampered by the need for multiple assays to detect genotypes in  
87 alpha, beta and gamma genera (5,6).

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Next generation sequencing (NGS) based methods have the promise to improve the spectrum of HPV  
genotypes detected. Some NGS methods used PCR with consensus primers targeting the L1 region for library  
preparation, restricting regions of the genome that could be analyzed (7-13). HPV genome sequencing to single or  
limited HPV genotypes was achieved using the standard protocol for library preparation without any target  
enrichment (14), or after whole genome amplification by rolling circle amplification (15) or highly multiplexed  
degenerate primers (16). There are a few reports of NGS using target enrichment to study the mechanistic  
signatures of integration of a limited number of HPV genotypes in cervical carcinomas (17-19). The goal of this  
study was to capitalize on the availability of new target enrichment technology to facilitate whole genome  
sequencing to detect, genotype and potentially characterize variant and integration status of all known HPVs  
belonging to alpha, beta and gamma genera using a single assay. Target enrichment technology uses  
hybridization to purify genomic fragments of interest with complementary DNA or RNA baits. DNA/RNA baits  
were initially used and proved to be highly effective to enrich human exomes for variant calling by deep  
sequencing (20,21). We developed custom RNA baits specific to all known HPV genomes to enrich the sample  
for sequencing of whole viral genomes. Here we describe and provide an initial evaluation of this whole genome  
sequence based approach for broad-spectrum HPV genotype determination in samples with single and multiple  
HPV infection using the Agilent SureSelect target enrichment technology.

103 **MATERIALS AND METHODS**

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105 **Samples**

106 DNA extracted from three cells lines (ATCC, Manassas, VA) known to include HPV 16 (Caski: ~500  
107 copies/cell, SiHa: 1-2 copies/cell) and HPV 18 (HeLa: ~50 copies/cell) served as HPV positive controls, and  
108 water and human placental DNA (Sigma-Aldrich Corporation, St. Louis, MO) were included as HPV negative  
109 controls. A panel of coded samples was prepared so that testing and analysis could be performed in a blinded  
110 fashion. The blinded set included 9 residual samples from past WHO validation panels [whole HPV genome  
111 DNA plasmids diluted to 50,000 copies/sample in human placental DNA (100 ng/50  $\mu$ L)] and 15 residual DNA  
112 extracts from anonymized cervical/vaginal samples previously typed using the Linear Array (LA; Roche  
113 Molecular Diagnostics, Indianapolis, IN). Two of the cervical/vaginal extracts were negative for the 37 genotypes  
114 targeted by LA and the other 13 samples were selected to include multiple HPV genotypes (median 5; range 2-  
115 12). DNA was quantified by fluorescence-based Picogreen assay (Molecular Probes, Inc., Eugene, OR) and  
116 volume was adjusted with a buffer (pH 8.0) composed of 10 mM Tris-HCl and 1 mM EDTA  
117 (ethylenediaminetetraacetic acid) so that a final concentration of 100 ng/50  $\mu$ L was used for library preparation.  
118 The exception was the water blank (0 ng DNA), and cell line samples that were also prepared at 10 ng DNA to  
119 test the effect of reduced DNA input on sequencing results. The final number of samples sequenced was 32: 8  
120 control samples [3 cell line controls (100 ng), 3 cell line controls (10 ng), human placental DNA (100 ng), water  
121 (0 ng)] and 24 blinded samples [9 plasmid samples and 15 cervical/vaginal extracts (100 ng)].

122 **HPV whole genome reference sequences**

123 We retrieved the whole genome HPV reference sequences for all 188 HPV genotypes that were available  
124 in the PapillomaVirus Episteme (PaVE) database (<http://pave.niaid.nih.gov/>) as of September 2014 (22). We  
125 also retrieved whole genome sequences for three HPV subtypes detected by LA (HPV55, a HPV44 subtype;  
126 HPV82A/IS39; and HPV68b) from the National Center for Biotechnology (NCBI) database  
127 (<http://www.ncbi.nlm.nih.gov/>). The 191 HPV reference genomes included in this study (Table S1) ranged in size  
128 from 7095bp – 8104bp and were distributed phylogenetically into 5 genera (67 alpha, 47 beta, 74 Gamma, 2 Mu  
129 and 1 Nu). The complete reference sequences were used for RNA bait design as well as for sequence mapping.

### 130 **Design and synthesis of RNA baits**

131 Agilent Technologies Inc. (Santa Clara, CA,) designed a custom RNA bait library using eArray software  
132 comprised of 120 bases RNAs complementary to one strand of the whole genomes of all 191 HPV genotypes at  
133 two fold coverage (60 bases overlapping). The resulting 23,941 RNA sequences comprised a bait library size of  
134 1.442 (M)bp. The bait library sequences were subjected to BLAST (Basic Local Alignment Search Tool) search  
135 against NCBI human genome database, and 21 fragments were identified to have homology to human sequences.  
136 Removing these 21 fragments from RNA bait synthesis resulted in 100% coverage for 182 HPV genotypes and  
137 99.17%-99.25% coverage for 9 HPV genotypes (HPV 6, 19, 34, 71, 82A/IS39, 142, 171, 172, 175), for overall  
138 design coverage of 99.96%. A BLAST search of the final synthesized bait sequences against the full NCBI  
139 database identified 15, 669 unique hits, 99.94% specific to HPV sequences. The other 10 hits (0.064%) shared  
140 identity to chimpanzee, feline and macaque papillomaviruses. As an internal control for the quality of samples,  
141 we included 12 RNA fragments covering nt2041 to 3480 of the coding sequence of human beta-globin (HBG)  
142 gene (1439bp, GenBank #GU324922.1). The custom biotinylated RNA bait library (HPV and HBG) was  
143 synthesized at Agilent Technologies. Upon request to the authors, the custom design ID may be shared with those  
144 who want to synthesize these probes through Agilent Technologies.

### 145 **Target enrichment library preparation and sequencing**

146 The work-flow for the preparation of the sequencing library followed Agilent Technologies'  
147 SureSelect<sup>XT2</sup> target enrichment protocol (Version D3) optimized for 100 ng DNA (Figure 1). Briefly, DNA  
148 samples (50  $\mu$ L in 96 well microTUBE plate) were sheared using Covaris LE220 Focused-ultrasonicator with the  
149 software SonoLab 7.3.2.4 (Covaris, Inc., Woburn, MA). The conditions used for shearing (duty factor, 15%; peak  
150 incident power, 450; treatment time, 490 seconds) generated DNA fragments with a peak around 150bp as  
151 determined by the high sensitivity DNA kit and Bioanalyzer 2100 (Agilent Technologies). DNA fragments were  
152 end-repaired, 3' A-tailed, and underwent pre-capture indexing whereby each sample was barcoded with an index  
153 of 8bp sequence. Indexed libraries were amplified by PCR with limited number of cycles (8 cycles), followed by  
154 purification and assessment of the quality and quantity of each library by Bioanalyzer 2100 (Agilent  
155 Technologies). Indexed libraries with unique barcodes were pooled (16 samples/pool) for overnight hybridization

156 with custom RNA bait, followed by capture of hybridized fragments and 14 cycles of PCR to amplify indexed  
157 libraries. Following further purification, the quality and quantity of post-capture HPV enriched pooled libraries  
158 were assessed by Bioanalyzer 2100 and qPCR using KAPA DNA library quantification kit (KAPA Biosystems,  
159 Wilmington, MA) and LightCycler 480 (Roche Diagnostics, Indianapolis, IN). Pooled libraries were paired-end  
160 sequenced on a two-lane flow cell on Illumina HiSeq 2500 at the Centers for Disease Control (CDC) Core  
161 Facility using TruSeq Rapid SBS Kit HS (200 cycle) (Illumina, San Diego, CA,) with Rapid run mode according  
162 to the following settings: Read1, 100 cycles; index (i7), 9 cycles; read 2, 100 cycles. For each DNA library, a  
163 seeding concentration of 5.33pM was applied for cluster generation with 5% PhiX virus genome library added as  
164 sequencing control.

### 165 **Bioinformatics**

166 The raw sequence data were de-multiplexed, and the adaptors and barcodes were removed using Illumina  
167 BCl2fastq V1.8.4. Reads with base quality Q score were exported as fastq files for batch mapping to reference  
168 sequences using CLC genomics workbench 7.5 (CLCbio, Waltham, MA). The reference sequences of 191 HPV  
169 types/subtypes and HBG that were used for bait design were imported to CLC genomics for mapping.

170 The analysis was directed to HPV detection and typing so duplicate reads were not removed. We used  
171 reads with 0 mismatches in the index sequence for analysis in this report. Two parameters, read lengths (L0.5 or  
172 L1) and similarity scores (S0.8 or S1.0), were used to adjust mapping stringency. BAM (Binary Alignment/Map)  
173 files were analyzed to generate mapping statistics by CLC genomics. Three additional parameters were evaluated  
174 in the process of differentiating signal from noise: number of reads mapped to the HPV type-specific reference  
175 sequence, average depth of coverage for the mapped sequences and fraction of HPV genome covered by mapped  
176 reads. HPV types in 8 control samples detected by enriched whole genome sequencing (eWGS) with different  
177 mapping stringency and acceptance parameters were recorded prior to un-blinding expected HPV results from 24  
178 blinded samples. Subsequent matching of HPV genotype calls by WGS to expected results was used to evaluate  
179 performance of the assay and analysis.

180 RNA bait performance was evaluated based on results from single HPV plasmids: percentage HPV  
181 reference genome covered by sequenced reads, percentage of reads that mapped to predicted types (compared to

182 total HPV reads), and uniformity of coverage. To evaluate the uniformity, the reference sequences were divided  
183 into bins consisting of 300bp, and the average mapping depth within each bin calculated by Strand NGS software  
184 (<http://www.strand-ngs.com/>) using BAM files generated by CLC genomics under L1-S1 mapping stringency.  
185 The mean and the standard deviation (SD) of the average mapping depths among the bins were calculated. The  
186 uniformity of coverage was calculated as the percentage of bins with coverage within the average read depth  
187  $\pm 2SD$  for all bins. The target enrichment factor for a sample was calculated using formula: (Total HPV mapped  
188 reads/Total HPV and human mapped reads)/(HPV genome size/human diploid genome size) (23). HPV and  
189 human diploid genome sizes were considered 8 (k)bp and 6.6 (G)bp respectively for this calculation.

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## 191 **RESULTS**

### 192 **Assessment of sequence data quality**

193 DNA sheared approximately to 150bp is expected to increase in size to around 300bp in the indexed  
194 library after ligation of adaptors that enable limited PCR amplification and sequencing of the library. As expected,  
195 bioanalyzer analysis of the indexed pooled libraries prepared for sequencing indicated the size distribution with  
196 peak around 300bp. The seed concentration of 5.3pM generated cluster densities of 1173K and 1183K/mm<sup>2</sup> in  
197 pool 1 (samples 1-16) and 2 (samples 17-32), respectively. All raw reads from both pools passed the default  
198 filtering of the Illumina BC12fastq V1.8.4 software (pool 1- 268,096,524 and pool 2 -226,336,668). The average  
199 number of reads per sample in pool 1 was 8,538,564 and those from Caski 100 ng (sample 11) with ~500 HPV16  
200 copies/cell dominated, comprising 21.9% of the total (Figure 2A). The average number of reads per sample in  
201 pool 2 was 11,224,892. The mean base quality Q score for each sample (excluding sample 9, water control)  
202 ranged from 34.57 to 36.87 (mean=Q35.6), and 88% of the bases had quality scores greater than 30 (Figure 2B).  
203 The water control generated 3,692 reads with Q scores ranging from 2 to 40 (mean=Q25) with only 47 % of bases  
204 having Q score greater than 30.

### 205 **Mapping results for internal control HBG**

206 The fraction of the globin reference sequence mapped in all samples with genomic DNA (all except water  
207 control, sample 9) ranged from 93-100% (mean 98%). Samples with 100 ng genomic DNA (samples 1-8, 10-11,

208 13, 15, and 17-32) generated a mean of 7406 reads (range 1903–11225) that, using L1-S1 stringency, mapped to  
209 the globin reference (HBG nt2041-3480) with an average depth of coverage ranging from 132.2–779.5 (mean  
210 coverage 514.3) (Figure 3). The number of mapped reads (range 902-3327, mean 2000.3) and coverage (range  
211 62.6 - 231, mean 138.9) were reduced in samples with 10 ng genomic DNA (samples 12, 14 and 16). Only 2 reads  
212 from the water control (sample 9) mapped to beta globin (depth of coverage 0.1 and fraction of reference  
213 sequence mapped 12%).

#### 214 **Evaluating cut-off to improve signal/noise ratio for HPV genotyping from whole genome sequence data**

215 We evaluated mapping results for the 8 control samples (Table 1) in terms of the number of reads,  
216 average coverage and the fraction of genome covered using different stringencies to differentiate signal from  
217 noise for determination of HPV genotype. Caski (100ng) generated a total of 50,890,604 reads mapped to any  
218 HPV reference sequence, of which 99.94% of reads (50,861,070 reads) mapped specifically to HPV16 under the  
219 less stringent L0.5-S0.8 mapping condition. WGS also detected HPV 16 in SiHa (134,664 out of 164,138 total  
220 reads, 82.04%) and HPV 18 in HeLa (314,969 out of 342,887 total reads, 91.86%) as the most dominant types.  
221 Without any cut-offs for non-specific signal, all positive controls detected additional HPV types under all three  
222 mapping stringencies. The presence of non-specific signal was also seen in water and placental DNA negative  
223 controls. For example, WGS detected HPV 16 with 1800 reads in the negative placental DNA using L0.5-S0.8.  
224 With increased mapping stringency (L1-S1), placental DNA still detected HPV 16 with 861 reads and 10.9  
225 average coverage. Based on this, we selected a cut-off of  $\geq 1000$  mapped reads and average coverage  $\geq 20$  for  
226 reliable sequence assignment. We also added the fraction of reference genome covered  $\geq 0.5$  (to indicate that at  
227 least 50% of viral genome is retained, allowing for loss due to potential integration events) to the cut-off  
228 parameters. With L1-S1 mapping stringency and the selected cut-offs (number of mapped reads  $\geq 1000$ , average  
229 coverage  $\geq 20$  and fraction of genome covered  $\geq 0.5$ ), HPV genotyping results for the 8 controls were concordant  
230 with the expected results (Table 1). The control cell line samples, Caski (~500 HPV16 copies/cell), HeLa (~50  
231 HPV 18 copies/cell), and SiHa (1-2 HPV16 copies/cell), vary in known copy numbers of HPV and were analyzed  
232 at two concentrations of input DNA (100 ng, 10 ng). In each case, the number of mapped reads under stringent  
233 conditions roughly correlated with copy number (Table 1) and type assignment could be made with input of 10 ng



234 DNA. The fraction of genome covered for HPV18 in HeLa cell line was only 63%. No reads mapped to a 2.6  
235 (k)bp central region (nt3100-5730), compatible with a deletion (Figure 4).

### 236 **Determination of HPV types from whole genome sequence data**

237 eWGS data from the remaining 24 samples were analyzed using criteria set in the control samples to  
238 assign WGS HPV results without knowledge of prior HPV data. Comparing eWGS typing results with prior LA  
239 results, type-specific HPV detection was at least partially concordant in 18 of 24 samples (75%) (Table 2).  
240 Genotype determinations for the 9 HPV plasmids (HPV types 45, 58, 31, 33, 52, 6, 18, 11 and 16) were 100%  
241 concordant. The average depth of coverage for the 9 WHO plasmids ranged from 1,210 to 3,751 with a mean of  
242 2,837. Among the 15 cervico-vaginal swab samples, typing results for 9 samples were fully or partially  
243 concordant. Of these 9, full concordance was found in 5 samples, 2 negative for HPV and 3 samples with  
244 multiple types (3, 4 and 8 types). Among the 6 discordant samples, 4 were negative by eWGS but positive for  
245 multiple types by LA whereas 2 samples were HPV positive by both methods but for different types. The overall  
246 type-specific concordance for types included in the LA assay was 95.83%, and for LA targeted types, there were  
247 no instances of samples positive by eWGS and negative by LA. In six samples, eWGS identified HPV types not  
248 included in the LA assay (HPV 30, 43, 68a, 87, 90, 91, and 114). In cervico-vaginal samples with multiple HPV  
249 types, the average depth of coverage varied greatly from as low as 27 to 129,998 (Table 2), probably a result of  
250 varying viral loads.

251 While some LA types were not detected by WGS in this small sample set (HPV 26, 35, 53, 72, 81, and  
252 84), other LA types were detected in some, but not all samples (HPV 18 and 66). In some instances, eWGS  
253 detected types but did not meet the criteria for number of reads or depth of coverage. For example, in sample 25,  
254 eWGS failed to call HPV 66 and 18 which were detected by LA, but 1478 reads mapped to HPV66 with 99% of  
255 genome covered but failed because average depth of coverage (18.9) was just below cutoff (20); and HPV 18 had  
256 a subthreshold 234 reads mapped with 71% genome coverage. In sample 20, eWGS failed to call HPV 72,  
257 although 1412 reads mapped to HPV 72 with 87% genome coverage but average depth of coverage 17.7 was  
258 below cutoff 20.

259 In sample 29, LA detected HPV84 but eWGS detected HPV 114. HPV84 and HPV114 have 84%  
260 identity over the whole genome, suggesting the possibility of misclassification based on genomic fragments. To  
261 explore this, sample 29 reads were mapped with HPV84 as the only reference sequence. While no reads mapped  
262 to HPV 84 under stringent conditions, under L0.5-S0.8 conditions 62,203 reads mapped to HPV84 with 94% of  
263 the genome covered. HPV 53 was not identified in any of the 4 samples that were LA positive for this type,  
264 however HPV 53 sequences were found in one of four samples (number of reads, 1438; average coverage, 18; and  
265 genome coverage, 99%) if mapping was done under less stringent conditions (L0.5-S0.8).

266 LA includes probes for HPV 64 which has been reclassified as a subtype of HPV 34. For sample 17,  
267 16,120 reads mapped to HPV34 with 16% coverage (compared to whole genome), whereas 1736 reads mapped to  
268 458 bp HPV64 L1 partial sequence (GenBank# AJ81226.1) with 85% coverage. As the full length reference  
269 sequence for HPV 64 is not available and HPV 64 is reclassified as a subtype of HPV34, the eWGS results were  
270 assigned to HPV 64 based on post-hoc assessment, giving partial concordance with LA results

#### 271 **Evaluation of Custom HPV Bait**

272 The performance of the HPV custom RNA bait pool for the 9 HPV types included as individual plasmids  
273 is shown in Table 3. One HPV 6 bait sequence had to be removed due to homology with the human genome,  
274 resulting in 99.2% design coverage for that type; design coverage was 100% for other 8 types. The mean coverage  
275 of mapped reads to reference sequences was 99.8% (range 99.2% – 100%) and 99.2% to 100% of HPV reads  
276 mapped to predicted type (mean 99.60%). The mapped reads gave 95.9% uniform mean coverage (range 92.6 %–  
277 96.3%) suggesting that the bait resulted in unbiased enrichment of the whole HPV genome. The eWGS method  
278 averaged 184,483-fold enrichment for HPV sequences in HPV positive samples (range 3,294–914,377).

279

#### 280 **DISCUSSION**

281 This is the first application of NGS for whole genome identification of essentially all known HPV types  
282 (191 HPV types in the alpha, beta and gamma genera) using RNA baits of the Agilent SureSelect target  
283 enrichment technology. The target enrichment method avoids the limitations of targeting only limited areas of the  
284 genome, minimizes the potential for PCR bias and significantly increases the potential to increase the number of

285 types identified in a single assay. A recent report of using Roche NimbleGen DNA bait based target enrichment  
286 largely focused on understanding the mechanistic signatures of integration of HPV types (87 types, 63% alpha) in  
287 cervical carcinomas (17) with limited data on NGS-based HPV type determination and concordance with a  
288 current HPV typing assay. In comparison, we mainly focused on the development of a universal HPV typing  
289 assay to detect all known HPV types in epidemiologic studies that increasingly examines a broad-spectrum of  
290 HPV in alpha, beta and gamma genera in both mucosal and cutaneous specimens for their disease association  
291 (5,6,11,24). Towards this goal, we evaluated our method in terms of the performance of RNA baits for whole  
292 genome identification, level of enrichment, and a number of read mapping metrics for determination of HPV  
293 types under single and multiple infection.

294 The custom RNA bait library exhibited excellent performance in terms of the fraction of genome  
295 coverage, percentage of on-target reads mapped to predicted HPV types, uniformity of coverage for the types that  
296 we evaluated, and by the observed average level of target enrichment (184,483) by a factor of nearly 5 log. Our  
297 results support the concept of specific capture and whole genome sequencing of viruses from clinical samples  
298 through target enrichment technologies (18,25,26). Sequencing the target enriched libraries generated high quality  
299 reads with more than 88% of bases having Q scores greater than 30. Mapping results revealed that this method  
300 could detect single and multiple infections of HPV along with HBG as an internal control for cellularity.

301 The mapping stringencies and threshold for the number of mapped reads required to avoid “bleed  
302 through” of non-specific HPV signals was established using 8 control samples ([3 cell line controls (100 ng), 3  
303 cell line controls (10 ng), human placental DNA (100 ng), water (0 ng)]. The most stringent L1-S1 mapping  
304 condition along with a cut-off of  $\geq 1000$  mapped reads, average coverage  $\geq 20$  and fraction of genome covered  
305 greater than 50% allowed reliable sequence assignment. The control cell lines, Caski (~500 HPV16 copies/cell),  
306 HeLa (~50 HPV 18 copies/cell), and SiHa (1-2 HPV16 copies/cell), vary in copy numbers of HPV and were  
307 analyzed at two concentrations of input DNA (100 ng, 10 ng in 50  $\mu$ L). In each case, the number of mapped reads  
308 under stringent conditions roughly correlated with copy number, and type assignment could be made with an  
309 input of 10 ng DNA. In agreement with previous reports by other methods (27,28), the fraction of genome

310 covered for HPV18 in HeLa was only 63% since no reads mapped to a 2.6 (k)bp central region (nt3100-5730),  
311 compatible with a deletion due to integration.

312 The bleed-through of reads from dominant sequences in adjacent clusters has been reported in  
313 multiplexed samples sequenced using the Illumina platform (29,30). This might happen more frequently under  
314 relatively high seeding density for cluster generation and when one sequence dominates. These two conditions  
315 seemed to have taken place in this study. At the seeding density of 5.3 pM used in this study, pool 1 and pool 2  
316 libraries generated cluster densities of 1173K and 1183K respectively which is slightly higher than the  
317 recommended cluster density of 850-1000K. Additionally, over 31 million reads of HPV 16 from Caski 100 ng  
318 dominated sequences and corresponded to the HPV16 bleed-through found in other samples in the same pool. The  
319 HPV 16 sequences in the placental DNA negative control in pool 1 had the unique 29 nucleotide substitutions  
320 identical to Caski HPV16 sequences (data not shown). It is unlikely that misidentification of barcode sequences  
321 contributed to bleed-through since we restricted the analysis to reads with 0 mismatches in the index reads.

322 We applied the mapping stringency and cut-off determined with the control samples for determination of  
323 HPV types in 24 samples blinded to their HPV status. The HPV types determined by the eWGS were 100%  
324 concordant with LA in the 9 plasmid samples but concordance was lower in the cervi-covaginal samples (Table  
325 2). Nine of 15 samples (60%) showed complete or partial concordance and type-specific concordance for the 37  
326 LA types was 95.83%. It could be anticipated that biologic samples from exfoliated cells would present greater  
327 challenges to HPV detection than purified plasmid DNA. These samples vary significantly in the amount of  
328 cellular and viral material. Given the relation between viral copy number and number of reads noted in the  
329 plasmid and cell line results, it could be suspected that low copy numbers may contribute to failure to detect types  
330 found by LA. Additionally, LA used 10  $\mu$ l of extract regardless the DNA concentration, while the eWGS method  
331 used 100 ng. Of the 4 samples that were LA positive but eWGS negative, three had 6-10 times (600 ng – 1030  
332 ng) more DNA input for LA. On the other hand, even with only 15 epidemiologic samples, eWGS detected 7  
333 HPV types (HPV 30, 43, 68a, 87, 90, 91, and 114) not targeted by LA. The ability of this eWGS method to detect  
334 HPV types not targeted by current assays indicates it could be useful in evaluation of HPV negative cervical

335 lesions (31) and in epidemiologic studies of HPV in geographic regions that may have uncommon types in  
336 circulation (32).

337         Some HPV types detected by LA were not detected by eWGS in this small sample set (HPV 26, 35, 53,  
338 72, 81, 84). HPV 84 was also reported discordant between NGS and LA in earlier studies (10,15). Further  
339 evaluation is needed to determine explanations for the failure to detect these types. Reduced mapping stringency  
340 did detect HPV 53 and 84 in some instances, and there could be unreported variants affecting mapping, and/or  
341 these types may be present at low copy numbers. We were able to confirm the specificity of RNA baits for  
342 HPV53 at the highest stringency of reference mapping by detecting HPV53 plasmid DNA (data from subsequent  
343 study not shown).

344         In conclusion, we developed and provided results from the initial evaluation of a non-PCR whole genome  
345 sequence based approach which is as possibly close to a “gold standard” for broad-spectrum HPV genotype  
346 determination. The method relies on a custom-made RNA bait library that showed excellent performance in  
347 terms of genome coverage, percentage of reads mapped to predicted HPV type, uniformity of coverage, and the  
348 level of target enrichment. Further optimization and evaluation of this eWGS method for HPV detection is  
349 required in terms of cost/sample and throughput. Additional studies with samples including more types and  
350 optimization of sequencing conditions to minimize the bleed-through are needed. Studies evaluating the  
351 reproducibility and lower limits of detection are planned. Finally, it should be noted that despite enrichment with  
352 RNA bait by a factor of 5 log, 75% of the total reads (average) were human (off-target), with the exception of the  
353 sample with the highest viral load (100 ng Caski) in which 36% of total reads were off-target (data not shown).  
354 This suggests adjustment in the conditions for on-target enrichment could enhance results. Future laboratory and  
355 bioinformatics efforts are focused on determining the sensitivity and reproducibility of this HPV genotyping  
356 method, along with expanding its application for HPV detection and genotyping in samples for a variety of  
357 anatomical sites, and development of a bioinformatics pipeline for automatic determination of HPV types and  
358 variants. This methodology is conceptually suitable for detecting both known and unknown HPV types through  
359 adjusting stringencies at the level of hybridization to RNA bait and reference mapping parameters. The current  
360 reference mapping stringency used to detect known HPV genotypes would likely miss unknown types.

361

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473 **Table 1. Determination of cut-off to differentiate signal from noise in HPV whole genome sequence data**  
 474 **based using control samples**

Control samples	L0.5-S0.8*			L0.5-S1*			L1-S1*			HPV type	
	No of reads mapped	Average coverage	Fraction of genome covered	No of reads mapped	Average coverage	Fraction of genome covered	No of reads mapped	Average coverage	Fraction of genome covered	Without Cut-off	With Cut-off**
Caski (100 ng)	50,861,070	637,921.0	1.00	49,687,563	624,209.5	1.00	31,807,177	402,316.9	0.99	16	16
	187	2.1	0.63	139	1.7	0.57	116	1.5	0.50	33	
Caski (10 ng)	9,020,079	113,103.0	1.00	8,807,582	110,605.2	1.00	5,582,647	70,612.8	0.99	16	16
	37	0.4	0.16	14	0.2	0.12	10	0.1	0.10	45	
SiHa (100 ng)	134,664	1,679.0	1.00	130,955	1,638.0	1.00	87,797	1,110.5	0.98	16	16
	75	0.9	0.22	65	0.8	0.19	47	0.6	0.12	18	
SiHa (10 ng)	68,146	850.0	1.00	66,413	831.1	1.00	44,921	568.2	0.98	16	16
	94	1.0	0.33	66	0.8	0.30	59	0.7	0.29	31	
Hela (100 ng)	314,969	3,953.7	0.67	298,994	3,760.7	0.67	183,032	2,329.5	0.64	18	18
	1,339	16.0	1.00	1,100	13.8	1.00	659	8.3	0.90	16	
Hela (10 ng)	83,489	1,049.0	66.49	78,847	992.9	66.49	48,305	614.8	0.62	18	18
	610	7.3	0.99	409	5.1	0.98	225	2.8	0.77	16	
H <sub>2</sub> O	115	1.5	0.29	110	1.4	0.27	69	0.9	0.18	18	Negative
	12	0.1	0.13	2	0.0	0.02	1	0.0	0.01	16	
Placenta	1,800	21.3	1.00	1378	17.3	1.00	861	10.9	0.88	16	Negative
	46	0.5	0.26	37	0.5	0.24	23	0.3	0.16	18	

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477 \* Three mapping stringencies (L0.5-S0.8, L0.5-S1 and L1-S1) were evaluated using combination of parameters L  
 478 and S that represent read length (0.5 or 1 and similarity score (0.8 or 1), respectively.

479 \*\*HPV types determined following cut-off on number of mapped reads  $\geq 1000$ , average coverage  $\geq 20$  and the  
 480 fraction of genome covered  $\geq 0.5$  under L1-S1 mapping stringency.

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485 **Table 2. HPV genotype determination in blinded samples based on target enrichment and whole genome**  
 486 **sequencing**

Sample No <sup>s</sup>	Sample type	No of reads mapped	Average coverage	Fraction of genome covered ( $\geq 0.5$ )	HPV type (WGS result) <sup>a</sup>	HPV type (LA result)	Concordance <sup>b</sup>
1	Plasmid	282773	3598.5	0.99	45	45	Yes
2	Plasmid	293528	3751.6	1.00	58	58	Yes
3	Plasmid	210381	2659.0	1.00	31	31	Yes
4	Plasmid	190903	2413.7	0.99	33	33	Yes
5	Plasmid	237806	2994.3	1.00	52	52	Yes
6	Plasmid	268460	3357.4	1.00	6	6	Yes
7	Plasmid	95100	1210.4	1.00	18	18	Yes
8	Plasmid	169355	2135.4	1.00	11	11	Yes
17 <sup>#</sup>	Cervicovaginal	16120	208.7	0.16	(34)		Yes, partial
		1736	379	0.85	64 (34 subtype)	64 (reclassified as 34 subtype)	
						81	
18	Cervicovaginal	7873	100.9	0.99	67	67	Yes
		10820	139.5	0.97	54	54	
		8012	101.4	0.91	70	70	
		3967	49.4	0.99	90 <sup>+</sup>		
19	Cervicovaginal	667344	8554.6	0.90	67	67	Yes
		675774	8535.7	0.97	42	42	
		680615	8425.5	0.91	89	89	
		345249	4372.5	0.82	59	59	
		170609	2105.2	0.53	83	83	
		36981	472.7	1.00	66	66	
		32800	419.2	0.72	58	58	
		13844	176.5	0.91	56	56	
20	Cervicovaginal				HPV-	72, 35, 52, 53, 54, 62, 81	No
21	Cervicovaginal				HPV-	HPV-	Yes
22	Cervicovaginal				HPV-	6, 53, 56, 62, 70	No
23	Cervicovaginal	135138	1711.5	0.73	59	59	Yes, partial
		110107	1392.2	0.99	40	40	
		95442	1193.2	0.99	87 <sup>+</sup>		
		73386	940.7	0.84	67	67	
		49125	638.0	0.87	73	73	
		36101	452.7	0.98	43 <sup>+</sup>		
		29721	375.9	0.97	16	16	
		13891	171.4	0.73	83	83	
		9380	119.9	0.99	66	66	
		3091	39.5	0.99	68a*		
		2121	27.0	0.85	56	56	
						45, 51, 61, 89	
24	Cervicovaginal	2503879	31916.9	0.99	56	56	Yes, partial
		2221727	27974.4	1.00	52	52	
		1054024	13048.1	0.99	89	89	
		598662	7506.7	0.90	43 <sup>+</sup>		
		266588	3318.7	0.98	90 <sup>+</sup>		
		213130	2692.1	0.95	42	42	
		50171	628	0.66	61	61	
		40905	523.9	0.99	51	51	
		31221	390.3	0.98	87 <sup>+</sup>		
						53, 68b	
25	Cervicovaginal				HPV-	66, 18, 31	No

26	Cervicovaginal	10277696	129998.7	1.00	<b>16</b>	<b>16</b>	Yes, partial
		428009	5411.7	0.97	<b>40</b>	<b>40</b>	
		184266	2310.5	0.86	<i>43*</i>		
		127862	1609.9	0.99	<b>52</b>	<b>52</b>	
		118695	1490.0	0.83	<i>91*</i>		
		90888	1148.0	0.99	<b>42</b>	<b>42</b>	
		3722	46.0	0.66	<b>62</b>	<b>62</b>	
		2610	33.3	0.96	<b>39</b>	<b>39</b>	
				72, 83			
27	Cervicovaginal				HPV-	26, 42, 58, 83, 84	No
28	Cervicovaginal	9041	115.1	0.99	<i>30*</i>	35, 53	No
29	Cervicovaginal	23813	295.1	0.76	<i>114*</i>	40, 54, 66, 84, 89	No
30	Cervicovaginal	2102063	26836.0	1.00	<b>39</b>	<b>39</b>	Yes
		1743872	22288.8	0.99	<b>66</b>	<b>66</b>	
		81139	1039.2	0.85	<b>51</b>	<b>51</b>	
		49403	617.8	0.62	<b>6</b>	<b>6</b>	
		12102	152.6	0.96	<b>11</b>	<b>11</b>	
31	Cervicovaginal				HPV-	HPV-	Yes
32	Plasmid	270183	3417.4	1.00	<b>16</b>	<b>16</b>	Yes

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488 Bold font = types detected by both assays; Italics font = type not included in LA assay

489 <sup>§</sup>Sample numbers 1-8 multiplexed with control samples in pool 1 and samples 17-32 multiplexed in pool 2.

490 Sequence information is given for only those passed the signal/noise cut-off for HPV type determination

491 <sup>\*</sup>HPV types not included in LA but detected by WGS492 <sup>&</sup>HPV concordance based on WGS and LA results.493 <sup>#</sup>Note, HPV 64 has been reclassified as subtype of HPV 34. The only reference sequence available for HPV 64 is

494 an L1 fragment. On post-hoc assessment, results for this sample are assigned to HPV 64 based on reads mapped

495 to L1 fragment with good coverage, and reads mapping to HPV 34 with low genome coverage.

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497 **Table 3: Performance of different RNA bait evaluation metrics based on WGS mapping results**  
 498 **under L1-S1 from plasmid samples**

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Sample No	HPV type (eWGS result)*	Bait design coverage (%) <sup>@</sup>	HPV genome coverage by mapped reads <sup>§</sup> (%)	HPV reads mapped to predicted type (%) <sup>#</sup>	Uniformity of coverage (%) <sup>&amp;</sup>
1	45	100	99.0	99.8	96.3
2	58	100	100	99.7	96.3
3	31	100	100	99.7	96.3
4	33	100	99.2	99.2	96.3
5	52	100	100	99.7	96.3
6	6	99.2	100	99.8	96.3
7	18	100	100	100	96.3
8	11	100	100	99.5	92.6
32	16	100	100	99.8	96.3
Mean		99.9	99.8	99.6	95.9

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501 \*HPV type determined by eWGS for the corresponding samples numbers (see Table 2)

502 <sup>@</sup>Proportion of the HPV genome covered by the bait design criteria

503 <sup>§</sup>Proportion of the HPV reference genome covered by the mapped reads

504 <sup>#</sup>Proportion of reads that mapped to HPV predicted type compared to the total HPV reads

505 <sup>&</sup>Uniformity of coverage across the genome was calculated as the percentage of bins with coverage within the  
 506 average read depth  $\pm 2SD$  for all bins (see methods for details).

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515 **Figure legends**

516 **Figure 1:** Laboratory work-flow for HPV genotyping following RNA bait-based target enrichment and whole  
517 genome sequencing.

518 **Figure 2:** Number of reads (A) and mean base quality (Q) score of reads (B) passing the default filtering of  
519 Illumina BCL12fastq V1.8.4 software. Reads restricted to 0 mismatches in 8bp index reads.

520 **Figure 3.** Performance of RNA baits for internal control human beta-globin gene based on number of reads (A),  
521 average coverage (B) and fraction of reference sequence covered by the reads (C).

522 **Figure 4.** Mapping results showing high specificity of RNA baits restricted to the HPV 18 genome integrated to  
523 the HeLa genome. Reduced fraction of HPV genome covered by the sequenced reads due to deletion of central  
524 region of HPV 18 genome (the central region indicated within the two dashed vertical lines), compatible with a  
525 deletion due to integration, is shown reproducibly with 100 ng (A) and 10 ng (B) input DNA. The stringent L1-S1  
526 mapping conditions result in small gaps in the consensus sequence due to mismatched reads. Mapping results  
527 schematically aligned to HPV genome with the location of “early” and “late” region genes (C).

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