RAPID AND AFFORDABLE HPV GENOTYPING USING CAPILLARY GEL ELECTROPHORESIS

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ABSTRACT:

Cervical cancer, the second most prevalent cancer in women worldwide, is linked to Human Papillomavirus (HPV). Early detection, like most cancers, increases the success of treatment by orders of magnitude. PAP tests at best indicate the possibility of cervical cancer (sensitivity of 51%). Organizations like ACOG, ASCP and USPSTF have come up with guidelines for Cervical cancer screening which recommend HPV co-testing for women in the age group of 30 to 65 years old. These guidelines further suggest analysis for HPV types 16 and 18 in samples that are positive for HPV and negative for PAP. To address these testing requirements we have developed a rapid, low cost and accurate genotyping methodology that not only addresses the typing of HPV types 16 and 18, but also allows identification of all high risk, and if the need arises, low risk HPV genotypes. Identification of genotypes besides types 16 and 18 has multiple advantages. All FDA approved high risk tests on the market have been shown to amplify low risk HPV types at higher probability and therefore produce false positive results for high risk HPV type. This test can be used as confirmation of high risk types if clinically indicated. Furthermore, studies have shown that certain types of HPV (16, 18, 52 and 58) have a much higher rate of re-occurrence after colposcopy than other types, providing a prognostic indicator for the physician relative to patient follow-ups. Identification of other types besides 16 and 18 can also have epidemiological value such as studies of the effectiveness of the current HPV vaccines.

Our criteria for development of the test were for it to be rapid, accurate, low cost and easy to perform. We decided to utilize a PCR/RFLP methodology combined with Capillary Gel Electrophoresis (CGE) for accurate size determination and

automation. PCR/RFLP methodologies are cumbersome and time consuming using agarose or acrylamide gel electrophoresis and gel interpretations. CGE allows an automated approach that addresses the shortcomings of slab-gel electrophoresis by offering fast separations with high sensitivity, excellent resolution, and ease of operation. However, CGE is usually avoided due to high cost of instrumentation and per sample cost. We employed the BiOptic's Qsep100 CGE system as an economical alternative with an instrumentation cost well below similar units and a cost per run of 20 cents per sample, bringing down the cost per sample to less than $7.00.The use of this system allows sample analysis time of 7 hours for 24 genotypes, which includes a total of 1.5 hours of technician time.

30 HPV positive samples by Gen-Probe Aptima HPV with varying RLU's were chosen for this study. All samples were genotyped by both PCR/RFLP and Innogenetics HPV LIPA assay. 11 samples, including discrepant ones were independently genotyped by Hologic/Gen-Probe for comparison and validation purposes. We observed 87% concordance between the Line Probe assay and our methodology, with a cost savings of over 80% and time savings of over 50% for 24 samples.

METHODS:

SAMPLES: Residual cell suspension from cervical specimen collected in either Hologic's PreservCyt® or BD's Surepath® for thin cell layer preparation was used in this study. After the samples were processed for thin-layer slide preparation and HPV detection, positive samples were split into 2 parts for detection of HPV by our PCR/RFLP method or Line Probe Assay

EXTRACTION: We used the MagnaPure LC automated nucleic acid isolation platform (Roche Applied Sciences) in conjunction with Magna Pure LC DNA Isolation Kit for isolating the viral DNA. 1 ml of either PreservCyt or Surepath liquid based cervical cell suspension was centrifuged at 5000g for 5 minutes and the cell suspension was brought up in 250ul of PBS. The extraction volume was always 200ul of cervical cell suspension in PBS and the final elution volume was 65ul.

SET-UP AND CYCLING PARAMETERS:

Set-up		**Cycling Parameters**	
MgCl2	4ul	a. Initial PCR activation:	95 °C, 4 min 1 cycle
Master Mix	11ul	b. cycling:	94 °C, 30 sec 35 cycles
HPV Primers (MY09/11)	6ul		55 °C, 30 sec
DNA	2ul		72 °C, 30 sec
Water	2ul	c. Final extension:	72 °C, 8 min 1 cycle
RXN Total	25ul		

DIGESTION AND CLEAN-UP:

10ul PCR products were digested with 4 different endonucleases (Pst I, Hae III, Rsa I, and DdeI) for 1 hour at 37 degrees and subsequently cleaned by using Edge Biosystem's Quick step 2 PCR purification kit primarily to remove the salts from samples. All samples were then diluted three fold in BiOptic's DNA Dilution Buffer prior to Capillary Gel Electrophoresis.

Qsep100 Capillary Gel Electrophoresis (Figure 2):

The Gel-Cartridge used was a 50 μm I.D. Capillary with S1 BiOptic gel-matrix (Linear Polymer + EtBr), 15 cm total length and 12 cm to the detector window. The samples were run with 4 KV, 4 second injection and 8 KV for 220 seconds of Separation. BiOptic LED-Induced Fluorescence detector Excites at 520nm and detects between 590 to 650 nm Emission wavelengths.

INNO-LIPA HPV Genotyping RUO kit (Figure 1):

We followed the manufacturer's suggested protocol with the exception of using Magna Pure

RESULTS AND DISCUSSION:

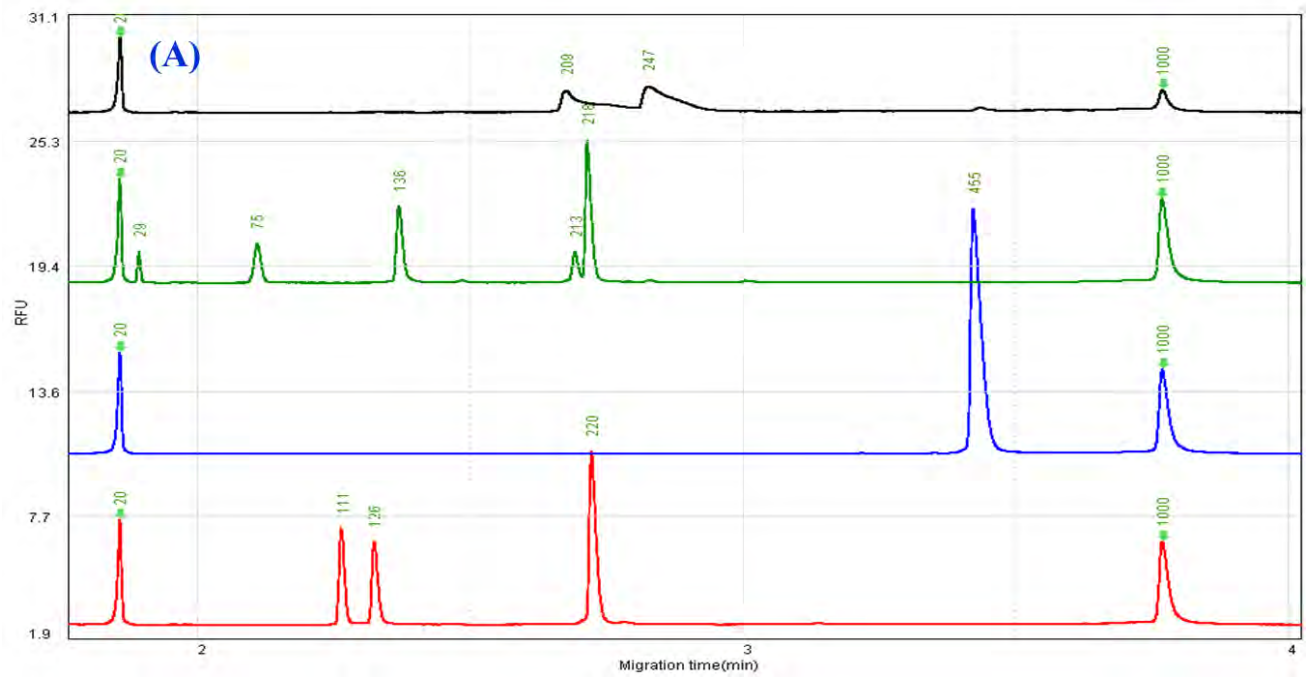
Four different restriction endonucleases were chosen to discriminate between all High Risk HPV types (Table 1). Positive samples with varying RLUs by Aptima HPV were used for all genotyping methods to ascertain the effects of low RLUs on different methodologies. The results of the genotyping are detailed in Table 2. 24 of the 30 samples tested by our new methodology agreed with either the INNO-LIPA assay or the Roche LIPA assay performed at Hologic. There were four samples with no PCR products by either our assay or the LIPA that were positive by Aptima HPV and Negative by cytology. There was one sample with different results by all three methodologies. In all, we were able to demonstrate that the methodology provided a fast, economical and accurate alternative to commercially available assays. The use of the Capillary Gel Electrophoresis unit proved to provide an automated easy to use system by generating clear peaks with accurate size calls (Figure 1). One of our concerns was the versatility of our methodology in distinguishing multiple HPV types from single samples but we were pleasantly surprised to see that the system was capable of distinguishing at least two different types as demonstrated in Figure 3. One area of concern was the number of samples that did not yield any PCR products either by our assay or by either LIPA assays. In particular there were four samples that were clearly positive by Aptima HPV assay and negative by our assay or the LIPA. Curiously, the cytology result for the same samples were negative. Assuming the sensitivity of the Aptima HPV to be superior to the cytology results, the only difference that could be identified was the extraction methodology. We intend to further investigate by utilizing different extraction methodologies.

HPV TYPE	AMPLICON (bp)	Pst I	Hae III	Dde I	Rsa I
16	452	216,210,26	444,8	452	310,72,70
18	455	242,213	455	432,23	135,125,85,72,38
18a	455	242,213	455	432,23	260,85,72,38
31	452	216,210,26	328,14	283,167,2	380,72
31a	452	216,210,26	328,15	283,90,77,2	380,73
33	449	242,207	449	320,77,52	236,102,72,39
35	458	426,26	261,180,8,3	294,135,23	177,161,72,42
45	452	242,213	447,8	324,131	338,72,45
45a	455	242,214	447,9	324,132	237,101,72,45
45b	455	242,215	447,10	324,133	203,180,72
45c	455	242,216	447,11	324,134	180,158,72,45
51	455	452	379,73	362,90	380,72
52	452	423,26	258,183,8	357,92	449
52a	449	423,27	258,183,9	246,107,96	449
56	449	242,207	275,166	307,142	310,72,49,18
58	449	216,207,26	449	348,101	306,111,32
59	455	429,26	399,56	455	455
66	449	207,150,66,26	449	291,158	449
66a	449	207,150,66,27	441,8	291,128,30	449
66b	449	207,150,66,26	449	291,158	377,72
68	455	455,386,69	455	455	260,85,72,38

TABLE 1. This table provides fragment sizes for different HPV types by restriction enzyme

ACCESION NUMBER	Aptima-result	Genotyping-Armalil	Genotyping- Innogenetics	Genotyping-Genprobe	Cytology-Primex
4306698	2.6	66	66	Not Tested	NEG
4313611	12	16	No PCR Product	Not Tested	ASCUS
4314430	11.4	31	31,58	Not Tested	ASCUS
4315199	12.5	No PCR Product	No PCR Product	Not Tested	NEG
4315942	4.6	No PCR Product	No PCR Product	Not Tested	NEG
4316049	2.2	No PCR Product	indeterminate	Not Tested	NEG
4317148	3	No PCR Product	No PCR Product	Not Tested	NEG
4352381	11.2	31	31	No PCR Product	ASCUS
4352497	16.6	56	51	51,56	ASCUS
4352994	8.7	52	18,51	No PCR Product	NEG
4353534	9.4	11	11,44	QNS	ASCUS
4353942	24.4	45,52	16,31,58	45,52	ASCUS
4354786	11.8	58	58	58	N/A
4355042	4.6	52	52	51,52	ASCUS
4355092	11.5	18	18	18,84	ASCUS
4355213	8.9	66	66	Not Tested	ASCUS
4355701	18.5	66	66	Not Tested	ASCUS
4357069	9	No PCR Product	68	Not Tested	NEG
4402052	0.5	66	6,66	66	ASCUS
4403200	11.9	16	16,18	16,18	ASCUS
4405070	16.7	18	18	18	AQU. CELL. CARC.
4405828	10.5	16	16	Not Tested	ASCUS
4406354	21	52	31	Not Tested	Cin II-II,HSIL
4407649	8.6	6,66	6,66	Not Tested	ASCUS
4407652	2.8	82	82	Not Tested	ASCUS
4407654	11.4	53	53	Not Tested	ASCUS
4408326	6.5	No PCR Product	35	Not Tested	ASCUS
4409190	11.1	No PCR Product	31	Not Tested	ASCUS
4409964	6.7	16	16	Not Tested	ASCUS
4411356	11.9	31	31	Not Tested	NEG

TABLE 2. This table details all the genotyping and the cytology results for the 30 samples tested. Samples highlighted in Red show non-concordance



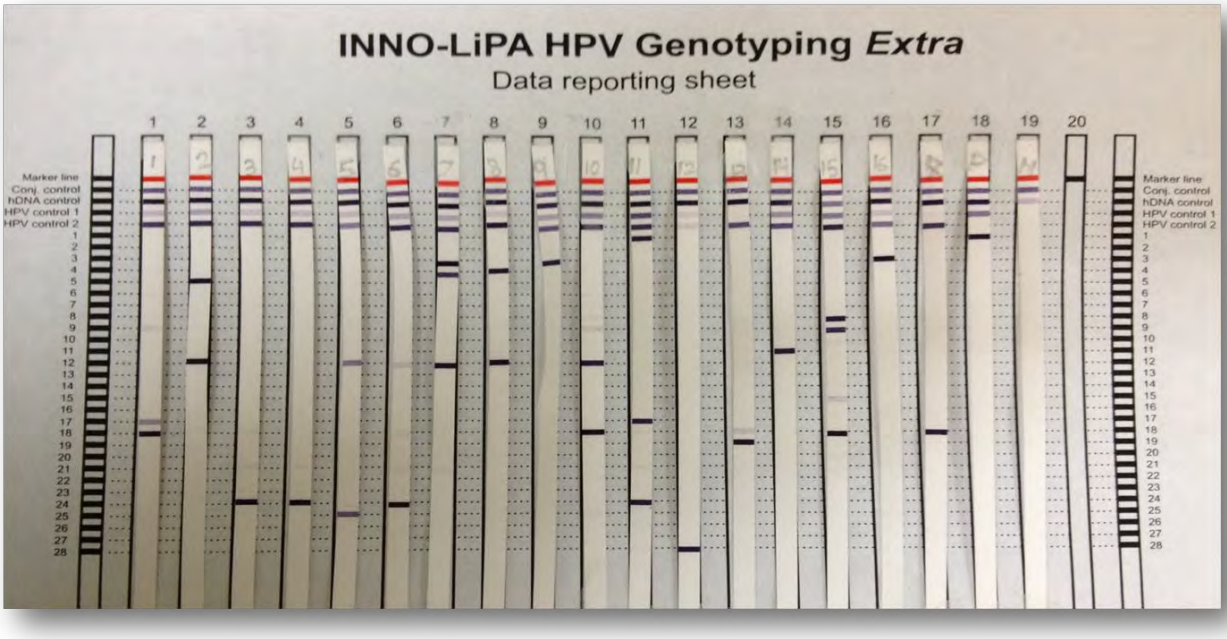
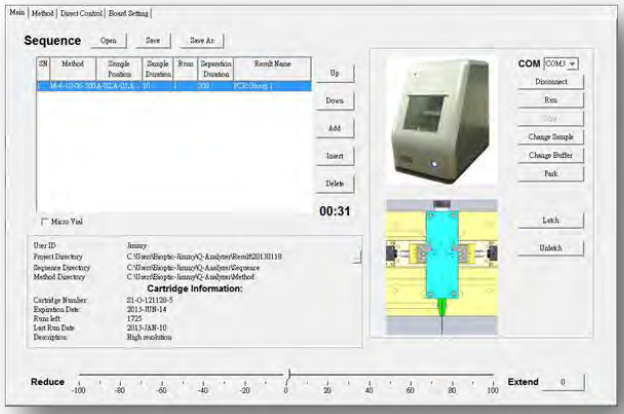


FIGURE 1. Genotyping results by PCR/RFLP (**A**) and LIPA assay. Interpretation of the results from the PCR /RFLP can be automated and is objective while The LIPA assay results interpretation is subjective.


(A)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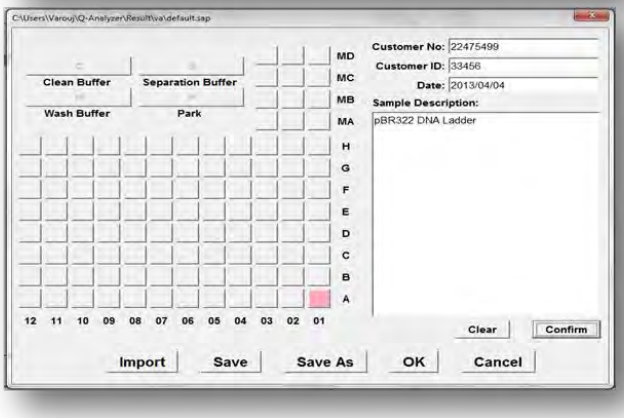
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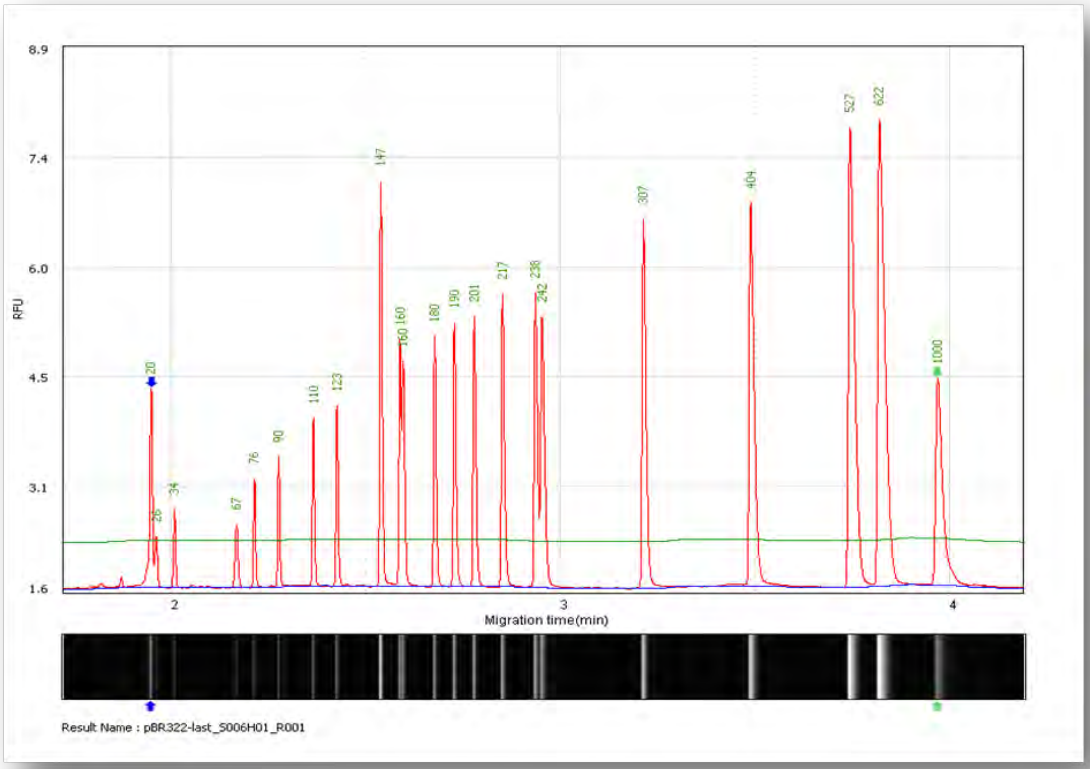
(B)



(C)

FIGURE 2. The Qsep100 system is the basis of Capillary Gel Electrophoresis (CGE) technology providing post PCR separation and detection of DNA Fragments. The system is comprised of the Detection Engine (LED-Induced Fluorescence), Analysis Engine, the Sample Loader and the Disposable Pen-Shaped Gel-Cartridge. The pen-shaped gel-electrophoresis cartridge can perform 200 tests at 2-7 minutes per sample run. Novel reagent packaging and intuitive software provides ease of operation, automated tracking of consumables and overall management of the genotyping system.Qsep100 (**A**) was used for detection of the type specific HPV beads. The Q-Analyzer software (**B**) allows for easy interface and analysis. DNA Ladder result (**C**) demonstrates the resolution power of the system.

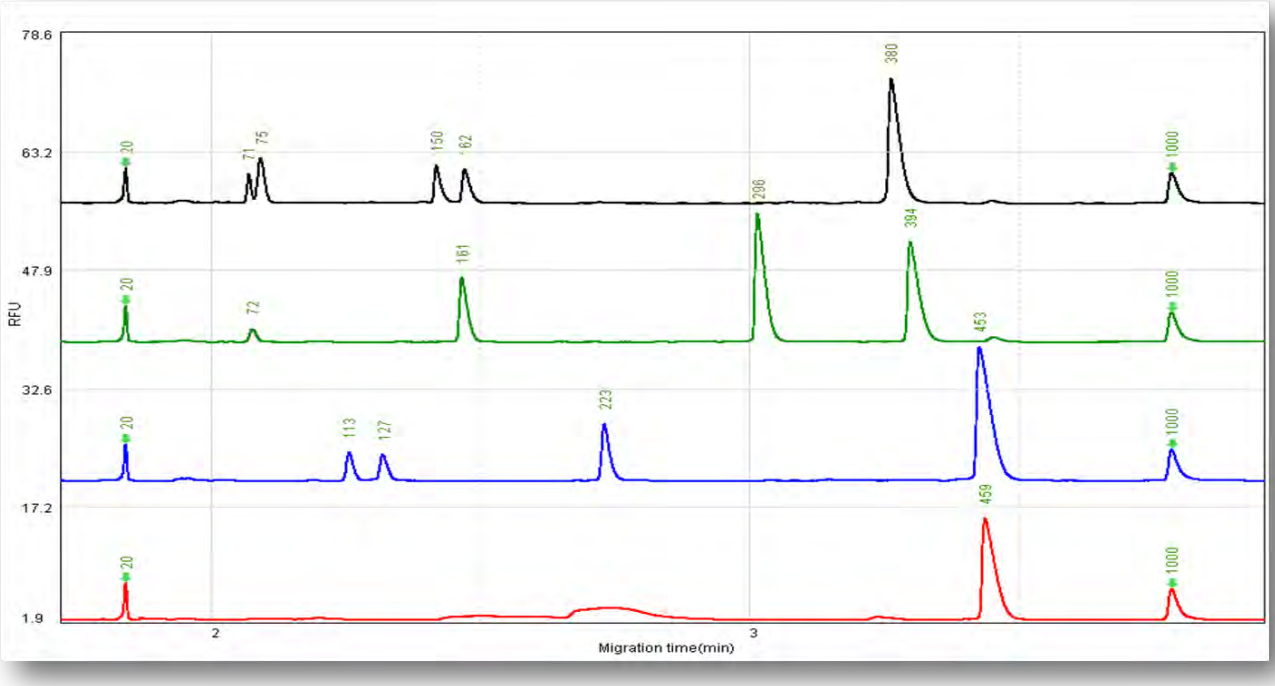


FIGURE 3. This figure demonstrates the capability of the PCR/RFLP methodology in determining co-infections of different HPV types. The above sample was determined to have a high risk Genotype HPV (type 66) and a low risk genotype HPV (type 6).

SUMMARY:

- Qsep100 PCR/RFLP HPV Typing system can be effectively used for genotyping of HPV from Liquid PAP media.
- This methodology has the advantage of detecting expanded panel of both High risk and Low risk genotypes and co-infections; a coverage lacking from current FDA approved assays.
- This methodology is cost efficient with reasonable throughput, 30 samples in 24 hours.