CHAPTER 4

High-throughput genotyping of High-Risk HPV by the digene HPV Genotyping LQ Test using GP5+/6+-PCR and xMAP technology

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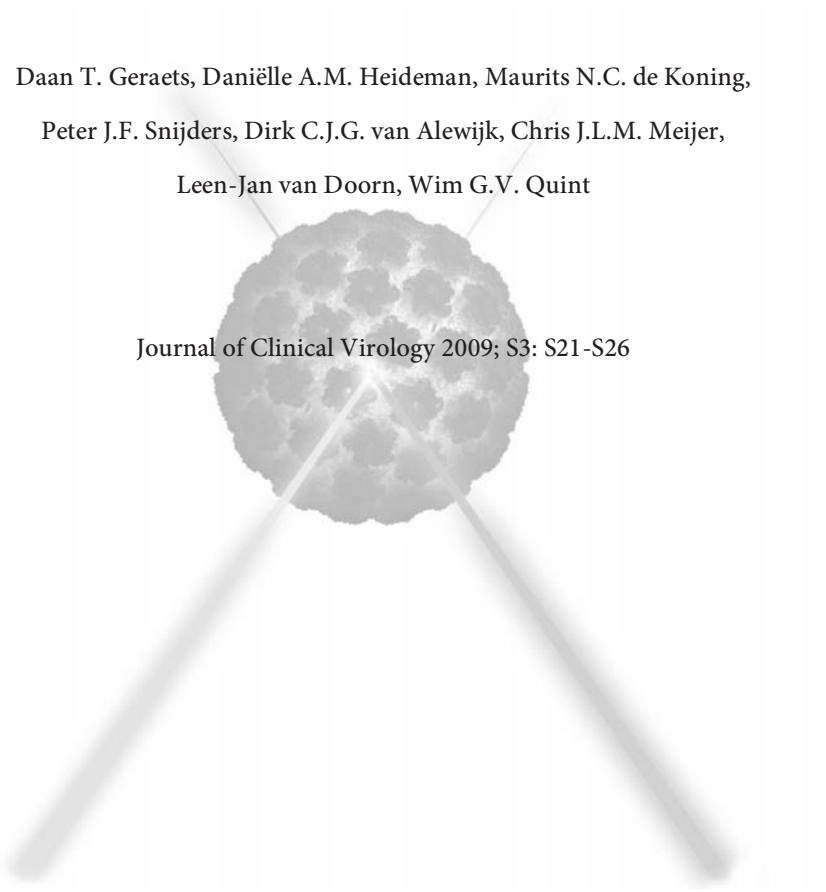

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High-throughput genotyping of high-risk HPV by the *digene* HPV Genotyping LQ Test using GP5+/6+-PCR and xMAP technology

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SUMMARY

Background: Epidemiologic studies have classified 18 genotypes of the human papillomavirus (HPV) as (probably) high-risk (HR) based on their association with cervical cancer, i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. Given the fact that certain HR HPV types confer an increased risk of cervical (pre)cancer, type-specific identification might aid clinical management of women tested positive for HR HPV. Therefore, the development of robust, high-throughput genotyping assays is important.
Objectives: An analytical comparison of the *digene* HPV Genotyping LQ Test (*digene* LQ Test), capable of identifying 18 HR types using bead-based xMAP suspension array technology, with the established Reverse Line Blot (RLB) genotyping assay was carried out on amplimers generated with the clinically validated GP5+/6+-PCR method.
Study design: GP5+/6+ amplimers, generated from 434 *digene* High Risk HPV HC2 DNA Test (HC2)-positive and 95 HC2-negative cervical smears, were genotyped by both the *digene* LQ Test and the RLB genotyping assay.
Results: The genotyping assays revealed high agreement for overall HR HPV detection ($\kappa = 0.884$) and type-specific identification of the 18 HR HPV types (overall $\kappa = 0.958$, individual κ range 0.795 to 1.000). The *digene* LQ Test demonstrated a very good inter-laboratory reproducibility ($\kappa = 0.987$). Among the HC2-positive women, the *digene* LQ Test revealed positivity for one or more HR HPV type(s) in 85.9%, and negativity was observed in 97.9% of the HC2-negative women.
Conclusions: The *digene* LQ Test demonstrated a high genotyping agreement with the established RLB genotyping assay on GP5+/6+ amplimers. This novel assay allows for high-throughput genotyping following HR HPV testing by HC2.

Abbreviations

HPV:	human papillomavirus
HR:	high-risk
RLB:	reverse line blot
PCR:	polymerase chain reaction
HC2:	Hybrid Capture 2
DNA:	deoxyribonucleic acid
FDA:	Food and Drug Administration
RCS:	Rapid Capture System
EIA:	enzyme immunoassay
CIN:	cervical intraepithelial neoplasia
UCM:	Universal Collection Medium
RNA:	ribonucleic acid
RLU/CO:	relative light unit per cutoff value
LR:	low-risk
MFI:	median fluorescent intensity

1. Introduction

Epidemiologic research has classified 18 genotypes of the human papillomavirus (HPV) as (probably) high-risk (HR), based on their association with cervical cancer, i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82.[1] Results from several randomized trials indicate that HR HPV testing has a higher sensitivity for the detection of cervical (pre)cancer, than current cytology-based cervical screening. This argues for implementation of HR HPV testing to increase the effectiveness of screening programs. Until recently, based on these trials, two HPV assays could be considered as clinically validated for the detection of cervical high-grade precursor lesions and cancer (≥CIN 2).[2] One is the FDA-approved *digene* Hybrid Capture 2 High-Risk HPV DNA Test (HC2) (QIAGEN, Gaithersburg, USA). This assay uses chemiluminescent signal amplification for the simultaneous detection of 13 HR types and can be applied in a high-throughput format using an automated Rapid Capture System (RCS) (QIAGEN, Gaithersburg, USA). The other assay involves the home-brew

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HR HPV GP5+/6+-PCR EIA,[3] which uses target amplification by PCR and subsequent detection with a probe cocktail for 14 HR types. Recently, the FDA approved two more assays, the Cervista HPV HR (detection of 14 HR types) and Cervista HPV 16/18 (genotyping of HPV 16 and 18) tests.

Since the oncogenic potential differs between the HR HPV types,[4–6] viral genotyping following HR HPV detection may be useful for better risk stratification of HR HPV-positive women. Genotyping technologies additionally allow monitoring of viral persistence, a condition known to be relevant for cervical (pre)cancer development.[7]

Given the fact that the HC2 and GP5+/6+-PCR EIA HR HPV detection systems are clinically compatible, a scenario for a combined test has been proposed. The (automated RCS format of) HC2, being robust and ideal for high-throughput screening, is used first, and the GP5+/6+-PCR functions as a reflex test for genotyping of HC2-positive specimens.[8]

To that end, a novel, commercial reverse hybridization assay (*digene* HPV Genotyping RH Test) for the identification of 18 HR HPV types on GP5+/6+-PCR products was developed and analytically compared to the established Reverse Line Blot (RLB) genotyping assay,[3] as described in an accompanying paper.[9] The *digene* HPV Genotyping RH Test can be carried out manually as well as in an automated procedure.

A next step in automation is the use of high-throughput read-out systems for genotyping. The novel *digene* LQ Test presented in this study is such a method. This assay uses multiplex, bead-based xMAP technology and an automated, high-throughput read-out by either the LiquiChip 200 workstation (QIAGEN, Hilden, Germany) or Luminex 100 IS System (Luminex Corporation, Austin, TX, USA). The test was developed for identification of the 18 HR HPV genotypes associated with cervical cancer[1] using GP5+/6+-PCR products. Read-out of the *digene* LQ Test is expressed as the median fluorescent intensity (MFI) of the reporter fluorescence for each genotype. Positivity for the respective types is calculated from the MFI over a defined threshold level, and can provide a semi-quantitative numerical output.

In this study, an analytical comparison between the *digene* LQ Test and the established RLB genotyping assay was performed using the same GP5+/6+-PCR products. Cervical specimens of women tested by HC2 in a population-based cervical screening were amplified by the GP5+/6+-PCR. Generated amplimers were genotyped by both the *digene* LQ Test and the RLB assay in a blinded manner and genotyping results were compared.

2. Materials and methods

To investigate the performance of the *digene* LQ Test (QIAGEN), GP5+/6+-PCR products were available from an evaluation study of the *digene* HPV Genotyping RH Test (QIAGEN). PCR products were derived from clinical samples collected during the course of a population-based controlled intervention trial. The trial was approved by the national ethical committee (Ministry of Public Health) and all women enrolled had given informed consent. From 434 HC2-positive and 95 HC2-negative cervical samples sufficient amounts of amplimers were present. GP5+/6+-PCR products from an HPV cloned plasmid test panel were also included in this study. Details on the plasmid panel, clinical specimens, HC2 testing, processing, DNA amplification, and RLB assay analysis are provided in an accompanying paper.[9]

2.1. digene HPV Genotyping LQ Test (digene LQ test) analysis

The *digene* LQ Test utilizes probes for 18 HR HPV types (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) that are the same as the respective RLB-probes, with minor modifications, and are immobilized on color-coded beads. The *digene* HPV Genotyping LQ Test, Detection Kit was performed in the Luminex 100 IS System (Luminex Corporation) according to the manufacturer's instructions. In brief, 3B Buffer was added to the High-Risk HPV Beads to minimize background in the final Luminex read-out. Subsequently, GP5+/6+-PCR products were added. Next, heat-denaturation, hybridization under stringent conditions, and incubation with streptavidin-conjugated R-phycoerythrin detection conjugate were followed by read-out according to the specified instrument settings, resulting in MFI levels per HPV type for each specimen.

Testing with both the RLB assay and the *digene* LQ Test was performed in parallel by different technicians who were unaware of each other's test results and blinded to HC2 status of the samples.

2.2. Assessment of viral load for HPV 52

HPV 52 type-specific DNA load as well as the β-globin DNA load were determined by real-time PCR assays on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) in separate reactions to quantify the number of HPV 52 copies and the number of cells, respectively. The LightCycler assays and the nucleotide sequences of primers and probes have been described in detail elsewhere.[10] The LightCycler reactions were run in duplicate and values obtained were averaged. The viral load for each sample was calculated by dividing the HPV copy number by the number of cells and expressed as copies per cell.

2.3. Data analysis and statistics

For analytical comparison of the *digene* LQ Test to the RLB assay, two threshold levels for type-specific positivity, i.e., 100 MFI and 30 MFI, are presented in this study. The *digene* LQ Test kit insert recommends using a cut-off of 100 MFI. However, due to the very low background MFI signals, a lower threshold of 30 MFI was also evaluated to demonstrate the potentially higher sensitivity of the *digene* LQ Test. For determining the agreement of overall HR HPV detection, both assays were considered HR HPV-positive when genotyping revealed one or more of the 18 HR HPV genotypes that can be detected by both assays (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). Furthermore, for comparison to HC2 a distinction was made between HR HPV positivity for the 13 HR HPV types targeted by HC2 (i.e., HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and the complete group of 18 HR HPV types targeted by the *digene* LQ Test.

To investigate the agreement between both genotyping assays for one or more of the 18 HR HPV types, results were considered either as concordant (both methods yielded completely identical genotyping results), compatible (both methods showed one or more of the same genotype[s]), or discordant (no similarity between genotypes detected by both methods).[11] Since the *digene* LQ Test does not distinguish between IS39 and MM4 subtypes of HPV 82, positive RLB results for these subtypes were regarded as HPV 82-positive.

The two-tailed McNemar's test was used for mutual comparison of the positivity rates of the RLB assay and the *digene* LQ Test. The level of agreement was determined using Cohen's kappa statistics. Level of statistical significance was set at 0.05. All analyses were performed using SPSS version 15.0.

3. Results

3.1. Analytical performance of the digene LQ Test

The specificity of the *digene* LQ Test was evaluated with GP5+/6+-PCR products derived from a panel of cloned HPV types

Table 1
Type-specificity of the *digene* LQ Test[a]

Probe for	Amplimers for HPV type																			
	16	18	26	31	33	35	39	45	51	52	53	56	58	59	66	68	68a	73	82	
																			IS39	MM4
CC	9014	9046	8660	8863	9465	8941	9242	8968	9211	8577	9327	9057	9067	8935	8828	8613	8715	8623	9049	8485
HPV 16	2268	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HPV 18	0	2396	1	0	0	1	0	1	1	1	1	0	1	1	1	1	0	1	0	1
HPV 26	1	1	1255	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
HPV 31	1	1	1	1822	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1
HPV 33	0	1	1	1	2651	1	1	1	1	2	1	0	1	1	1	1	1	1	0	1
HPV 35	1	1	1	1	1	1748	5	1	1	1	1	1	1	2	2	2	1	1	2	2
HPV 39	1	0	1	1	1	1	2599	1	1	1	0	0	0	1	1	2	1	1	1	1
HPV 45	1	1	1	1	1	1	1	1667	1	1	1	1	1	1	1	1	1	1	1	0
HPV 51	1	1	1	1	1	1	1	1	3233	1	1	1	1	1	1	1	1	1	1	1
HPV 52	1	1	1	1	1	1	1	1	1	3143	1	1	1	1	1	1	1	1	1	1
HPV 53	1	1	1	1	1	1	1	1	1	1	3695	1	1	1	1	1	1	1	1	1
HPV 56	1	1	1	1	1	1	1	1	1	1	1	1804	1	1	1	2	1	1	1	2
HPV 58	1	0	1	1	1	1	1	1	0	1	1	1	1906	1	1	1	1	1	1	1
HPV 59	1	1	1	1	1	1	1	1	1	1	1	1	1	2481	1	1	1	1	2	1
HPV 66	2	2	1	2	1	1	2	2	2	1	2	2	2	2	941	2	2	9	2	2
HPV 68	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2064	2	1	1	1
HPV 68	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	2	2784	1	1	1
HPV 73	1	2	2	2	1	2	2	2	1	2	1	1	1	2	1	2	1	2483	2	2
HPV 82	1	1	2	1	1	2	1	2	1	2	2	2	2	2	1	2	2	1	1999	2
HPV 82	1	2	2	1	2	1	2	2	2	2	1	1	2	2	1	2	2	1	2	2190

[a] Indicated are the MFI read-outs (without background substraction) of GP5+/6+ amplimers generated from different HPV plasmids (listed on top) in relation to the probes (listed on the left) included in the *digene* LQ Test. The conjugate control (CC) serves as the positive control for correct incubation with the detection conjugate for each separate specimen.

(i.e., HPV 6, 11, 13, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 68a, 69, 70, 73, 74, 82IS39, 82MM4, and 85)[a]. Amplimers of the targeted types demonstrated specific hybridization with MFI values between 941 and 3695, while background read-out varied between 0 and 9 (Table 1). None of the untargeted types had an MFI value above 11 (data not shown).

3.2. Inter-laboratory reproducibility of the digene LQ Test

digene LQ Test analysis of the same GP5+/6+ amplimers derived from a series of cervical samples (n = 92) at two different locations revealed excellent inter-laboratory reproducibility at a cut-off of 100 MFI ($\kappa = 0.987$). The only discrepancy was in the recognition of HPV 52 for two samples (MFI = 122 and 105 vs. 65 and 49; P = 0.500, Table 2). At 30 MFI, inter-laboratory agreement was slightly higher ($\kappa = 0.994$), with only one discordant result for HPV 16 (MFI = 19 vs. 30; P = 1.000, Table 2).

3.3. Comparison of the digene LQ Test with the RLB assay for HR HPV detection

For comparison of overall HR HPV detection between the *digene* LQ Test and the RLB assay, we analyzed the same GP5+/6+ amplimers derived from a series of 529 cervical smears, previously testing positive (n = 434) and negative (n = 95) for HR HPV DNA by HC2. Results for the HC2-positive specimens are given in Table 3 in relation to the two thresholds for assay-positivity applied to the *digene* LQ Test (i.e., 100 MFI and 30 MFI, respectively). The genotyping assays demonstrated a high agreement in overall HR HPV detection, ranging from $\kappa = 0.884$ (95% CI: 0.820–0.949) at 100 MFI to $\kappa = 0.850$ (95% CI: 0.773–0.926) at 30 MFI. When applying a threshold of 100 MFI the detection rates were similar (P = 0.774), whereas at a cut-off of 30 MFI, the *digene* LQ Test detected significantly more samples positive for HR HPV than the RLB assay (P = 0.002).

At a cut-off of 100 MFI, the RLB identified 7 samples additionally positive, which included one triple infection with HPV 45, 52 and 58, as well as 6 single infections revealing HPV 39, 52 (3×), 56 and 59, respectively. The 5 samples that were scored positive for HR HPV only by the *digene* LQ Test contained single infections with types HPV 16, 39, 53 (2×) and 68, respectively. When considering a cut-off of 30 MFI, the additional 14 specimens recognized positive by the *digene* LQ Test as compared to the MFI 100 cut-off included HPV types 18, 31, 39, 51, 52, 59 and 68. In 6 of these samples the RLB assay confirmed HR HPV presence. Furthermore, RLB detected single infections with LR types (HPV 42 and 67, respectively) in 2 samples, whereas the remaining 6 samples were RLB-negative.

Irrespective of the cut-off level used, the *digene* LQ Test and RLB were able to confirm HR HPV negativity in 93/95 (97.9%) HC2-negative samples and found single infections with either HPV 16 or 45 in two respective cases (data not shown).

When restricting the analysis to the 13 HR HPV types detected by HC2 in the 434 HC2-positive cervical smears, and applying cut-offs of 100 and 30 MFI, the *digene* LQ Test scored 79.5% and 83.6% as HR HPV positive, respectively. Another 6.5% (100 MFI cut-off) and 5.5% (30 MFI cut-off) could be attributed to positivity for one or more of the five additional HR HPV types targeted by the

[a] Underlining indicates HPV types targeted by the *digene* RH Test.

Table 2
Inter-laboratory comparison of the *digene* LQ Test for 92 samples[a]

Genotype	Threshold 100 MFI					Threshold 30 MFI				
	No. of samples found positive in			κ	P-value[b]	No. of samples found positive in			κ	P-value[b]
	Laboratories I and II	Laboratory I	Laboratory II			Laboratories I and II	Laboratory I	Laboratory II		
HPV 16	20	0	0	1.000	1.000	20	0	1	0.969	1.000
HPV 18	6	0	0	1.000	1.000	8	0	0	1.000	1.000
HPV 26	0	0	0			0	0	0		
HPV 31	11	0	0	1.000	1.000	11	0	0	1.000	1.000
HPV 33	3	0	0	1.000	1.000	3	0	0	1.000	1.000
HPV 35	1	0	0	1.000	1.000	1	0	0	1.000	1.000
HPV 39	3	0	0	1.000	1.000	4	0	0	1.000	1.000
HPV 45	2	0	0	1.000	1.000	2	0	0	1.000	1.000
HPV 51	8	0	0	1.000	1.000	9	0	0	1.000	1.000
HPV 52	9	2	0	0.888	0.500	11	0	0	1.000	1.000
HPV 53	3	0	0	1.000	1.000	4	0	0	1.000	1.000
HPV 56	3	0	0	1.000	1.000	3	0	0	1.000	1.000
HPV 58	4	0	0	1.000	1.000	4	0	0	1.000	1.000
HPV 59	1	0	0	1.000	1.000	1	0	0	1.000	1.000
HPV 66	4	0	0	1.000	1.000	5	0	0	1.000	1.000
HPV 68	1	0	0	1.000	1.000	1	0	0	1.000	1.000
HPV 73	0	0	0			0	0	0		
HPV 82	1	0	0	1.000	1.000	1	0	0	1.000	1.000
Any type	80	2	0	0.987	0.500	88	0	1	0.994	1.000

[a] Data are presented as the number of each genotype detected on each location (i.e., laboratory I and/or II) and given for two general thresholds for positivity of the *digene* LQ Test, i.e., 100 MFI and 30 MFI.
[b] McNemar's test.

Table 3
Comparison of HR HPV detection by the RLB assay and the *digene* LQ Test in 434 HC2-positive cervical smears[a]

RLB assay	*digene* LQ Test			
	Threshold 100 MFI		Threshold 30 MFI	
	HR HPV+[b]	HR HPV−[c]	HR HPV+[b]	HR HPV−[c]
HR HPV+[b]	368	7	374	1
HR HPV−[c]	5	54	13	46

[a] Data are shown for two general thresholds for positivity of the *digene* LQ Test, i.e., 100 MFI and 30 MFI.
For threshold of 100 MFI: κ = 0.884 (95% CI: 0.820–0.949), P = 0.774.
For threshold of 30 MFI: κ = 0.850 (95% CI: 0.773–0.926), P = 0.002.
[b] HR HPV+ is defined as positive for HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and/or 82.
[c] HR HPV− is defined as negative for HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82.

digene LQ Test (i.e., HPV 26, 53, 66, 73 and 82). Amongst the 54 (100 MFI cut-off) or 46 (30 MFI cut-off) HC2-positive samples that were HR HPV-negative by both the *digene* LQ Test and the RLB assay, the latter detected 13/54 or 11/46 positive for LR HPV, including HPV types 6, 30, 42, 67 and 70 or types 6, 30, 67 and 70, respectively.

3.4. Comparison of HR HPV genotyping results of the digene LQ Test with the RLB assay

Next, the *digene* LQ Test and the RLB assay were compared for identification of individual HR HPV genotypes in HC2-positive women. In 434 HC2-positive cervical smears, both assays revealed genotyping results that were, depending on thresholds of 100 and 30 MFI, completely concordant in 399 (91.9%) and 380 (87.6%) samples, and compatible in 23 (5.3%) and 40 (9.2%) specimens, respectively. Discordance was observed in 12 (2.8%, 100 MFI) and 14 (3.2%, 30 MFI) smears.

The individual genotyping findings among HC2-positives are shown in Table 4. Genotyping agreement between the *digene* LQ Test and the RLB typing procedures was dependent on the choice of the cut-off level for positivity. Despite an overall high kappa value of 0.935, the *digene* LQ Test detected more genotypes at a threshold of 30 MFI than the RLB assay ($P < 0.001$). At a cut-off of 100 MFI the assays demonstrated a better agreement (overall $\kappa = 0.958$, and individual kappa values ranging from 0.795 to 1.000) and both assays detected a similar number of genotypes ($P = 0.143$).

Nonetheless, when using a threshold of 100 MFI, the RLB assay and *digene* LQ Test revealed a significant difference in the detection of HPV type 52 ($P = 0.004$). Interestingly, both assays were in better agreement for HPV 52 when the cut-off of 30 MFI was used ($\kappa = 0.949$, 95% CI: 0.900–0.999, Table 4). The RLB assay scored 9 additional HPV 52 as compared to the *digene* LQ Test at a cut-off of 100 MFI. Eight of these demonstrated MFI above 30 (range 33–96 MFI), and 6 comprised multiple infections.

Subsequent viral load analysis for HPV 52 revealed on average a low copy number (median of 0.6 copies/cell, range 0.0–3.2) in specimens demonstrating a low MFI (below 100) in the *digene* LQ Test, whereas samples with a high MFI contained on average high HPV 52 copy numbers (median of 29.6 copies/cell, range 4.5–304.6; Table 5).

4. Discussion

This study examined the *digene* LQ Test, a novel assay for fast, high-throughput genotyping of 18 HR HPV types yielding semi-quantitative results. The test was compared with the established RLB genotyping assay on the same group of GP5+/6+ amplimers generated from cervical samples derived from a screening cohort.

Table 4
Comparison of genotyping findings of the *digene* LQ Test and the RLB assay in 434 HC2-positive cervical smears[a]

Genotype	Threshold 100 MFI					Threshold 30 MFI				
	No. of samples found positive by			κ	P-value[b]	No. of samples found positive by			κ	P-value[b]
	digene LQ Test and RLB	*digene* LQ Test only	RLB only			*digene* LQ Test and RLB	*digene* LQ Test only	RLB only		
HPV 16	119	3	0	0.983 (0.963–1.000)	0.250	119	11	0	0.938 (0.902–0.974)	0.001
HPV 18	27	2	0	0.962 (0.909–1.000)	0.500	27	7	0	0.877 (0.787–0.967)	0.016
HPV 26	0	0	0			0	0	0		
HPV 31	63	1	1	0.982 (0.956–1.000)	1.000	64	2	0	0.982 (0.957–1.000)	0.500
HPV 33	16	0	0	1.000 (1.000–1.000)	1.000	16	2	0	0.939 (0.854–1.000)	0.500
HPV 35	9	0	1	0.946 (0.841–1.000)	1.000	10	1	0	0.951 (0.856–1.000)	1.000
HPV 39	26	1	2	0.942 (0.876–1.000)	1.000	28	5	0	0.912 (0.835–0.988)	0.063
HPV 45	22	0	2	0.954 (0.891–1.000)	0.500	23	0	1	0.978 (0.934–1.000)	1.000
HPV 51	40	0	1	0.986 (0.960–1.000)	1.000	40	4	1	0.935 (0.878–0.992)	0.375
HPV 52	34	0	9	0.872 (0.790–0.954)	0.004	42	3	1	0.949 (0.900–0.999)	0.625
HPV 53	8	4	0	0.795 (0.600–0.991)	0.125	8	5	0	0.756 (0.550–0.962)	0.063
HPV 56	22	0	1	0.977 (0.931–1.000)	1.000	22	1	1	0.954 (0.891–1.000)	1.000
HPV 58	24	0	2	0.958 (0.899–1.000)	0.500	24	0	2	0.958 (0.899–1.000)	0.500
HPV 59	8	1	2	0.839 (0.659–1.000)	1.000	9	3	1	0.813 (0.635–0.992)	0.625
HPV 66	26	0	3	0.942 (0.876–1.000)	0.250	29	5	0	0.914 (0.840–0.989)	0.063
HPV 68	5	1	0	0.908 (0.728–1.000)	1.000	5	3	0	0.766 (0.509–1.000)	0.250
HPV 73	6	1	0	0.922 (0.770–1.000)	1.000	6	2	0	0.855 (0.656–1.000)	0.500
HPV 82	2	0	0	1.000 (1.000–1.000)	1.000	2	0	0	1.000 (1.000–1.000)	1.000
Any type[c]	457	14	24	0.958 (0.944–0.971)	0.143	474	54	7	0.935 (0.919–0.952)	<0.001

[a] Data are shown for two general thresholds for positivity of the *digene* LQ Test, i.e., 100 MFI and 30 MFI.
[a] McNemar's test.
[c] Restricted to assay-common types (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82).

Table 5
HPV 52 type-specific viral load analysis in relation to the *digene* LQ Test and RLB assay findings

MFI *digene* LQ Test probe HPV 52	Result RLB (HR types)	Result viral load analysis HPV 52 (copy/cell)
Low MFI		
33	45 52 58	1.8
55	16 52	3.2
82	16 45 52	0.0
44	16 52	0.5
86	52	1.8
7	52 66	0.0
96	52	1.2
65	52	0.6
49	45 52 66	0.6
High MFI		
4297	52	14.1
3223	52	13.3
4968	52	29.6
3131	31 52	4.5
4402	52	304.6
3528	52	10.5
3190	52	29.6
3763	52	113.9
3828	52	272.0
3625	51 52	132.2

A high level of agreement in detection and genotyping of 18 HR HPV types in HC2-positive specimens between the *digene* LQ Test and the RLB assay was observed in this study. When compared to the strip-based *digene* RH Test, the *digene* LQ Test also reveals an excellent genotyping concordance (overall $\kappa = 0.955$ at cut-off 100 MFI; data not presented). Together with high inter-laboratory agreement, these data indicate that this assay provides an accurate and reproducible high-throughput alternative for the RLB genotyping assay or the *digene* RH Test.

The use of the recommended cut-off of 100 MFI revealed few discrepancies with the established RLB assay, and 85.9% of the HC2-positive women revealed HR HPV positivity by the *digene* LQ Test, indicating suitability of the assay as follow-up test for HC2-positive women. Lowering the cut-off to 30 MFI resulted in a slightly higher HR HPV positivity (89.2%).

A partial explanation for non-detection of HR HPV genotypes by the *digene* LQ Test in HC2-positive samples could be the cross-reactivity of the HC2 assay with LR HPV types that are targeted by the RLB assay, as has been reported previously.[12–14] On the other hand, it might reflect background noise using the standard threshold for positivity (RLU/CO ≥ 1), which supports a cut-off adjustment that has been proposed earlier.[8] Otherwise, insufficient amplification by the GP5+/6+-PCR, either or not influenced by inadequate DNA extraction or PCR-inhibiting factors in these samples, might have resulted in false-negativity by the PCR-based genotyping assays.

At the type-specific level, the *digene* LQ Test and the RLB assay differed only in the detection of HPV 52, when applying a 100 MFI threshold. The samples additionally positive by the RLB showed low viral loads for HPV 52. It remains to be determined whether the HPV 52 infections missed by the *digene* LQ Test at a cut-off of

100 MFI are clinically relevant. In that case, type-specific adaptation of the recommended threshold for HPV 52 may be considered.

Taken together, data from this study indicate that the novel *digene* LQ Test provides a high-throughput alternative for the RLB and *digene* RH Test to genotype GP5+/6+-PCR amplimers. While the *digene* RH Test offers a format that is easily implemented in routine facilities, the xMAP bead-based LQ Test is very effective for laboratories specialized in large cervical screening programs where high-throughput genotyping of HC2-positive specimens is required.

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