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TITLE PAGE

Title: Development and Validation of a Wart-Associated Human Papilloma Virus (HPV) Genotyping Assay for Detection of HPV in Cutaneous Warts

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manuscript was written by Nina Redzic and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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None required since procedure was part of clinical patient care.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

ABSTRACT

Background

Cutaneous warts are infectious disorders caused by human papillomavirus (HPV). A recent study revealed that the HPV genotype influences the natural course and response to treatment for plantar warts, suggesting that HPV genotyping could potentially be used to optimize wart treatment schemes. For this purpose, a wart-associated HPV genotyping assay was developed.

Methods

The assay was subjected to an intensive validation process including i.a. empiric determination of the annealing temperature, primer-probe optimization, evaluation of the analytical specificity and sensitivity, viral load quantification, and qualitative as well as quantitative analysis of intra-run repeatability and inter-run reproducibility. The newly developed assay was employed in a small-scale HPV genotyping study of wart biopsies (n=50).

Results

The assay exhibited an analytical type-specific sensitivity and specificity of 100% [95%CI83.9-100%]. The limit of quantification of the tested sequences corresponded to less than 17 viral copies/µl, while the limit of detection was less than 5 copies/µl. Very good to excellent agreements were gained between intra- and inter-run measurements (κ=0.85-1.00) and coefficients of variation of the quantitative agreements were less then 3%. 22.5% [95%CI11-39%] of the analysed biopsies were negative for the tested HPV types, while 35% [95%CI21-52%] contained multiple infections.

Conclusions

The wart-associated HPV qPCR assay was proven to be highly sensitive and specific. Multiple HPV infections were detected in 35% of lesions, contradicting the current literature claiming that in immunocompetent patients only 0-16% of warts exhibit multiple HPV infections. This assay is qualified to be implemented in development of future genotype specific wart treatment strategies.

KEY WORDS

PCR; HPV; cutaneous warts; HPV genotyping

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
bp	Base pairs
CIN	Cervical intraepithelial neoplastic lesions
CV	Coefficients of variation
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin-fixed, paraffin-embedded
HE	Haematoxylin-eosin
HPV	Human papilloma virus
IDT	Integrated DNA Technologies
LOD	Limit of detection
LOQ	Limit of quantification
LNA	Locked nucleic acids
SCC	Squamous cell carcinoma
Tm	Melting temperature
WHO	World Health Organization

Introduction

The family of the human papillomaviruses (HPVs) includes over 200 different genotypes, classified into different genera according to their DNA sequence. Members of the genus alpha-HPV have a specific tropism for mucosal epithelium, while beta-, gamma-, mu- and nu-HPVs most frequently cause cutaneous lesions and are commonly referred to as cutaneous HPV types.[1] HPV is capable of causing a wide range of diseases from benign lesions to invasive tumors, and although it is most commonly known for its involvement in the development of cervical cancer, there are also other mucosal as well as cutaneous disorders where HPV plays a role, one of the best known being cutaneous warts.

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Cutaneous warts are caused by HPV infection of keratinocytes. They are a common, infectious and sometimes very painful problem, with a varying worldwide prevalence of 0.84-12.9%.[2] The prevalence rate in children and young adults is even higher and reported to reach 30%.[3] Although most warts have a benign nature, they can have a substantial impact on quality of life. This becomes apparent when lesions are painful, persistent or interfering with natural function. An armamentarium of wart treatments is currently at our disposal, starting from folk remedies to over-the-counter medications and more aggressive clinic-based treatments. Unfortunately, none of these treatments seem to produce consistent results and reported efficacies often vary widely depending on several factors (e.g. age, compliance, immunocompetence).

Interestingly, a study by Bruggink et al. revealed that the HPV genotype influences the natural course and response to treatment in plantar warts.[4] This finding was also confirmed in two additional studies that concluded that from all patient- and wart-specific characteristics analyzed, HPV genotype most strongly predicted treatment response in warts.[5,6] These studies suggested that HPV genotyping could potentially be used to optimize wart treatment schemes by determining which warts will clear spontaneously as opposed to which do need treatment. For this purpose, we developed a wart-associated HPV genotyping PCR assay capable of detecting the most prevalent HPV types in cutaneous warts. In this article we describe in detail not only the design of the assay but also the rigorous validation process and employ it in a small-scale pilot study considering HPV-genotyping in wart biopsies.

Method

Literature review

A profound literature review was performed considering HPV genotype-specific prevalence in cutaneous warts. The HPV detection methods examined included i.a. general primer-mediated PCR followed by HPV typing by direct sequencing or by restriction enzyme digestion, southern blot, dot blot, reverse hybridization, in situ hybridization, xMAP Luminex technology, and enzyme-linked immunosorbent assay (ELISA). This review demonstrated that the most prevalent HPV types in cutaneous warts are HPV 1, 2, 3, 4, 10, 27 and 57. Other frequently found types are HPV 7, 41, 60, 63 and 65.[2,6–12]

Assay design

The genomic stability of these types was examined by alignment of all known NCBI sequences of each type and localization of conserved regions and mutational hotspots using a bioinformatic software i.e. CLC Genomics Workbench 9.0 (CLC Bio, QIAGEN, Aarhus, Denmark). Based on this analysis, a wart-associated HPV genotyping qPCR assay was developed, able to detect all the above-mentioned cutaneous HPV types. The assay involves a TaqMan real-time PCR containing type-specific primers and consensus probes capable of detecting multiple HPV types. The PCR reactions are performed in a 15 µl volume containing LightCycler 480 Probes Master (Roche Applied Science, Basel, Switzerland), primers and probes and 3 µl

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template DNA. The analysis is executed in 5 parallel multiplex or 12 singleplex reactions on the LightCycler 480 system (Roche Applied Science, Basel, Switzerland). The assay is not only able to detect multiple HPV types in one reaction due to the unique design of the probes (i.e. consensus probes that can detect two or more types) but can also be used as a type-specific PCR that only detects one HPV type per reaction due to type-specific primers (see Table 1). The most cost-efficient way of utilizing this unique design is by firstly analyzing each sample with five multiplex reactions. Multiplexing is performed by addition of multiple type-specific primer pairs and one consensus probe. If the sample is found to be positive for a specific multiplex reaction, further HPV typing is performed by two to three singleplex reactions containing one specific primer pair and the consensus probe for which the sample was previously found positive. By this approach the consumption of the most expensive PCR reagent (i.e. probe) is limited, since each sample is only analyzed with a minimum of five multiplex reactions, instead of 12 singleplex reactions. The PCR-program involves (1) pre-incubation: 10 minutes at 95°C; (2) temperature adjustment: 2 minutes at 60°C; (3) 50 two-step cycles of amplification: 10 seconds at 95°C and 30 seconds at 60°C; and (4) cooling: 10 seconds at 40°C. The assay was subjected to an intensive validation process including i.a. empiric determination of the annealing temperature, optimization of primer-probe concentrations, evaluation of the analytical sensitivity and specificity, viral load quantification, and qualitative as well as quantitative analysis of intra-run repeatability and inter-run reproducibility.

Controls and clinical materials

Several types of synthetic controls were used during validation i.e. gBlock® gene fragments, containing only target sequences of relevant HPV types (Integrated DNA Technologies, IDT, Coralville, IA, USA), and HPV constructs, containing whole genome reference sequences, provided by the World Health Organization (WHO) and the International HPV Reference Centre (Karolinska Institute, Stockholm, Sweden). Following validation, the wart-associated HPV PCR assay was implemented in a small-scale study considering characterization of genotype-specific HPV prevalence in cutaneous wart biopsies. A total of 50 formalin-fixed, paraffin-embedded (FFPE) biopsies were included in this study. Before and after slicing of the sections (10x5µm) predetermined for DNA extraction, additional sections were made for haematoxylin-eosin (HE) staining to ensure that these were derived from wart epithelium. All samples were extracted according to the previously described optimized DNA extraction protocol.[14] The newly developed wart-associated HPV qPCR assay, together with the in-house HPV Riatol genotyping assay,[15] capable of detecting the most relevant mucosal types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68), was used to determine the HPV prevalence.

Results

Validation of the Wart-Associated HPV qPCR Assay

All primers and probes were designed to be highly specific for their respective targets. The OligoAnalyzer Tool from IDT (Inc. Coralville, IA, USA) was employed to assure that no secondary structures (e.g. hairpins, homo- and hetero-dimers) would

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inhibit assay performance *in silico*. A temperature gradient was performed to empirically evaluate the annealing temperature. Eight different annealing temperatures were analyzed (58.0, 58.4, 59, 59.5, 60, 61, 61.5 and 62°C) on the iCycler system (Bio-Rad, Hercules, CA). All sequences showed optimal results at the predetermined assay annealing temperature. Subsequently primer and probe concentrations were optimized using several primer and probe concentration ratios (see Table 1). Whole genome HPV constructs were used to evaluate the analytical specificity and sensitivity. The constructs were diluted in different concentrations in order to create both weak and strong positive samples (concentrations from 1×10^5viral copies/µL to 1×10^2 viral copies/µL). Each sequence was evaluated with ten samples positive for the HPV type to be tested and 20 samples positive for other wart-associated HPV types. Each assay exhibited an analytical sensitivity of 100% [95%CI 83.9-100%] for their respective HPV type, and a corresponding analytical specificity of 100% [95%CI 83.9-100%] i.e. cross-reactivity with other HPV genotypes did not occur. Furthermore, absolute quantification was achieved by development of a standard curve with serial dilutions of gBlock gene fragments with known concentrations (dilutions from 1×10^(-2) ng/µL to 1×10^(-8) ng/µL were analyzed in triplicate). All standard curves showed acceptable correlation coefficients ($0.990 \leq R2 \leq 1$), slopes ($-3.8 \leq \text{slope} \leq -2.9$) and PCR efficiencies ($0.830 \leq E \geq 1.210$) in accordance with the guidelines for absolute quantification using qPCR.[16] Further dilutions were made in order to determine the limit of quantification (LOQ; the lowest DNA concentration that falls within the linear range) and detection (LOD; the lowest DNA concentration that can be detected in at least 85% of the cases; see Table 2). In order to analyze the intra-run repeatability, ten samples positive for the specific type to be tested and ten samples positive for the other types were used. The samples consisted of gBlocks as well as constructs in different dilutions (strong and weak positives) and were tested in duplicate. This run was repeated once more on the same day, and at one day and at seven days after the first run to determine the inter-run reproducibility. Kappa statistics were used to qualitatively analyze the repeatability and reproducibility of each HPV assay. Very good to excellent agreements were gained between intra- and inter-run measurements with kappa coefficients ranging from 0.85 - 1.00. Quantitative agreement was assessed by calculating coefficients of variation (CV) between Ct values as well as between viral copy numbers obtained from different PCR reactions. The obtained CV values were in accordance with predetermined criteria (CV% < 15%; see Table 2).[16]

Implementation of the Wart-Associated HPV qPCR Assay

In regard to the study evaluating the genotype-specific HPV prevalence in cutaneous wart biopsies, the wart diagnosis was confirmed by HE staining in 80% (40/50) of the samples. Only confirmed wart samples were included in further analysis. All samples tested positive for β-globin. 22.5% [95%CI 11-39%] of the samples were negative for the above mentioned cutaneous as well as mucosal HPV types. 42.5% [95%CI 27-59%] were positive for only one HPV type and 35% [95%CI 21-52%] contained multiple infections (see Figure 1). Cutaneous HPV types 3, 41, 60 and 63 were not detected. 5% [95%CI 0.8-18%] of the samples was infected with mucosal low-risk (HPV11) and high-risk (HPV16) types (see Figure 2).

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Furthermore, the degree of correlation between the multiplex and singleplex PCR reactions was also evaluated per HPV type in wart biopsies. There were no significant differences in Ct-values between the two reaction types ($p>0.05$) and an average CV of ≤5% was observed. This trend was also observed for the most abundant HPV type in samples where multiple HPV types of the same multiplex where present, indicating that the simultaneous co-amplification of different HPV types in biopsies infected with multiple types did not significantly influence the PCR efficiency of the most abundant HPV type. However, the degree of correlation could not be calculated for all HPV types considering that some cutaneous HPV types were not detected in the biopsies (i.e. HPV type 3, 41, 60 and 63).

Conclusion

Although to date several detection methods exist that are able to detect most of the wart-associated HPV types, these methods are generally time-consuming, laborious and rely on sequencing for HPV genotyping making them rather expensive and therefore not suitable for large epidemiological studies and daily routine diagnostics (e.g. Southern blot hybridization, PCR followed by sequencing).[10] The newly designed wart-associated HPV PCR assay fulfilled all the predetermined validation criteria and was able to amplify HPV DNA from various sources. The assay exhibited an analytical type-specific sensitivity and specificity of 100% [95%CI83.9-100%]. The LOQ of the tested sequences corresponded to less than 17 viral copies/µl, while the LOD was less than 5 copies/µl. Very good to excellent agreements were gained between intra- and inter-run measurements (κ=0.85 - 1.00) and CV of the quantitative agreements was less then 3%. In addition, the current assay was also capable of absolute quantification of the type-specific viral load by use of calibration standards, as well as being easily adaptable for automatic processing, making it an high throughput assay and an excellent tool for use in large epidemiological studies.[15]

As regards to the application of the test on a cohort of FFPE wart biopsies, multiple HPV infections were detected in 35% [95%CI 21-52%] of biopsies. This is in contrast to the current literature claiming that in immunocompetent patients only 4-16% of cutaneous warts exhibit multiple HPV infections. [4,10,17,18,19] The observed occurrence of multiple infections is probably due to the high sensitivity of the newly developed HPV qPCR assay. Our results confirm earlier findings by Schmitt et al., comparing HPV detection rates of two different xMAP Luminex technology based assays in 100 swab samples of cutaneous warts i.e. BSwart and HSL-PCR/MPG HPV genotyping assays.[8] Schmitt et al. demonstrated that the BSwart assay exhibited superior ability of detecting multiple infections in the same sample due to its higher sensitivity. Analogous to our findings BSwart was able to detect 51 (53.6%) single, 34 (35.7%) double, 6 (6.3%) triple, 3 (3.2%) quadruple, and 1 (1.1%) sextuple infections in 95 samples.

It is hypothesized that if multiple HPV types are detected in a wart, usually only one HPV type will be responsible for the development of the wart.[6] This is supported by evidence on the clonal origin of warts and in analogy with cervical HPV infections where it was demonstrated that within a defined cervical intraepithelial neoplastic lesion (CIN), only one HPV type is present.[6,20] The most abundant HPV type is

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biologically most active and proposed to be causing the lesion, while the other types are just transient, passenger infections.[8] Our findings confirm that this theory is also valid for cutaneous types i.e. biopsies with multiple infections generally exhibit one highly abundant HPV type accompanied by one or more HPV types characterized by a lower viral load. Alternatively, another explanation for some warts displaying multiple HPV types could be the co-infection of single cells with diverse HPV types.[6]

Furthermore, although it is common knowledge that warts are caused by HPV, 22.5% [95%CI 11-39%] of the tested biopsies were HPV negative. HPV molecular testing is challenging in FFPE samples due to poor DNA quality in the embedded tissue, resulting in lower HPV detection rates. Especially formalin fixation can cause extensive DNA damage i.a. cross-linking and fragmentation.[21] In addition, the newly developed assay is limited in detection of only 12 HPV types. It is possible that another type, that is not included in the assay, is responsible for the formation of the wart. Ideally, a more ubiquitous approach to HPV detection should be applied in order to ensure detection of all HPV types present e.g. sequencing. However, sequencing remains to date rather expensive and cutaneous warts do not pose a serious treat in immunocompetent population. Nevertheless, this is contradicted in immunocompromised subjects, where up to 50% of renal transplant recipients have cutaneous warts at one year post-transplant, and 77–95% have warts at five years after surgery.[22] Progression of these warts to dysplastic lesions and squamous cell carcinoma (SCC) has been described, and HPV is proposed to have a possible cofactor role in immunosuppression-associated skin cancer. Therefore, information gained from HPV genotyping of warts can also be used for development of prophylactic or therapeutic vaccines.[8] Identification of the wart-associated HPV types is necessary in order to determine which types should be included in future vaccines, that would make eradication of these types in the population possible, hereby protecting the weakest members i.e. transplant patients and other immunocompromised patients from serious illnesses (e.g. SCC). Nevertheless, before this line of reasoning can be tested we need to ascertain that the same HPV types responsible for the benign lesion in the general population are causing malign lesions in immunocompromised patients. In general, it has already been demonstrated that cutaneous warts from immunocompetent and immunocompromised patients exhibit same genotype distributions.[23–25] However, the number of detected types per lesion seems to differ between the two groups.[26] These findings have to be confirmed in large-scale epidemiological studies comparing the HPV type-specific distribution in the two populations with the same techniques. It should also be interesting to compare the average viral load per HPV type in both groups and examine the potential use of viral load as a biomarker for malignancies. At present, cutaneous warts are again the focus of attention with the increasing number of chronically immunosuppressed patients.[10] A straightforward, highly sensitive, high-throughput HPV genotyping technology such as that described in this study will be crucial for future research in immunocompromised patients, as well as for the previously mentioned prognosis of natural course and treatment efficacy of warts in immunocompetent individuals.

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Figure 1: HPV infection in cutaneous wart biopsies. 42.5% [95%CI 27-59%] of the samples exhibited single infectionsand 35% [95%CI 21-52%] multiple HPV infections. Of the multiple infections, 25% [95%CI 13-42%] were double infections, 7.5% [95%CI 2-21%] triple and 2.5% [95%CI 0.1-15%] quadruple. 22.5% [95%CI 11-39%] of the samples were negative for the tested cutaneous as well as mucosal HPV types.

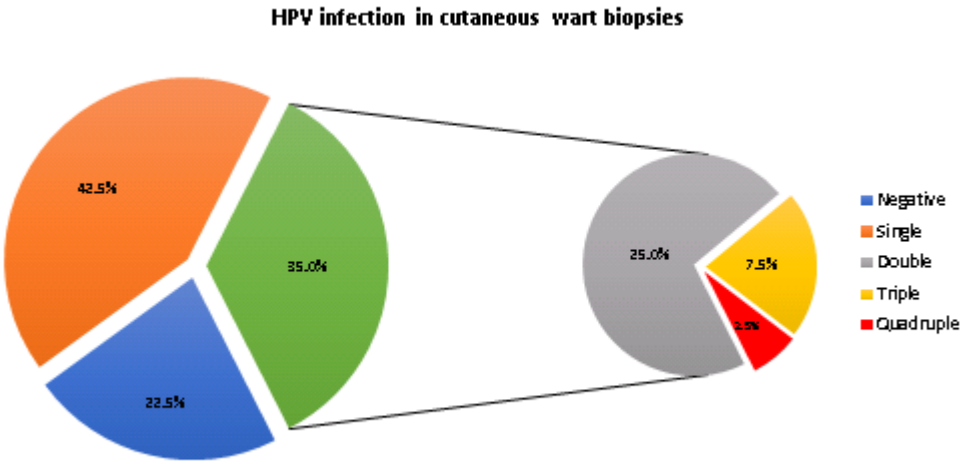

Figure 2: HPV type-specific prevalence in cutaneous warts with 95% confidence intervals. Cutaneous HPV types are depicted in dark grey, mucosal HPV types in light grey. The most prevalent HPV types in the defined population were cutaneous types HPV1 (37.5% [95%CI 23-54%]) and HPV57 (25% [95%CI 13-42%]).

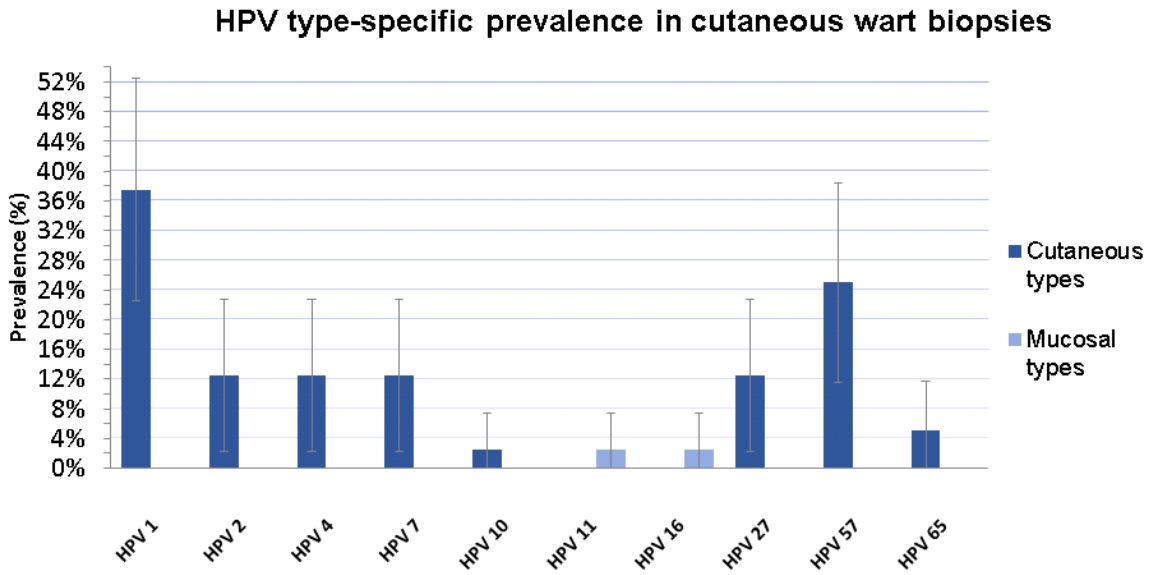

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Table 1: Overview of the multiplex PCR assays, the sequences of the primers and probes, amplicon sizes and optimized primer/probe concentrations. Two out of five probes contain Locked Nucleic Acids (LNAs; **+**). LNAs are nucleic acid analogues which are constrained in the ideal conformation for Watson-Crick binding. This conformation makes the pairing with a complementary nucleotide strand more rapid and increases the stability of the resulting duplex. The higher stability causes an increase in the duplex melting temperature of 2-8 °C per LNA monomer, making it possible to use shorter probes but still retain a relatively high melting temperature (Tm) necessary for effective annealing.[13] FP: forward primer; RP: reverse primer; bp: base pairs.

Table 2: Results of qPCR assay validation. Limits of quantification (LOQ) and detection (LOD) per type-specific assay are depicted in the table below, together with the average intra- and inter-run coefficients of variation (CV%) and kappa values (κ) per HPV type in their respective linear range. Ct: cycle threshold.

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Multiplex	Oligonucleotide	Sequence (5'-3')	Amplicon (bp)	Optimized concentration (nM)
1	HPV1/63 probe	GAA+CCAAGCGTC+CTAGAT		100
	HPV1 FP	GCCAGCACTAAAGGACC	74	200

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	HPV1 RP	CCTCCTCGTAACAATAAAGATCTAA		200
	HPV63 FP	GAGCAGCCAAATATAGGTGATT	85	200
	HPV63 RP	CCTCAGCAGGTATATCCTCA		200
2	HPV2/27/57 probe	GGTTGCGTGCAGTTTCGGT		50
	HPV2 FP	CAGATTTCTGCACCCCATC	163	200
	HPV2 RP	CCAAAATCCAAACTCATCGTC		200
	HPV27 FP	TCTGCACCCTATTGTCCTT	141	600
	HPV27 RP	TTGTTGCTGGCACAGC		600
	HPV57 FP	GCACTCTGTAATTGTCCCC	118	200
	HPV57 RP	AGTGTGCTGGCAGCA		200
3	HPV3/10 probe	TGCTGGTCACGATGCACG		100
	HPV3 FP	TCATTGGAGGGGGAGC	108	200
	HPV3 RP	GCATAGTGCAGGGACG		200
	HPV10 FP	AGGGTACTGGAGAGGTAGT	115	200
	HPV10 RP	GCACATTGCATACAGGGATA		200
4	HPV4/60/65 probe	AATAGA+CA+C+CTGTTGC		100
	HPV4 FP	GGAGTCGGTGGTTCCA	78	200
	HPV4 RP	GCAGCATACAATGTAATTCTTACAG		200
	HPV60 FP	GAGGAGTTGTCACCCGA	107	200

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	HPV60 RP	GCAATAATAGTAAATCTAACACCTGC		200
	HPV65 FP	AGAGGAGGAGCTTTTTCCT	90	200
	HPV65 RP	CCAAATTCCACAGCAAACAG		200
5	HPV7/41 probe	CA+TAA+CAA+T+G+GCAT		50
	HPV7 FP	GGCTCTCTAGTTACCTCTGATT	162	200
	HPV7 RP	CGATTGTGTAGCAGCACAT		200
	HPV41 FP	TAGCTACTGAGCAGCAGC	114	200
	HPV41 RP	TGGTAGTGTCAACCAATGTTAC		200

Table 2: Results of qPCR assay validation. Limits of quantification (LOQ) and detection (LOD) per type-specific assay are depicted in the table below, together with the average intra- and inter-run coefficients of variation (CV%) and kappa values (κ) per HPV type in their respective linear range. Ct: cycle threshold.

HPV type	**Absolute quantification**		**Intra-run**			**Inter-run**		
	LOQ (copies/µl)	**LOD (copies/µl)**	**Average CV%**		**κ**	**Average CV%**		**κ**
			Ct	**log copies**		**Ct**	**log copies**	
1	4,06	4,06	0,25	0,02	1,00	0,98	0,08	1,00
2	8,13	1,02	0,74	0,10	1,00	1,25	0,13	1,00
3	8,13	2,03	0,69	0,05	1,00	0,69	0,05	0,90
4	8,13	2,03	0,53	0,07	1,00	1,05	0,12	1,00

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7	4,06	4,06	0,53	0,06	1,00	2,87	0,30	1,00
10	16,26	2,03	0,32	0,03	1,00	0,54	0,05	1,00
27	8,13	4,06	0,67	0,08	1,00	1,74	0,20	1,00
41	8,13	2,03	0,52	0,06	1,00	2,02	0,22	1,00
57	8,13	2,03	0,78	0,09	1,00	1,67	0,19	0,85
60	2,03	2,03	0,29	0,03	1,00	0,91	0,09	1,00
63	8,13	2,03	0,49	0,04	1,00	0,59	0,06	1,00
65	4,06	2,03	0,46	0,05	1,00	1,29	0,14	1,00

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