Quantification of beta-human papillomavirus DNA by real-time PCR

Sönke J Weissenborn, Ulrike Wieland, Monika Junk & Herbert Pfister

Institute of Virology, German National Reference Centre for Papilloma and Polyomaviruses, University of Cologne, Koeln, Germany. Correspondence should be addressed to H.P. (herbert.pfister@uk-koeln.de).

Published online 10 December 2009; doi:10.1038/nprot.2009.153

Quantitative PCR with hybridization probes allows the reliable quantification of viral DNA sequences in clinical samples with a dynamic range and sensitivity that cannot be achieved with other methods. The technical background for the establishment of protocols is described and established protocols are presented to estimate the viral load per cell of frequently occurring betapapillomaviruses (HPV5, -8, -15, -20, -23, -24, -36 and -38) in skin tumors, healthy skin and hair bulbs. This approach accurately adjusts dilution series of reference DNA of different viral types relative to pUC18, which is crucial for comparative analyses and for interlaboratory standardization. The type-specific determination of beta-HPV DNA loads is an important research tool toward discrimination between low-level persistence and activated possibly pathologically relevant infections. The analysis of 24 samples, starting with DNA extraction and followed by HPV typing and quantification of—on average—three of the described HPV types takes about 2 d.

INTRODUCTION

Betapapillomaviruses

Human papillomaviruses (HPV) from genus beta have been discovered first in patients suffering from epidermodysplasia verruciformis (EV). Owing to a specific, genetically determined susceptibility to beta-HPV, EV patients develop disseminated keratotic lesions during childhood. These lesions display a high rate of progression to squamous cell carcinomas in the fourth to fifth decade of life, mainly in sun-exposed areas of the skin. The patients are usually infected with several betapapillomavirus types, whereas only a subset (mainly HPV5 or some related types as HPV8, -17, -20 and -47) is detected in the cancers. Detection of HPV DNA in a cancer is a first step to indicate an etiological role of the virus. HPV5 and -8 are widely accepted as carcinogenic in the skin of EV patients[1,2].

The detection of betapapillomaviruses by highly sensitive (nested) PCR completely changed our view of these viruses. The DNA of these HPV could be detected in 30–90% of actinic keratoses and squamous cell carcinomas of non-EV patients depending on the type of tumor, the immune status of the patients and the sensitivity of the detection technique[3–6]. Betapapillomavirus DNA was also frequently detected in swab and biopsy samples from healthy skin and in plucked hairs from healthy individuals[7–9].

Betapapillomaviruses turned out to be acquired soon after birth, probably through direct contact with the infected skin of adults[10,11]. Most HPV types in skin swabs from infants could be found in one or both parents. This argues for a predominantly intrafamilial transmission[10]. Type-specific persistence has been shown in two-thirds of children and in 74–90% of adults over 7 years, frequently concerning multiple beta-HPV types[10,12,13]. In a recent large cross-sectional study of 845 immunocompetent and 560 immunosuppressed individuals from six countries of different latitudes, the prevalence of beta-HPV in eyebrow hairs reached 91% in the immunocompetent and 98% in the immunosuppressed population[14]. The median number of infecting beta-HPV types ranged from three to six.

Aware of the oncogenic potential of HPV in anogenital cancer and in skin cancer in EV, case-control studies were initiated on the association between beta-HPV infection and nonmelanoma skin cancer in non-EV patients. In view of the high prevalence of beta-HPV in the general population, it is not surprising that case–control studies comparing the prevalence of viral DNA in skin cancer tissue and healthy skin or in eyebrow hairs of skin cancer patients and controls showed only slightly positive associations with odds ratios in the range of 1.7–9.2 (refs. 15–19). Recognizing the need to monitor the activity of HPV rather than infection *per se* only, we focused on viral DNA load as a quantitative parameter of viral DNA copy numbers per cell and/or the number of infected keratinocytes. Viral load is regarded as a surrogate marker of viral replication and is possibly associated with an increased concentration of viral oncoproteins[20].

Using quantitative, type-specific real-time PCR protocols, we have shown that HPV DNA loads in actinic keratoses and cutaneous squamous cell carcinomas ranged between 50 HPV DNA copies per cell and 1 HPV DNA copy per 14,200 cell equivalents (median: 1 copy per 344 cells)[20]. Because viral loads in actinic keratoses were significantly higher than those in squamous cell carcinomas, beta-HPV may have a carcinogenic role particularly in the early steps of tumor progression[20]. Compared with the general population, much higher viral loads were observed in premalignant and malignant skin tumors of EV patients and in their plucked eyebrow hair bulbs, frequently ranging between 10 to more than 400 HPV DNA copies per cell[21]. These data strongly support the hypothesis that a larger number of HPV-positive cells and/or higher viral copy numbers per cell account for an increased risk for skin cancer development as observed in EV patients.

Epidemiological studies based on qualitative HPV DNA assays should take into account the low beta-HPV DNA loads that are usually found in non-EV patients. Small amounts of cellular DNA input into qualitative PCR may become limiting for virus detection and affect data on prevalence and multiplicity of infection. To achieve comparability between different studies, standardized amounts of cellular DNA should be used or data on prevalence and multiplicity should be stratified by cellular DNA input.

TABLE 1 | A selection of PCR methods for the detection of beta-HPV DNA.

PCR name	Primers	Length of PCR product (bp)	Analytical sensitivity	HPV target gene	Detectable HPV types and detection system	References
M/H[a] nested PCR	Degenerate primers	780 (ext) 422 (int)	1–10 fg	L1	Beta-HPV Agarose gel electrophoresis Typing by (cloning and) sequencing	Boxman *et al.*[7]
PM	Composition of 2 forward and 7 backward primers	117	10–100 viral genomes	E1	Beta-HPV Reverse hybridization assay (25 types)	de Koning *et al.*[23]
FAB	Degenerate primers	480	1–10 viral genomes	L1	Beta/gamma/(alpha)-HPV Agarose gel electrophoresis Typing by (cloning and) sequencing	Forslund *et al.*[4]
BGC	Composition of 5 forward and 8 backward primers	72	10–100 viral genomes	L1	Beta/gamma-HPV Reverse line blot assay (25 beta, 5 gamma types)	Brink *et al.*[22] and Nindl *et al.*[24]
Multiplex PCR	27 type-specific forward and 26 backward primers	186–283	10 viral genomes	E7	Beta-HPV DNA microarray primer extension (25 types)	Gheit *et al.*[25]

bp, base pair; ext, external PCR product; int, internal PCR product.

HPV typing

Coinfections with multiple beta-HPV types occur very frequently[14]. This led to the development of PCR protocols with degenerate primers[4,7] or primer combinations[22–25] that are able to detect the broad spectrum of all currently known beta-HPV types. The characteristics of these primer combinations, including binding regions, reported sensitivities and the associated typing procedures, are given in more detail in **Table 1**. Typing by direct sequencing or after cloning is laborious and may lead to an underestimation of the spectrum of occurring types[4,19,25]. More recent assays therefore used beta-HPV-type-specific probes for reverse hybridization assays[22–24] or an array primer extension assay[25]. They are able to detect most beta-HPV fully characterized at present, however, with partly different detection limits for the different types and with differing ability to detect multiple infections. Thus, the spectrum of HPV types detected will to some degree still depend on the applied method[25]. The assays described by Brink *et al.*[22], Nindl *et al.*[24] and Gheit *et al.*[25] may be individually extended to detect other, newly characterized beta-HPV. The assay developed by de Koning *et al.*[23] is so far the only commercially available test and offers a high grade of interlaboratory reproducibility. In our laboratory, we are using the latter test for beta-HPV typing. An advantage of this test is its easy technical performance without the need of special laboratory equipment. A disadvantage is that the user cannot extend the kit to include more beta-HPV types than the 25 beta-HPV types included.

Quantitative PCR

Real-time PCR or quantitative PCR (qPCR) allows the quantification of viral nucleic acids in biological specimens[26]. Real-time PCR offers several technical advantages, including online monitoring, no need for postreaction analyses and consequently reduced risk of contamination. Fluorescent signals are monitored as they are generated. Initial template levels can be calculated automatically by analysis of the shape of the curve or by determination of the threshold cycle, i.e., the cycle when the signal rises first above the threshold of background fluorescence. Different types of fluorescent probes are used. Dyes that bind preferentially to double-stranded DNA such as SYBR Green 1 may be used as well as target sequence-specific reagents such as exonuclease probes, hybridization probes or molecular beacons[27]. Sequence-specific probes are more expensive but add specificity to the assay and enable multiplexing applications. We have developed qPCR protocols for quantification of the DNA of beta-HPV types 5, 8, 15, 20, 23, 24, 36 and 38 in skin tumor specimen, normal skin and in hair bulbs[20,21,28]. These types were chosen because of their relatively high prevalence in skin and hair bulb samples[14]. Protocols for beta-HPV types 92, 93, 96, 107, 110 and 111 were described by Forslund and colleagues[29,30].

The protocol described here starts with extraction of DNA from different specimens such as paraffin-embedded biopsies, fresh tissues or plucked hair bulbs. The presence of a single-copy gene (e.g., beta-globin) is measured by qPCR to assess the quality and quantity of the extracted DNA. The presence of HPV is then detected in the samples using a commercially available kit. Later, the viral load of the HPV types detected in the samples is measured using qPCR and compared with the results of a standard curve prepared with plasmids for the specific HPV types. The HPV plasmid dilution series have to be standardized relative to a fixed standard. This step is crucial for comparative analyses and, furthermore, important for interlaboratory standardization. Ideally, to do this, reference

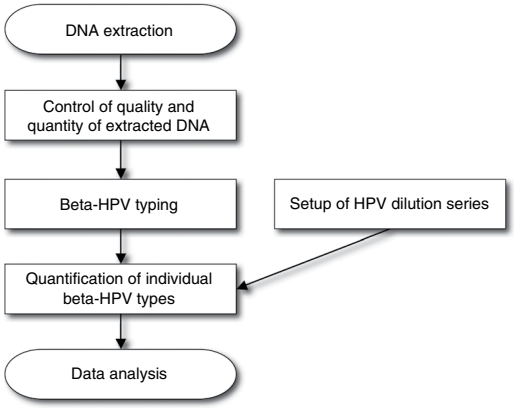

Figure 1 | Flowchart for quantification of beta-human papillomavirus DNA by real-time PCR. The setup of HPV DNA dilution series indicated by the box to the right is necessary only once at the beginning of the study.

plasmid dilution series are adjusted to a commercially available plasmid (pUC18 in this protocol) by quantification of the *TEM-1 beta-lactamase gene* by qPCR, a gene that is found in numerous vector plasmids[31]. The HPV dilution series standardization does not need to be carried out for every experiment. Finally, data analysis is described and expected results are given. A troubleshooting for the most important steps is indicated. A flow diagram outlining all major steps in the protocol is shown in **Figure 1**.

The established beta-HPV protocols should be of interest to researchers involved in pathogenesis and epidemiology of HPV in skin carcinogenesis. They are not meaningful for clinical diagnostics. Adjustment of dilution series by comparative qPCR is superior to absorption measurements in terms of sources of error and accuracy, and may also be used in quantitative DNA detection assays for other infectious agents. The viral-load determination described here can be time-consuming, if several beta-HPV types are found in a sample. It is not suitable for high-throughput studies with several thousand samples. However, if the protocols are adapted to plate-based cyclers, higher throughput should be feasible.

Experimental design

Controls and sample requirements. *Controlling for contamination*: PCR is a highly sensitive method and trace amounts of PCR products or cross-contamination may easily lead to false-positive results. Therefore, precautions should be taken to avoid cross-contamination of samples and chemicals as recommended by Kwok[32] and Kwok and Higuchi[33]. This includes a strict spatial separation of the PCR mixtures, nucleic acid extraction and reaction setup, and PCR execution and analysis to different rooms. At least one negative control as water or DNA extracted from human cells that are negative for HPV should be included in all steps to show possible cross-contaminations.

Single-copy gene determination: the protocol is suited for fresh frozen samples as well as for paraffin-embedded tissues; however, sensitivity is reduced in embedded materials and at least ten 10-µm sections of a 1-cm^2 tissue sample will be needed. Biopsies with a maximum of 25 mg of tissue may be used with the described procedure. Care should be taken that 10–20 hair bulbs are present for extraction when using plucked eyebrow hairs. DNA extracted from specimens of different sources, such as hair bulbs, fresh, frozen or paraffin-embedded tissues, and of varying age and storage conditions may yield highly varying amounts and qualities of extracted nucleic acids. Therefore, to rely on tissue weight or the count of hair bulbs may result in significant errors in determining viral load. Viral DNA loads can be defined as viral DNA copies per cell equivalent; however, it is crucial to control for variations in DNA quantity and for DNA integrity when performing these calculations. Furthermore, a correction for PCR efficiency is mandatory; this is performed by determining the number of input cell equivalents through quantification of a single-copy gene. To define HPV DNA loads as one HPV DNA copy per *x* cell equivalents, two copies of the single-copy gene *beta-globin* are taken as a cell equivalent. Beta-globin copies can be quantified in a single replicate using a commercially available qPCR kit (LightCycler Control Kit DNA, Roche; the manufacturer does not reveal the primer sequences used). However, other single-copy genes such as *beta-actin* or *alpha-TOP3* may be used and ready-to-use kits are sold by several suppliers, e.g., by Qiagen. Suitable primers may also be found on the following website: http://lpgws.nci.nih.gov/cgi-bin/PrimerViewer. To generate standard curves for the quantification of the single-copy gene, a ten-fold dilution series of control human genomic DNA is set up. One copy of a single-copy gene is found in 3.5 pg of human genomic DNA (3.2×10^9 bp per haploid genome multiplied by 660 (average molecular weight of one mole base pair in grams), divided by the Avogadro constant $N_A = 6.023 \times 10^{23}$). The threshold cycles C_t observed with the ten-fold dilution series are used by the LightCycler software to calculate the slope and the *y*-intercept of the standard curves. The LightCycler then uses this to automatically calculate the copy number of the single-copy gene present in a sample.

Determining the presence of HPV. *DNA input in the beta-HPV typing assay*: Beta-HPV DNA loads in clinical materials are usually low with median values of one beta-HPV DNA copy per 300–400 cells[20]. In view of these low viral loads, amounts of the cellular sample DNA input may become limiting for virus detection. Assuming a sensitivity of two HPV DNA copies per assay, ideally around 2,000 beta-globin gene copies of each DNA sample should be added to each assay to get positive HPV DNA results in more than half of the samples.

Detecting HPV using the PM-PCR/reverse hybridization assay: to identify human samples that are positive for quantifiable beta-HPV DNA, the specimens are analyzed by the qualitative PM-PCR/reverse hybridization assay as described by the manufacturer[23]. The PM-PCR generates a biotinylated short amplimer (117 bp) from the E1 region of the HPV genome, with a primer set consisting of two forward and seven reverse, nondegenerate primers. PCR products are then denatured and hybridized to genotype-specific probes covalently bound to the strip. After an enzymatic coloring reaction, line patterns allow the simultaneous identification of up to 25 HPV genotypes in a single hybridization step within 3 h. Other typing methods are discussed in the INTRODUCTION and the general steps of typing by PCR amplification and cloning[7] are shown in **Box 1**.

Plasmid dilution series: once a sample has been demonstrated to be positive for HPV, the viral load of the corresponding HPV type needs to be assessed. To quantify the HPV DNA load of a sample, a standard HPV plasmid dilution series needs to be included in each assay (i.e., the HPV plasmid should be of the same type as the HPV

BOX 1 | HPV DETECTION AND TYPING THROUGH PCR WITH DEGENERATE NESTED PRIMERS AND SEQUENCING

HPV detection can also be carried out using degenerate PCR. The design of degenerate primers is based on alignments of HPV DNA sequences and identification of identical and differing nucleotide positions. If there is, e.g., at a specific sequence position, a cytosine in some HPV types and a thymine in other HPV types, two primers are synthesized with the respective bases. To account for all variations within the primer sequence, a mixture of primers is synthesized, which corresponds to all permutations. To compensate for the loss of specificity and sensitivity, which is frequently associated with the use of degenerate primers, PCR products obtained with the first set of primers are amplified with a second set of degenerate primers, which bind within (nested) specific products of the first PCR. The probability that unwanted PCR products contain binding sites for both primer sets is very small, and thus products from the second PCR contain little contamination from unwanted products. We used degenerate, nested PCR primers CP62–70 (refs. 7,46) and 25 ng purified total DNA from clinical specimen in each PCR.

1. Extract total DNA from clinical specimens (see Step 1 of the Procedure).
2. To ensure the presence of adequate DNA and the absence of substances inhibitory to PCR, perform a beta-globin PCR with all biopsies[47].
3. For HPV DNA detection, use degenerate, beta-HPV-specific, nested PCR primers. Three negative controls, which consisted of water that was processed like samples during DNA extraction, should be included in each PCR run.
4. Following PCR, separate the internal PCR products obtained with the CP65/CP69 primers (421 bp)[7] on 2% agarose gels and visualize by ethidium bromide staining.
5. If single bands of the expected size were observed and only the predominating HPV type is of interest, purify the PCR products (QIAquick PCR Purification Kit, Qiagen) and sequence directly. Compare the resulting sequence with all known HPV sequences using BLAST.
6. To detect multiple infections, clone the internal PCR products into the pCR-Blunt II-Topo, using the ZeroBlunt-Topo PCR Cloning Kit (Invitrogen). Sequence 4–12 bacterial clones that carry an insert of the expected size.

detected in the sample). These standard curves are then used to estimate the number of HPV copies present in experimental samples. To avoid variations by the preparation of new reference plasmid dilution series, stocks for standard dilution series prepared with Tris-EDTA buffer with single-stranded salmon sperm DNA (TE-SS) should be prepared in sufficient amounts for a complete study and stored in aliquots at −80 °C, which are reliable for several years. Concentration measurements of the plasmid DNA by absorbance at 260 nm are, among others, sensitive to the applied DNA concentration (OD range should be between 0.1 and 1), solvents, pH values and temperature. Furthermore, large variations between different samples, measurements and devices may occur. Therefore, adjustment of the HPV dilution series relative to a commercially available plasmid (e.g., pUC18 plasmid) by quantification of the beta-lactamase gene by qPCR is superior. The *beta-lactamase* gene, encoding the *TEM-1 beta-lactamase*, is the most encountered AmpR marker used in molecular biology and found in numerous plasmids[31].

For the setup of a dilution series, the molecular weight of the cloned HPV DNA (vector plus insert) is calculated by multiplying the number of nucleotides by 660 (average molecular weight of one mole base pair in grams). To obtain the mass of 5×10^{10} molecules, divide the above calculated molecular weight of the cloned HPV DNA by the Avogadro constant N_A (6.023×10^{23}) and multiply by 5×10^{10}. Absorbance measurements of HPV DNA plasmid solutions may be used to determine the appropriate amount needed for the initial dilution steps. These are then amplified by PCR and compared with a reference dilution series prepared with pUC18 plasmid DNA. Deviations of mean threshold cycle numbers of the individual HPV dilution series should not exceed 0.4 cycles compared with the reference plasmid DNA (pUC18), which equals an error of about 32%. Assuming a PCR efficiency of 100% with a doubling of target DNA in each cycle, the relative content of plasmid DNA may be calculated by the formula 2^n, where n is the deviation of cycle numbers relative to the standard pUC18 plasmid. For example, a deviation of +0.7 cycles indicates an amount of 162.5% relative to the standard pUC18 plasmid, i.e., 62.2% excess plasmid DNA. Dilution series may then be adjusted accordingly and retested.

qPCR primer and probe design. To quantify different viral genotypes, unique regions of the viral genome need to be amplified first using type-specific primers and the resulting PCR products need to be detected by hybridization of type-specific probes to the generated amplimers. Genome regions with a maximum number of nucleotide sequence variations among different HPV types are ideal candidates for PCR primer and probe design. Such regions can be identified by a sequence alignment of all known viral genotypes (for a collection of complete HPV genome sequences, see http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=Papillomaviridae), maximizing mismatches to assay of different types, especially in the 3′-region of the primer and within the probes. General criteria for PCR primer design are the following: (i) length of primers should be 18–30 nt; (ii) G/C content should be between 40 and 60% without GC-rich clusters; (iii) the T_m (melting point temperature) of both primers should not be lower than 50 °C and should not differ by more than 5 °C; (iv) hairpin structure formation should be avoided; (v) primers should not form homodimers and heterodimers; (vi) primers must have high 3′-thermal stability to improve priming efficiency; and (vii) amplimers should not exceed 200 bp. PCR primer design may be assisted by some easy-to-use programs such as Oligo (Med Probe), Clone Manager Professional Suite (Scientific & Educational Software) and Primer Premier (PREMIER Biosoft). A unique sequence within the amplimer obtained with the chosen primer pair must be defined as target for the type-specific probe. Similar criteria regarding the design of primers apply for the design of probes. Our primers and probes bind in the L1 gene region of the respective HPV types.

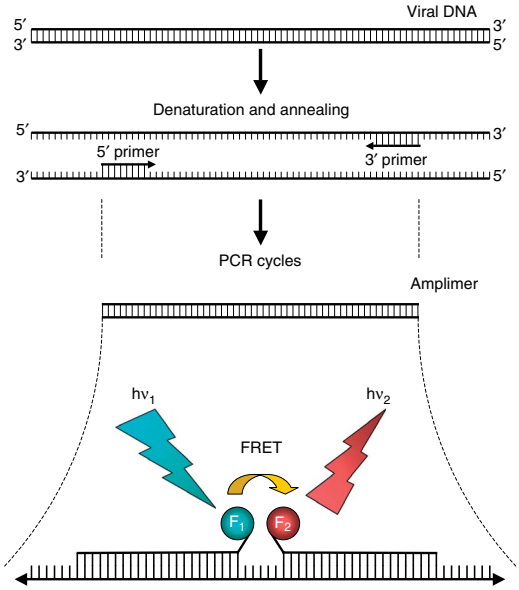

Figure 2 | Binding of primers and probes to viral DNA and amplimers. The fluorescence resonance energy transfer (FRET) between bound probes is indicated at the bottom hv1, light used to excite donor fluorophor F1; hv2, light emitted by the excited acceptor fluorophor F2.

Several probe formats are currently available. The rules given here especially apply for the hybridization probe format, which consists of two probes, which must bind with a gap of 1–5 nt and the fluorophores facing each other when bound to the single-stranded target sequence. A Förster resonance energy transfer, which is also known as fluorescence resonance energy transfer, can occur when both probes are bound simultaneously in close proximity to each other during the cooling phase after denaturation. This results in the emission of light by the acceptor fluorophor of a wavelength different from that used to excite the donor fluorophor (**Fig. 2**)[34,35]. The interaction between the electronic states of two fluorophores through dipole–dipole coupling is distance-dependent and takes place before the primers are extended by the polymerase. Therefore, the probes should have 5–10 °C higher melting temperatures than the primers, which are often attained by probe lengths between 28 and 35 nt. However, melting temperatures should not be too high (e.g., ≤80 °C) to prevent hindering of elongation and PCR inhibition. Probes should generally be placed as far away from the primer binding position as possible to prevent early probe displacement. When developing new assays, it may be expedient to start with the selection of adequate probes before selecting compatible primers. If primer/probe incompatibilities occur, shifting of the primers by a few bases may easily overcome problems. Experimental testing of two to three theoretically possible primer pairs is often the fastest and cheapest way to find optimal primer/probe combinations. Sequence complementarities between the 3′-ends of primers and probes should be avoided. The 3′-ends of the probes have to be blocked, usually by 3′-fluorescein in the case of the 5′-probe and by a 3′-phosphate in the case of the 3′-probe. Variants of HPV types may differ in one or two bases in the probe-binding sequence as observed here for HPV38a and HPV38b. In this case, variant-specific probes with perfect matches were designed to obtain highly comparable results (**Table 2**).

For the quantification of the beta-lactamase gene in plasmid solutions, amplimer detection was performed with the cheap dye SYBR Green I. SYBR Green binds sequence-independently to double-stranded DNA and the resulting DNA-dye complex emits green light upon excitation. SYBR Green is frequently used in real-time PCR applications, which do not require high specificity such as DNA mixtures of low complexity[36].

Optimization of reactions. *PCR parameters*: type specificity and sensitivity are the most important characteristics for beta-HPV quantification. They depend on an efficient amplification without unspecific by-products, and physical and chemical reaction conditions have to be established for most primer/probe combinations individually. The annealing temperature and the magnesium ion concentration are the most important parameters to vary, whereas primer concentrations, the number of amplification cycles, and the temperatures and duration of each step should be kept as standard. However, in some cases, the use of slightly higher primer concentrations may enhance sensitivity or lower primer concentrations may improve specificity. For any given primer pair, the choice of annealing temperature should be based on the calculated T_m of the primers. A preceding touchdown protocol, which includes several cycles with high stringency annealing conditions, may be required to obtain optimal specificity and sensitivity[37]. Furthermore, sensitivity may be enhanced using smaller temperature gradients from annealing to elongation temperature, and higher specificity may be achieved using larger temperature gradients. The protocol is started with primer concentrations of 0.5 µM, probe concentrations of 0.15 µM and Mg^{2+} concentrations of 3.0, 4.0 and 5.0 mM. Thereafter, 10^2–10^4 copies of HPV plasmid are amplified. The specificity of qPCR is verified by loading qPCR products on an agarose gel (a single band of the expected size should be seen) and/or by sequencing. The best Mg^{2+} concentration (which is the one delivering the first signal and the steepest slope of the amplification curve) is chosen. The Mg^{2+} concentration is varied by ±0.5 mM in 0.25-mM steps around the determined optimum level. Finally, the T_m is optimized for optimal sensitivity and specificity.

Controlling PCR specificity: to control the specificity of an optimized protocol, use 10^5 copies of heterologous HPV types (for example, HPV8, 15, 20, etc. for a quantification protocol for HPV5) instead of the optimized target HPV type. These assays should not yield signals above background fluorescence seen for a negative control with water instead of DNA. Test the reliability of the quantification in the presence of other HPV types by spiking six identical DNA samples containing 1,000 copies of the target HPV type with 10^5 copies of heterologous beta-HPV types. This test is successful, when spiked samples yield copy numbers within the three-fold standard deviation of six unspiked samples[20,38]. We perform all experiments on a LightCycler device with a carousel carrying 32 glass capillaries from Roche. This system has the advantage of short reaction times, which is especially useful in research settings with smaller sample numbers and if new protocols have to be established regularly. If high throughput and reduced consumable costs are more important, plate-based systems are available from several suppliers. For a detailed presentation and discussion of platforms, please refer to http://www.gene-quantification.info.

Data analysis

The software installed on real-time PCR systems is usually easy to use and standard settings yield correct results in the majority of cases. However, in cases of very low copy numbers, care

TABLE 2 | Primers, hybridization probes and PCR conditions for the quantification of DNA of HPV types 5, 8, 15, 20, 23, 24, 36 and 38 by real-time PCR.

HPV type	$T_{ann.}$ (°C)	Mg^{2+} (mM)	Sequences[a] (5′→3′)
5	54	4.5	fw: GGCTGGAGCACTAAAAGATG bw: CATTGATCTGTGCCAATACCT So1: GATATTTCATATTCTTCTACATGTCTTTGATA So2: TCTCTAAATTGATCTGCATTATAGTCTGCAA
8[b]	56	5.5	fw: GTTTACACTGAAAATGGGGAAC bw: AAACTTTGCATATGGATCAGGCT So1: ATTGTGTAAGATACCACTAAAGGCTGATGT So2: TTAGCACAAATCAATGCCATGAATTCA
15	55	5.0	fw: TTCCATGTTTAGGCGAACAC bw: CAGACTCACATCTGACTTAG So1: CCATCTTCAATAACTGTATTTTTAAGTTCC So2: AGGAGGACATTTTCCCGCCTGATT
20	55	3.25	fw: CAAGAATATTTAAGACACGTAG bw: CGAATCCTAACTGCCACTCC So1: TTACAGCTCTGTAAAGTTCCTTTAACAGCTGA So2: GTTTTAGCTCAAATTAATGCTATGAATTCAAATA
23[c]	56	3.5	fw: ACTACAGTTGTGCAGGATACCT bw: ATTTTGTGGCCTTTGAAGCC So1: CAGATATTTTAGAGAATTGGCAGTTAGGGT So2: TGTTCCTACACCAGATAATGCAGTTCA
24[d]	54	4.5	fw: GGAAGTAGCTGAGAGGTGTG bw: GATCTACTTTGTTGTAGTGTTC So1: ACCATCTTGAATTACTGAATTTACTAACTT So2: TAGGTGGACATCTACCAGCATCATTATT
36[b]	61	4.0	fw: CAATAATGGGGCACTAAAGGAC bw: TCAGGACAGCGAGTGGCTAAT So1: TAATATTACAGCTATGTAAGGTTCCTCTGAA So2: CAGAAGTATTGGCTCAGATAAATGCTATG
38[e]	56	3.5	fw: GTTACAGTCGCTGATAATACC bw: CTGTGTCAGCACTTCAGCA So1: GGTGCTCAAGAATATGATTCTGCAAAT So1b: GGTGCTCAGGAATATGATGCTACAAAT[F] So2: AGAGAATATTTAAGACATGTTGAGGAATACC

$T_{ann.}$, annealing temperature (a touchdown cycling program was used for HPV20, -24 and -38; see below under PCR cycling conditions). fw, forward primer; bw, backward primer; So1, 5′-hybridization probe (3′-end labeled with fluorescein); So2, 3′-hybridization probe (5′-end labeled with LightCycler Red 640 and 3′-end phosphorylated).

[a]If not stated otherwise, primers were used at 0.5 μM and hybridization probes were used at 0.15 μM. [b]Forward primer was used at a final concentration of 0.25 μM and backward primer of 0.375 μM. [c]Primers were used at a final concentration of 1 μM. [d]Primers were used at a final concentration of 0.25 μM. [e]Primers were used at a final concentration of 1 μM, probes So1 and So1b at 0.1 μM each. [f]A second probe was introduced to detect both HPV38a and HPV38b.

has to be taken, graphs for each sample should be evaluated individually and results should be checked for plausibility. When using the automatic 'second derivative maximum' analysis option in the LightCycler software, qPCR data may be misleading, if abnormal plots are not recognized and discarded. Normal plots as shown in **Figure 3** consist of a flat baseline, and a log phase of amplification, followed by a plateau. The LightCycler software indicates the parameters describing the standard curve as the slope and the *y*-intercepts, which, in combination with the threshold cycle C_t, serve to calculate the copy number of a nucleic acid present in a sample. Check whether the slope of the standard curve, which represents the efficiency of the amplification, is between −3.2 and −3.8 and the error of the standard curve is <0.1. If the error of the standard curve is larger than 0.1, the dilution series may be imprecise or pipetting errors may have occurred. In this case, dilution series should be checked and quantification of the samples should be repeated. Check controls for expected results such as regular spacing between the steps of the dilution series and similar steepness of the curves, sufficient sensitivity of the PCR run as represented by amplification of the ten copies per assay, and that there is no fluorescence increase for negative controls. Ensure that all data points to be recorded are within the dynamic range defined by the standard curve. Check replicates: all replicates should be within 0.5 C_t of each other. Viral loads of the samples are calculated as one HPV DNA copy/cell equivalent. A cell contains by definition two copies of a single-copy gene as beta-globin. Thus, two beta-globin gene copies are taken as a cell equivalent.

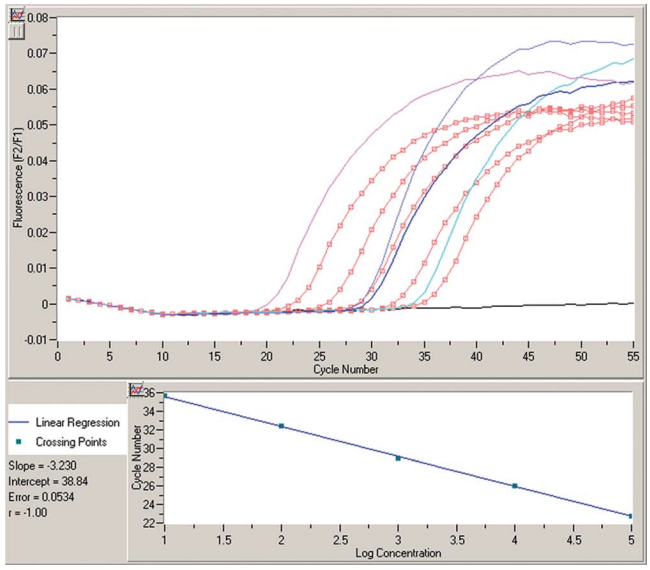

Figure 3 | Amplification plots of an HPV20 quantification experiment. Graphs of the dilution series of HPV20 (10^1–10^5) are given in red (with squares), the negative control in black (flat line). Plots of four samples are shown in different shades of blue and pink. In the field below, the parameters describing the standard curve as the slope and the *y*-intercept are given. All samples and controls were amplified as single replicates. All experiments were carried out with human tissues complied with institutional regulations.

MATERIALS

REAGENTS

- Fresh or formalin-fixed and paraffin-embedded samples **! CAUTION** Fresh tissue is a biohazard and samples should always be handled with gloves. All experiments carried out with human tissues must comply with institutional regulations.
- Primers and labeled oligonucleotides as specified in **Table 2**. All oligonucleotides are HPLC-purified (TIB Molbiol, Berlin, Germany)
- Plasmids containing HPV target sequences. Standard HPV DNA plasmids have to be obtained on request from the scientist who cloned the respective genomes (see **Table 3**). HPV5, -8 and -20 can be provided by this laboratory.
- pUC18 plasmid DNA (Sigma, cat. no. D4154)
- Human genomic DNA (Sigma, cat. no. D4642)
- BSA nonacetylated (Sigma, cat. no. B6917)
- Tween-20 10% (vol/vol) (Sigma, cat. no. P9416)
- DMSO (Sigma, cat. no. D9170)
- Sodium hypochlorite (Sigma, 239305) or ethanol (Sigma, E71489) **! CAUTION** May be harmful when inhaled, ingested or absorbed into the skin. Handle with gloves.
- QIAamp DNA Mini Kit (Qiagen, cat. no. 51304)
- RHA Kit Skin (beta) HPV (Diassay B.V.)
- Tris-EDTA buffer solution (1.0 M Tris-HCl and 0.1 M EDTA, Sigma, T9285)
- Salmon Sperm DNA (10 mg ml^{-1}, Invitrogen, cat. no. 15632011)
- dNTPs, 100 mM dNTP Set (Invitrogen, cat. no. 1029718)
- Platinum Taq DNA Polymerase delivered with 10× PCR buffer (without $MgCl_2$) and 50 mM $MgCl_2$ solution (Invitrogen, cat. no. 10966-034) (for Hot Start PCR)
- LightCycler Control Kit DNA (Roche-Molecular Biochemicals, cat. no. 12158833001) The manufacturer does not reveal the sequences of the primers used in the kit.
- SYBR Green (Invitrogen, cat. no. S7567) **! CAUTION** May be carcinogenic when ingested or absorbed into the skin. Wear appropriate gloves when working with solutions that contain this dye.

EQUIPMENT

- Microfuge
- Thermomixer
- LightCycler 1.5 (Roche-Molecular Biochemicals)
- LightCycler capillaries (Roche Molecular Biochemicals)
- LightCycler centrifuge adapters (Roche Molecular Biochemicals)
- Spectrophotometer for 260/280-nm measurements
- Scalpels

REAGENT SETUP

TE-SS buffer 10 mM Tris, 1 mM EDTA and Salmon Sperm DNA of 40 ng μl^{-1}. To prepare 10 ml of TE-SS buffer, pipet 100 µl Tris-EDTA solution and 40 µl of salmon sperm DNA solution into 9.86 ml of PCR-grade water. The buffer can be stored at −20 °C for years.

SYBR Green A 1:1,000 working solutions in TE buffer may be prepared in advance and are extremely stable to repeated freeze/thaw cycles. They may be stored at −20 °C and are reliably stable for up to 2 weeks at 4 °C.

Reaction mixtures for PCR can be prepared for several assays without polymerase and appropriate aliquots may be kept at −20 °C for months. The enzyme should then be added before use.

PROCEDURE

Preparation of samples ● TIMING 15 h

1| Human samples can be prepared for PCR amplification using option A for paraffin-embedded biopsies or option B for fresh biopsies and plucked eyebrow hair bulbs.

! CAUTION All experiments carried out with human tissues must comply with institutional regulations.

(A) Preparation of paraffin-embedded biopsy samples for PCR amplification

(i) Remove all paraffin that does not contain tissue using a scalpel. Cut at least ten 10-µm sections of a 1-cm^2 tissue sample with a microtome.
▲ CRITICAL STEP To avoid cross-contaminations in the microtome, the equipment should be cleaned with 1% (vol/vol) sodium hypochlorite, followed by 70% (vol/vol) ethanol for each biopsy. Use a new scalpel for each sample.

(ii) Extract DNA with the QIAamp DNA Mini Kit according to the manufacturer's instructions with the modifications stated below. Digest the sections in 180 µl of ATL buffer with 25 µl (instead of 20 µl) of proteinase K (20 mg ml^{-1}) overnight with an incubation temperature of 62 °C (instead of 56 °C). Briefly, centrifuge the tube to remove drops from the inside of the lid.

TABLE 3 | Standard HPV plasmids for dilution series.

HPV type	Reference	GenBank Accession Numbers
5	Kremsdorf *et al.*[40]	M17463
8	Pfister *et al.*[41]	M12737
	Steger *et al.*[42]	
15	Kremsdorf *et al.*[43]	X74468
20	Kremsdorf *et al.*[43]	U31778
23	Kremsdorf *et al.*[43]	U31781
24	Kremsdorf *et al.*[43]	U31782
36	Kawashima *et al.*[44]	U31785
38	Scheurlen *et al.*[45]	U31787

Add 200 µl of AL buffer, thoroughly mix by vortexing for 15 s and incubate for 10 min at 70 °C. Add 200 µl of ethanol (96–100% (vol/vol)), thoroughly mix by vortexing for 15 s, incubate for 1 min at 70 °C, vortex again and briefly centrifuge the tube at 1,000*g*, room temperature (RT, 18–25 °C).

(iii) Apply the digested sample onto a QIAamp Mini Spin Column (in a 2-ml collection tube) and immediately centrifuge for 1 min at 6,000*g*, RT, to prevent clogging. Place the column in a new 2-ml collection tube and wash DNA using buffers AW1 and AW2 according to the manufacturer's instructions.

(iv) Elute the DNA from the column using (according to sample size) 50–200 µl (prewarmed to 37 °C) of extraction buffer AE according to the manufacturer's instructions.

■ **PAUSE POINT** Samples can be stored at −20 °C for at least 1 year.

(B) Preparation of fresh biopsies and plucked eyebrow hair bulbs for PCR amplification

! **CAUTION** Fresh tissue is a biohazard and samples should always be handled with gloves.

(i) Mince the biopsies before digestion in a Petri dish at room temperature using a scalpel to pieces of <1 mm^3 or use a mixer mill.

▲ **CRITICAL STEP** To avoid cross-contaminations due to mincing, use new scalpels and Petri dishes for each biopsy.

(ii) Digest the samples in 200 µl of digestion buffer with proteinase K (supplied with the QIAamp DNA Mini Kit) overnight according to the manufacturer's protocol. Extract DNA according to the sample size and hair bulb numbers in 50–200 µl (prewarmed to 37 °C) extraction buffer supplied with the QIAamp DNA Mini Kit.

■ **PAUSE POINT** Samples can be stored at −20 °C for at least 1 year.

Control of quality and quantity of extracted DNA ● TIMING ~1.5 h

2| Prepare a ten-fold dilution series of control human genomic DNA starting with a concentration of 17.5 ng of human genomic DNA per µl down to 1.75 pg µl^{-1}. This covers a range from 10^4 copies of beta-globin gene/2 µl down to 1 copy/2 µl.

▲ **CRITICAL STEP** Prepare a fresh dilution series of human genomic DNA each day to ensure integrity.

3| Use the dilution series prepared in Step 2 and 2 µl of each sample prepared in Step 1 to quantify a single-copy gene, e.g., beta globin using the LightCycler control kit according to the manufacturer's instructions. Include a negative control of 2 µl of the water used to prepare the dilution series. The LightCycler software will calculate the slope and the *y*-intercept of the standard curves and then use this to automatically calculate the copy number of the single-copy gene present in the sample.

? TROUBLESHOOTING

Beta-HPV typing ● TIMING ~6 h

4| Using 10 µl of DNA sample in a 50-µl final reaction volume, type the beta-HPV using the PM-PCR/reverse hybridization assay[23] as described in the manufacturer's protocol.

▲ **CRITICAL STEP** Samples should contain at least 200 amplifiable copies of a single copy gene per µl as determined in Step 3.

? TROUBLESHOOTING

Setup of HPV dilution series ● TIMING ~ 4 h

5| Calculate the mass in grams of 5×10^{10} molecules of the cloned HPV DNA plasmid (vector plus insert) by multiplying the number of nucleotides by 660, dividing by 6.023×10^{23} and multiplying by 5×10^{10}.

6| Determine the concentration of the DNA (ng µl^{-1}) in the sample prepared in Step 1 by absorbance measurement at 260 nm[39].

7| Dilute the equivalent volume of 5×10^{10} copies of the cloned HPV DNA plasmid into 1,000 µl of TE-SS buffer (final concentration 5×10^7 copies of HPV DNA per µl). Dilute the 5×10^7 DNA solution in TE-SS buffer in 1:10 dilution steps to achieve two solutions with 5×10^6 and 5×10^5 copies of HPV DNA per µl.

8| Using pUC18 plasmid DNA, set up a reference dilution series resulting in four solutions containing 5×10^7, 5×10^6, 5×10^5 and 5×10^4 copies of DNA per µl.

9| Pipette 18 µl each of the reaction mix described in **Table 4** into separate LightCycler capillaries. Prepare four capillaries for HPV DNA dilutions prepared in Step 7, one for a negative control with 2 µl of water instead of DNA and eight capillaries for the pUC18 plasmid dilutions prepared in Step 8.

! CAUTION SYBR Green may be carcinogenic when ingested or absorbed into the skin. Wear appropriate gloves when working with solutions that contain this dye.

10| Pipette 2 µl of the dilution steps (5×10^6 and 5×10^5 copies of HPV DNA per µl) prepared in Step 7 and the pUC18 plasmid DNA dilution steps (5×10^7–5×10^4 copies of DNA copies per µl) prepared in Step 8 in duplicate and one sample of 2 µl of water into the capillaries prepared in Step 9.

11| Amplify the nucleic acids using the PCR cycling conditions given in **Table 5**.
? TROUBLESHOOTING

12| Compare the mean threshold cycle numbers of the 5×10^6–5×10^5 copies of DNA per µl of the cloned HPV plasmid with the mean threshold cycle numbers of the 5×10^6–5×10^5 copies of DNA per µl pUC18 plasmid standard dilution series. If a larger mean deviation than 0.4 cycles is observed for one of the HPV DNA dilutions, calculate the relative content of HPV plasmid DNA by using 2^n, where n equals the observed mean deviation. Then prepare a new 5×10^6 dilution step using the 2^{-n}-fold volume of HPV DNA plasmid solution used before. Prepare a 1:10 dilution step to obtain 5×10^5 copies of HPV DNA per µl TE-SS buffer and restart at Step 9.
? TROUBLESHOOTING

TABLE 4 | Reaction mix for beta-lactamase PCR.

Reagents	Volumes for one sample (µl)	Final concentration
PCR buffer (10×)	2	1×
Primer Blac-fw (10 µM)	1	0.5 µM
Primer Blac-bw (10 µM)	1	0.5 µM
SYBR Green (1/1,000)	2	1/10,000
BSA (20 mg ml^{-1})	0.5	500 ng μl^{-1}
dNTPs (10 mM, each)	0.4	200 µM
Platinum Taq (5 U μl^{-1})	0.25	1.25 U
DMSO	1	5% (vol/vol)
Mg^{2+} (50 mM)	1.6	4 mM
H_2O	8.25	

Primer sequences are Blac-fw 5′-AGCATCTTACGGATGGCATG-3′ and Blac-bw 5′-GTTGTCAGAAGTAAGTTGG-3′. The amplimer comprises 91 bp.

Quantification of individual beta-HPV types ● TIMING ~1.5 h

13| For beta-HPV PCR, prepare the reaction mix described in **Table 6**.
▲CRITICAL STEP The reaction mix without the polymerase can be prepared for several assays and appropriate aliquots kept at −20 °C. Add the polymerase immediately before use.

14| Pipette 18 µl of the reaction mix into a separate LightCycler capillary for each sample in duplicate and a negative control with 2 µl of water instead of DNA.

15| Set up the reference HPV dilution series with 2 µl of dilution steps (5×10^5–5×10^0 copies of HPV DNA per µl).

16| Pipette 2 µl of sample DNA into a separate LightCycler capillary in duplicate. Samples should contain at least 200 amplifiable copies of a single copy gene per µl.

TABLE 5 | Cycling conditions of the beta-lactamase PCR.

Step	Temperature (°C)	Temperature transition rate (°C/s)	Two target temperature (°C)	Step size (°C)	Time (s)
Predenaturation	95	20			60
Touch down (10 cycles)					
Denaturation	95	20			1
Annealing	68	20	58	1	5
Elongation	72	5			10
Amplification (20 cycles)					
Denaturation	95	20			1
Annealing	58	20			5
Elongation	72	5			10
Fluorescence acquisition	79	20			2

▲ CRITICAL STEP If viral load has been too low for HPV detection in a previous qPCR run, up to 8 µl of DNA preparation may be used instead of water in the PCR mixture.

17| Amplify the nucleic acids using the PCR cycling conditions given in **Table 7**.
Ensure that the ID numbers of all samples are entered correctly in the respective form of the LightCycler software before starting the run.
? TROUBLESHOOTING

Data analysis ● TIMING ~10 min
18| Read the HPV DNA copy number calculated by the software from the screen and calculate HPV DNA loads as one HPV DNA copy/cell equivalent. Two beta-globin gene copies are taken as a cell equivalent and beta-globin gene copy numbers were determined in Step 3.
▲ CRITICAL STEP Check for abnormal plots. The slope of the standard curve should be between −3.0 and −3.8 and error of the standard curve should be smaller than 0.1. Ensure that controls yield the expected results. Also ensure that all sample results are within the dynamic range defined by the standard curve. All replicates should be within 0.5 C_t of each other. If larger deviations are observed, measuring of the sample should be repeated.

Troubleshooting advice for qPCR is given in the Troubleshooting section. For further advice, see also Table 5 in reference 26.

TABLE 6 | Reaction mix for the beta-HPV PCR.

Reagents	Volumes for each sample (µl)	Final concentration
PCR buffer (10×)	2	1×
Primer	See **Table 2**	
Probe HPV-So1[a] (3 µM)	1	0.15 µM
Probe HPV-So2[a] (3 µM)	1	0.15 µM
BSA (20 mg ml^{-1})	0.5	500 ng $µl^{-1}$
dNTPs (10 mM, each)	0.4	200 µM
Platinum Taq (5 U $µl^{-1}$)	0.25	1.25 U
DMSO	1	5% (vol/vol)
Mg^{2+} (50 mM)	See **Table 2**	
Tween-20 (10%)	1	0.5% (vol/vol)
TE-SS (40 ng $µl^{-1}$)	1	2 ng $µl^{-1}$
H_2O	Add water to a final volume of 20 µl	

[a]For HPV38, different probe concentrations were used (see **Table 2**).

● TIMING
Step 1, Sample preparation (24 samples): 1 h hands-on time (plus 15 h (overnight) incubation time)
Steps 2 and 3, Control of quality and quantity of extracted DNA: 1.5 h (has to be performed just once for all HPV types in a sample)
Step 4, Beta-HPV typing: ~6 h
Steps 5–12, Setup of HPV dilution series: ~4 h (only once at the beginning of the study)
Steps 13–17, Quantification of individual beta-HPV types: 1.5 h per HPV type
Step 18, Data analysis: 10 min

TABLE 7 | Cycling conditions of the beta-HPV PCR.

Step	Temperature (°C)	Temperature transition rate (°C/s)	Two target temperature (°C)	Step size (°C)	Time (s)
Predenaturation	95	20			120
Touchdown[a] (10 cycles)					
Denaturation	95	20			1
Annealing	65/64/66[a]	20	55/54/56	1	10
Elongation	72	5			10
Amplification (40–45 cycles)[b]					
Denaturation	95	20			1
Annealing	See **Table 2**	20			12[d]
Elongation	72	Different[c]			10

[a]Only in the protocols for HPV20/HPV24/HPV38 is a touchdown program required before the amplification program. The different annealing temperatures for HPV20/HPV24/HPV38 are given. [b]Cycle numbers were 40 for HPV38, and 45 for HPV5, HPV8, HPV15, HPV20, HPV23, HPV24 and HPV36. [c]Temperature gradients (°C/s) from annealing to elongation were 2 °C/s for HPV8 and -36; 5 °C/s for HPV20, -23, -24 and -38; 10 °C/s for HPV5 and 20 °C/s for HPV15. Reasons for different temperature gradients are discussed in the Experimental design. [d]Fluorescence F2/F1 is acquired at the end of the annealing step.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 8**.

TABLE 8 | Troubleshooting table.

Step	Problem	Possible reason	Solution
3, 4, 11 and 17	No amplification detected (neither samples nor positive controls)	No amplification occurring or no detection of PCR product	Check amplification assay by agarose gel electrophoresis to see if a PCR product is present
		Probe-based detection failing	If a PCR product is visible on a gel, try fluorescent nucleic acid–binding dye (SYBR Green) detection
			Prepare/order new probes
		LightCycler hardware defect	Call LightCycler Service
		A reagent is missing from the PCR or its concentration is too low.	If no PCR product is visible on a gel, prepare a new reaction mix. Thaw stock solutions completely before pipetting. Vortex reaction mixes thoroughly
		Primer degraded	Order a new batch of primers
11 and 17	No amplification detectable in specific samples	Inappropriate primers/probes (mismatches with the sequence of the subtype in the sample)	Design additional primers or probes that cover the subtype of the respective HPV type
12	A larger mean deviation than 0.4 cycles is observed for one of the HPV DNA dilutions	Pipetting error or imprecise absorbance measurement	Prepare a new dilution series of HPV plasmid DNA starting from the 5×10^7 solution and restart at Step 9
3	No beta-globin gene amplification occurring in samples	The sample digestion is not efficient	Repeat digestion step (increase incubation time or proteinase K concentration)
		The target DNA is fragmented or degraded	Collect a new sample
		Not sufficient cells in the sample	
3, 4, 11 and 17	Amplification occurring in negative controls	PCR carryover contamination	Follow good laboratory practice for PCR, e.g., spatially separate pre-PCR setup, DNA extraction and amplification/detection. Do not move equipment such as pipettes, racks between laboratories. Always use filter pipette tips
			Reagents should be made up and stored in small aliquots so that they can be discarded if carryover contamination is suspected or observed
17	Problems during setup of new PCR protocols	The probe is not binding to the target efficiently because the annealing temperature is too high	Verify the calculated T_m, using appropriate software
		The probe is not binding to the target efficiently because the PCR product is folding in solution	Design new primers for another viral genome region
		The reaction is not optimized, and no or insufficient product is formed	Titrate $MgCl_2$ and primer concentration and test different annealing temperatures

ANTICIPATED RESULTS

A typical result is shown in **Figure 3**. On the *y*-axis of the upper graph, the fluorescence (F2/F1) is plotted and on the *x*-axis the cycle number. Graphs display the course of fluorescence during the PCR. Fluorescence starts to visibly increase from a baseline value (depending on the input number of target molecules) between cycle numbers 20 and 38. The graph of the negative control does not increase above background. Beta-HPV-DNA copy numbers are usually low and within the range of the suggested standard dilution series, which are shown in red and contain 10^1 to 10^5 copies of HPV20. The form of the graphs is used by the LightCycler software (second derivative maximum method) to define the cycle number when fluorescence may be first differentiated from background fluorescence. This cycle is called threshold cycle, C_t. In the field below, the standard curve is given to the right and the parameters of the standard curve as slope and *y*-intercept are listed to the left. These parameters are used by the LightCycler software in combination with the threshold cycle C_t to calculate the HPV-DNA copy number present in a sample. Note that one sample (pink) contains more HPV-DNA copies than mirrored by the dilution series. This sample has to be remeasured after dilution, e.g., 1:100.

ACKNOWLEDGMENTS This study was supported by EC Grant QLK-CT-200201179. S.J.W. is supported by the 'Deutsche Krebshilfe,' EMBO and the Köln Fortune Program of the University of Cologne. U.W. was supported by the German Federal Ministry of Education and Research (BMBF) (Grant no. 01 KI 0771 (TP7)).

AUTHOR CONTRIBUTIONS S.J.W. conceived and designed the protocol; S.J.W., M.J., U.W. and H.P. provided administrative, technical or material support; and S.J.W., U.W. and H.P. wrote the paper.

Published online at http://www.natureprotocols.com.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions.

1. IARC. Human papillomaviruses. *IARC Monogr. Eval. Carcinog. Risks Hum.* **90**, 245–259 (2007).
2. Orth, G. Genetics of epidermodysplasia verruciformis: insights into host defense against papillomaviruses. *Semin. Immunol.* **18**, 362–74 (2006).
3. Berkhout, R.J. *et al.* Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. *J. Clin. Microbiol.* **33**, 690–695 (1995).
4. Forslund, O., Antonsson, A., Nordin, P., Stenquist, B. & Hansson, B.G. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. *J. Gen. Virol.* **80** (Part 9): 2437–2443 (1999).
5. Pfister, H. *et al.* High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. *Arch Dermatol. Res.* **295**, 273–279 (2003).
6. Shamanin, V. *et al.* Human papillomavirus infections in nonmelanoma skin cancers from renal transplant recipients and nonimmunosuppressed patients. *J. Natl. Cancer Inst.* **88**, 802–811 (1996).
7. Boxman, I.L. *et al.* Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. *J. Invest. Dermatol.* **108**, 712–715 (1997).
8. Antonsson, A., Forslund, O., Ekberg, H., Sterner, G. & Hansson, B.G. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. *J. Virol.* **74**, 11636–11641 (2000).
9. Astori, G. *et al.* Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. *J. Invest. Dermatol.* **110**, 752–755 (1998).
10. Weissenborn, S.J., De Koning, M.N., Wieland, U., Quint, W.G. & Pfister, H.J. Intrafamilial transmission and family-specific spectra of cutaneous betapapillomaviruses. *J. Virol.* **83**, 811–816 (2009).
11. Antonsson, A., Karanfilovska, S., Lindqvist, P.G. & Hansson, B.G. General acquisition of human papillomavirus infections of skin occurs in early infancy. *J. Clin. Microbiol.* **41**, 2509–2514 (2003).
12. Hazard, K. *et al.* Cutaneous human papillomaviruses persist on healthy skin. *J. Invest. Dermatol.* **127**, 116–119 (2007).
13. de Koning, M.N. *et al.* Betapapillomaviruses frequently persist in the skin of healthy individuals. *J. Gen. Virol.* **88**, 1489–1495 (2007).
14. de Koning, M.N. *et al.* Prevalence and associated factors of betapapillomavirus infections in individuals without cutaneous squamous cell carcinoma. *J. Gen. Virol.* **90**, 1611–1621 (2009).
15. Harwood, C.A. *et al.* Increased risk of skin cancer associated with the presence of epidermodysplasia verruciformis human papillomavirus types in normal skin. *Br. J. Dermatol.* **150**, 949–957 (2004).
16. Struijk, L. *et al.* Presence of human papillomavirus DNA in plucked eyebrow hairs is associated with a history of cutaneous squamous cell carcinoma. *J. Invest. Dermatol.* **121**, 1531–1535 (2003).
17. Boxman, I.L. *et al.* Case–control study in a subtropical Australian population to assess the relation between non-melanoma skin cancer and epidermodysplasia verruciformis human papillomavirus DNA in plucked eyebrow hairs. The Nambour Skin Cancer Prevention Study Group. *Int. J. Cancer* **86**, 118–121 (2000).
18. Iftner, A. *et al.* The prevalence of human papillomavirus genotypes in nonmelanoma skin cancers of nonimmunosuppressed individuals identifies high-risk genital types as possible risk factors. *Cancer Res.* **63**, 7515–7519 (2003).
19. Asgari, M.M. *et al.* Detection of human papillomavirus DNA in cutaneous squamous cell carcinoma among immunocompetent individuals. *J. Invest. Dermatol.* **128**, 1409–1417 (2008).
20. Weissenborn, S.J. *et al.* Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J. Invest. Dermatol.* **125**, 93–97 (2005).
21. Dell'Oste, V. *et al.* High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis. *J. Invest. Dermatol.* **129**, 1026–1034 (2009).
22. Brink, A.A. *et al.* Development of a general-primer-PCR-reverse-line-blotting system for detection of beta and gamma cutaneous human papillomaviruses. *J. Clin. Microbiol.* **43**, 5581–5587 (2005).
23. de Koning, M. *et al.* Evaluation of a novel highly sensitive, broad-spectrum PCR-reverse hybridization assay for detection and identification of beta-papillomavirus DNA. *J. Clin. Microbiol.* **44**, 1792–1800 (2006).
24. Nindl, I. *et al.* Extension of the typing in a general-primer-PCR reverse-line-blotting system to detect all 25 cutaneous beta human papillomaviruses. *J. Virol. Methods* **146**, 1–4 (2007).
25. Gheit, T. *et al.* Development of a sensitive and specific multiplex PCR method combined with DNA microarray primer extension to detect betapapillomavirus types. *J. Clin. Microbiol.* **45**, 2537–2544 (2007).
26. Malnati, M.S. *et al.* A universal real-time PCR assay for the quantification of group-M HIV-1 proviral load. *Nat. Protoc.* **3**, 1240–1248 (2008).
27. Kubista, M. *et al.* The real-time polymerase chain reaction. *Mol. Aspects Med.* **27**, 95–125 (2006).
28. Kreuter, A. *et al.* Penile intraepithelial neoplasia is frequent in HIV-positive men with anal dysplasia. *J. Invest. Dermatol.* **128**, 2316–2324 (2008).
29. Vasiljevic, N. *et al.* Characterization of two novel cutaneous human papillomaviruses, HPV93 and HPV96. *J. Gen. Virol.* **88**, 1479–1483 (2007).
30. Vasiljevic, N., Hazard, K., Dillner, J. & Forslund, O. Four novel human betapapillomaviruses of species 2 preferentially found in actinic keratosis. *J. Gen. Virol.* **89**, 2467–2474 (2008).
31. Sutcliffe, J.G. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc. Natl. Acad. Sci. USA* **75**, 3737–3741 (1978).
32. Kwok, S. Procedures to minimize PCR-product carry-over. In *PCR Protocols—A guide to Methods and Applications* (eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J.) 142–145 (Academic Press, San Diego, California, 1990).

33. Kwok, S. & Higuchi, R. Avoiding false positives with PCR. *Nature* **339**, 237–238 (1989).
34. Wittwer, T.C. *et al.* Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques* **22**, 130–138 (1997).
35. Wittwer, T.C. *et al.* The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *BioTechniques* **22**, 176–181 (1997).
36. Mackay, J. & Landt, O. Real-time PCR fluorescent chemistries. In *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* 2nd edn. Vol. 353 (eds. Hilario, E.& Mackay, J.) 237–261 (Humana Press, Clifton, New Jersey, 2007).
37. Korbie, D.J. & Mattick, J.S. Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat. Protoc.* **3**, 1452–1456 (2008).
38. Weissenborn, S.J. *et al.* Oncogenic human papillomavirus DNA loads in human immunodeficiency virus-positive women with high-grade cervical lesions are strongly elevated. *J. Clin. Microbiol.* **41**, 2763–2767 (2003).
39. Sambrook, J. & Russell, D.W. *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2001).
40. Kremsdorf, D., Jablonska, S., Favre, M. & Orth, G. Biochemical characterization of two types of human papillomaviruses associated with epidermodysplasia verruciformis. *J. Virol.* **43**, 436–447 (1982).
41. Pfister, H., Nürnberger, F., Gissmann, L. & zur Hausen, H. Characterization of a human papillomavirus from epidermodysplasia verruciformis lesions of a patient from Upper-volta. *Int. J. Cancer* **27**, 645–650 (1981).
42. Steger, G., Olszewsky, M., Stockfleth, E. & Pfister, H. Prevalence of antibodies to human papillomavirus type 8 in human sera. *J. Virol.* **64**, 4399–4406 (1990).
43. Kremsdorf, D. *et al.* Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. *J. Virol.* **52**, 1013–1018 (1984).
44. Kawashima, M., Favre, M., Jablonska, S., Obalek, S. & Orth, G. Characterization of a new type of human papillomavirus (HPV) related to HPV5 from a case of actinic keratosis. *Virology* **154**, 389–394 (1986).
45. Scheurlen, W., Gissmann, L., Gross, G. & zur Hausen, H. Molecular cloning of two new HPV types (HPV 37 and HPV 38) from a keratoacanthoma and a malignant melanoma. *Int. J. Cancer* **37**, 505–510 (1986).
46. Wieland, U. *et al.* Communication: papillomavirus DNA in basal cell carcinomas of immunocompetent patients: an accidental association? *J. Invest. Dermatol.* **115**, 124–128 (2000).
47. Saiki, R.K. *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350–1354 (1985).