Qualitative PCR–ELISA protocol for the detection and typing of viral genomes

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PCR is an established technique providing rapid and highly productive amplification of specific DNA sequences. The demand for equally rapid, sensitive and objective methods to achieve detection of PCR products has led to the coupling of PCR with ELISA. PCR–ELISA involves direct incorporation of labeled nucleotides in amplicons during PCR-amplification, their hybridization to specific probes and hybrid capture-immunoassay in microtiter wells. PCR–ELISA is performed in 1 d and is very flexible, with the ability to process simultaneously up to 96 or 384 samples. This technique is potentially automatable and does not require expensive equipment, and thus can be fundamental in laboratories without access to a real-time PCR thermocycler. PCR–ELISA has mainly been used to detect infectious agents, including viruses, bacteria, protozoa and fungi. A PCR–ELISA protocol for the qualitative detection of papillomavirus genomes and simultaneous typing of different genotypes are detailed here as an example of the technique.

INTRODUCTION

The PCR, with its extraordinary sensitivity, is the method of choice for the detection of nucleic acids present in very low concentrations in biological specimens. PCR-based techniques are applied to gene analysis, to study gene expression and to detect etiological agents in infections. The need for a method to detect PCR-amplified products, which is able to offer sensitivity, specificity and an objective evaluation of results, has led to the coupling of PCR with ELISA.

PCR–ELISA was initially developed to allow the direct analysis of labeled amplicons using ELISAs[1] but, to improve the sensitivity and specificity of the detection, a hybridization step with specific probes has since been added[2]. A sensitive and specific PCR–ELISA is therefore based on the labeling of amplicons during PCR, their hybridization with probes specific for the target, capture of labeled hybrids onto microtiter plates or tubes and detection by immunoassay[3–5]. PCR–ELISA methods are normally developed using the conventional microtiter plate format and colorimetric detection, as detailed in **Figure 1**. However, chemiluminescent detection can also be performed using microplate luminometers or imaging systems, and other assay formats such as miniaturized plates and high-throughput assay formats can be used[6–8]. PCR–ELISA is a very versatile tool, able to process up to 96 or 384 samples simultaneously (depending on the number of wells of ELISA plates) in ~6.5 h (following preparation of the sample). It is also potentially automatable, due to robust standardization of the methods, reagents and samples.

Newer PCR techniques such as real-time PCR are now successfully used for the qualitative and quantitative evaluation of nucleic acids[9]. Real-time PCR shows similar sensitivity to the PCR–ELISA, but has the advantage of being a sealed system, decreasing the risk of carryover contamination during the reaction, which can be a problem during PCR–ELISA. Although the risk of carryover contamination with amplified products leading to false-positive results is greater with PCR–ELISA than real-time PCR, it can be prevented using several strategies such as physical separation of work areas, use of gloves, specialized pipetting devices and careful work habits (see TROUBLESHOOTING). Real-time PCR is also more rapid, since amplification and detection of target DNA can be completed within 1 h after DNA extraction. PCR–ELISA, however, does not require the use of expensive equipment, as real-time PCR presently does, and instead uses basic instruments present in every diagnostic laboratory. In addition, the typing of different genotypes of an infectious agent is more easily performed by PCR–ELISA rather than with real-time PCR, which would require multiple amplifications and/or different detector molecules. Another advantage of PCR–ELISA is that DNA purification step from serum samples, cytological and histological samples can be omitted.

In our laboratory, PCR–ELISA methods have been developed mainly for the detection of viral genomes in clinical samples such as cytological samples, biopsies, serum samples and plasma pools; however, PCR–ELISA can effectively be applied to amplify and detect any specific DNA sequence in every biological specimen. Recently, many PCR–ELISA-based assays have been developed for

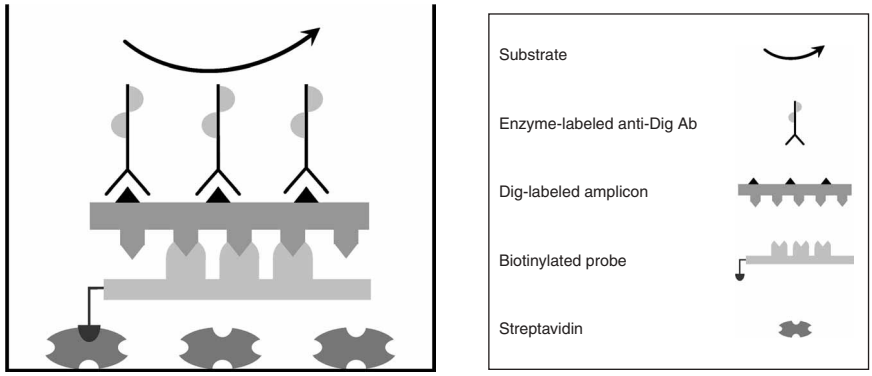

Figure 1 | Scheme of PCR–ELISA. PCR–ELISA for qualitative detection of viral genomes is performed by capture onto streptavidin-coated microtiter plates of biotinylated specific probes hybridized with digoxigenin (Dig)-labeled amplicons. The detection of immobilized hybridized amplicons is performed by anti-Dig antibodies conjugated to peroxidase or alkaline phosphatase with final colorimetric development.

the detection of viruses, bacteria, protozoa and fungi, in clinical, food and environmental samples, such as hepatitis B virus in blood[10], adenovirus[11] and rotavirus in stool samples[12]; *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk[13]; methicillin-resistant *Staphylococcus aureus* in nasal swabs[14]; *Salmonella* spp. in milk and meat samples[15]; *Pseudomonas aeruginosa* from blood culture bottles[16]; *Enterococcus faecalis* and *Pediococcus pentosaceus* in Nurmi-type cultures[17]; *Leishmania infantum* in pools of female sandflies[18]; *Plasmodium falciparum* types with single nucleotide polymorphisms in the *DHFR* and *DHPS* genes in different isolates[19]; *Trypanosoma congolense* type savannah in bovine blood samples[20]; *Wucheria bancrofti* in blood samples[21] and circulating *Aspergillus fumigatus* and *Aspergillus flavus* in serum samples[22]. The PCR–ELISA has therefore proved especially valid in the typing of different genomes.

Qualitative PCR–ELISA—the detection and typing of human papillomavirus

We present here a protocol for the qualitative detection and genotyping of papillomavirus genomes as an example for the use of PCR–ELISA technique. Human papillomavirus (HPV) infections are related to several cutaneous and mucosal dysplasias, including both benign and malignant lesions. To date, >100 HPV genotypes have been molecularly identified (~30 from the ano-genital tract) and the HPVs are commonly referred to as high- or low-oncogenic risk, depending on the frequency of their association with malignancy[23]. HPVs are now considered the causative agents of cervical cancer[24]. HPV genotyping is valuable for investigating the clinical behavior and epidemiology of particular types, for the characterization of study populations in HPV vaccination trials and for monitoring the efficacy of HPV vaccines.

Many PCR protocols use either primers recognizing specific HPV open reading frames or highly conserved common sequences among different HPV types. Typing of HPV DNA generated by PCR with consensus primers can be performed by restriction fragment length polymorphism (RFLP)[25,26], by direct DNA sequence analysis[27,28] and by line blot hybridization with type-specific probes[29]. For HPV genotyping, sequence analysis and RFLP analysis of PCR amplicons are not commonly used. Membrane-based genotyping methods (reverse line blot assay) are commonly used, commercially available, sensitive and well validated. However, line blot assays, in comparison with PCR–ELISA, provide only subjective results as read-out is visual, which may result in reproducibility problems and risks of mistakes during data entry.

PCR–ELISA, however, can be easily applied to the detection of HPV genomes and to the typing of different viral genotypes using type-specific probes, and offers the high sensitivity of the enzyme amplification and the specificity of the hybridization reaction. Moreover, results evaluation does not require subjective interpretation and semi-quantitative data can also be obtained. This assay can be performed in 1 d, is easily standardized and therefore seems to be a practical, sensitive and reliable diagnostic tool for the detection and typing of HPV genomes in cytological specimens and in biopsies in the routine diagnostic laboratory.

Experimental design

Primer and probe design. The approach to detect and type different viral genotypes is first to amplify conserved regions of the viral genome using consensus primers designed to cover a broad spectrum of viral types, and then to hybridize the PCR products with type-specific probes. The ideal candidate regions for PCR primer design are those identified as having the least number of nucleotide sequence variations among different genotypes, so that primers will cover a broad range of viral genotypes. Such consensus primers are designed on the basis of a sequence alignment of all known viral genotypes (from GenBank), minimizing any potential mismatches. In general, all PCR primers can be designed according to the following criteria: (i) primers should be 18–30 bp long; (ii) they should have a G/C content between 40 and 60%; (iii) they must have a T_m not lower than 50 °C and not differing by more than 10 °C; (iv) primers should not form possible hairpin structures; (v) they should not form homodimers and heterodimers; (vi) primers must have high 3′ −thermal stability to improve priming efficiency (see also TROUBLESHOOTING).

Afterward, for each viral type, a unique and specific sequence of interest within the target sequence amplified using the consensus primer pair must be clearly defined as the target for probe design. Several popular programs are available for PCR primer and probe design, such as Clone Manager Professional Suite (Scientific & Educational Software), Oligo (MedProbe) and Primer Premier (Premier Biosoft), which are easy to use.

For the detection and typing of HPVs, the most widely used PCR protocols employ consensus or degenerate primers that are directed toward a highly conserved region of the *L1* gene. Among these are the single pair of consensus primers $GP^{5+/6+}$ (see ref. 30) and the MY09/11 primers[31], along with the modified version PGMY09/11 (see ref. 32). Another multiple set of consensus primers (SPF) is available that amplifies a smaller fragment (65 bp compared with 150 bp for the GP primers and 450 bp for MY09/11) of the *L1* gene. This SPF-PCR[33] is designed to discriminate a broad spectrum of HPVs; however, because of the small size of the amplified fragment, one can anticipate reduced discrimination ability as compared with the other systems. Full distinction of ~40 HPV genotypes can be achieved by hybridization with type-specific probes[34], which can be performed in different formats, including line strip assays and microtiter plates that are amenable to automation.

In the following PCR–ELISA protocol, primers designed in a conserved region of the genome, such as consensus primer pair MY11/09 in the *L1* region, are suitable, but primers in the same or other conserved regions could also be employed. The specificity and typing of amplified products is assured by biotinylated oligoprobes specific for HPV 6, 11, 16, 18, 31, 33, 35, 45, 52, 58, which are considered the prevalent genotypes at low and high oncogenic risk. This method can identify multiple infections and can also be adapted for the detection of every HPV genotype[35,36].

Types of samples/specimens that can be used. For detecting and typing of viral genomes by qualitative PCR–ELISA, biological fluids, cytological and histological specimens can be analyzed. For HPV detection in particular, cytological and histological samples from HPV lesions at specific epithelial sites are used.

Optimization of reactions. The requirement of an optimal PCR is to amplify a specific sequence without any unspecific by-products. Therefore, annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA–DNA matches to

occur in the reaction. For any given primer pair, the PCR program can be selected based on the T_m of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small (from 0.1 to 2–3 kb). The activity of the *Taq* polymerase is ~2,000 nt min^{-1} at optimal temperature (72–78 °C), and the extension time in the reaction can be calculated accordingly. A number of parameters can be varied to optimize the reaction conditions for consensus or degenerate PCR. These include primer concentration, magnesium concentration, template concentration, number of cycles of amplification and the temperatures and times of each step in the amplification cycle. If each of these parameters is to be independently varied, the number of possibilities quickly reaches huge proportions. We suggest to fix almost all these parameters at the standard levels that have been successful for other people, and to vary only the parameter that is the most crucial: the temperature of the annealing step during amplification (see also TROUBLESHOOTING).

For the hybridization reaction, block titration of type-specific probes has to be performed: different concentrations (e.g., 2, 5, 10, 20, 50 pmol ml^{-1}) of probes can be hybridized with PCR-amplified products obtained from tenfold dilutions of positive reference controls, such as plasmids containing viral sequences, infected cells (for HPV, SiHa, HeLa or CaSki can be used) or positive reference samples. For the capture of biotinylated probes, commercially available streptavidin-coated microtiter plates guarantee well-standardized performances. For the immunoenzymatic revelation of hybridized amplicons, the optimal working dilution of each reagent used has to be determined by preliminary block titrations.

In order to determine the sensitivity of the reaction, end point dilutions of positive reference controls have to be performed; moreover, for the optimization of PCR–ELISA, it is desirable to test different samples that include those likely to be encountered in routine application.

In order to investigate the reproducibility of the PCR–ELISA method, at least two positive reference samples and two negative reference samples have to be amplified, and then assayed with type-specific immobilized oligoprobes in triplicate in three independent assays. Intraassay coefficient of variation (CV) $<5\%$ and interassay CV lower than 10% are desirable.

Choice of detection system. Many PCR–ELISA methods described in the literature employ streptavidin-coated wells to capture biotinylated probes; in fact a direct binding of the probes to polystyrene surfaces[2], although simpler, is very difficult to standardize in home-developed assays. Moreover, the flexibility of a streptavidin–biotin system able to capture every biotinylated probe cannot be provided by single probe binding methods. In PCR–ELISAs, immunoenzymatic detection of the hybrids is generally achieved by colorimetric reactions, but chemiluminescent detection can also be performed using microplate luminometers or imaging systems[7,8] which, up to now, are not commonly available in basic laboratories. PCR–ELISA using colorimetric detection for the detection and typing of HPVs has shown the same sensitivity range (50–100 genome copies)[35] as PCR–ELISA using chemiluminescent detection[8].

Nowadays, different commercial assays are designed to detect or type oncogenic HPVs present in genital specimens. Up to now, the only test currently approved by the US Food and Drug Administration for detecting HPV DNA is the Hybrid Capture 2 (HC2) system (Digene Corporation), which is based on a nucleic acid hybridization assay where specimens containing the target DNA hybridize with a specific HPV RNA probe mixture including probes for the prevalent high-risk HPV types. The cocktail approach of the HPV HC2 system cannot, however, determine the specific HPV type present in the sample; moreover, mixed infections cannot be diagnosed. Although this HC2 method is easy to use, some authors suggest that the number of false-positive samples in normal specimens due to cross-reactivity with low-risk HPVs needs to be reduced[37,38]. Other commercially available PCR-based assays that are CE-marked (i.e., approved to conform to health and safety standards set out in European directives) include the Amplicor HPV Test (Roche) designed to enable detection in microplates of the prevalent high-risk HPV genotypes using specific probes directly adsorbed to wells, the INNO-LiPA (Innogenetics) and the new LINEAR ARRAY HPV Genotyping Test (Roche). The latter two are membrane-based genotyping methods (reverse line blot assay) with visual read-out. These commercial systems, having a fixed and determined number of HPV type-specific probes for genital HPVs, are useful for screening HPVs in genital cancer, but are inadequate for research as they are not adaptable to study a wide range of different HPVs and can miss other potential pathogenic HPVs.

Controls and data analysis. To control variations in the efficiency of DNA extraction and PCR-amplification, a suitable housekeeping gene sequence such as β globin should be simultaneously analyzed; primers recognizing human β globin gene sequence should be used in each PCR, and amplified products have to be detected by a specific probe. At least one negative control (e.g., TF1 cells that are negative for HPVs), tested in duplicate, a substrate control without target (blank) and two positive controls (e.g., SiHa or CaSki cells and plasmid HPV DNA) should also be included in each assay.

Cut-off (CO) values for the ELISA reaction are then derived from the negative control readings. In general, the two main formulae used for calculating CO values for ELISAs are (i) the mean of the negative controls plus three s.d. (see ref. 39); or (ii) from two to four times the mean of the negative controls[40]. To detect HPV-positive experimental samples, the sensitivity and specificity at various CO values can be evaluated against a panel of reference HPV-positive and negative samples by using the receiver–operator characteristic (ROC) analysis[41]. The ROC curve helps to visualize and understand the trade-off between high sensitivity and high specificity when discriminating between positive and negative laboratory values. Accuracy level of 90% is selected for evaluation of the negative and positive values. As a result of ROC curve analysis, the resulting CO value, for each probe, is equivalent to two times the mean of the negative control. Samples can be considered negative if the OD is less than the CO value minus 20%, and samples are considered positive if the OD is greater than the CO value plus 20%. When the OD value is estimated to be within 20% of the CO value, the sample must be tested once again[42]. During the assay standardization, the working concentration for each probe in the PCR–ELISA is determined using a dilution series ranging from 10^0 to 10^6 molecules of the PCR-amplified digoxigenin (Dig)-labeled target HPV DNA plasmid for the hybridization reactions.

MATERIALS

REAGENTS

- Mineral oil (Applied Biosystems, cat. no. 1862302)
- Fresh or cryopreserved SiHa cell line (ATCC number HTB-35), known to contain one to two HPV 16 DNA copies per cell ▲ CRITICAL An alternative cell line, CaSki (ATCC number CRL-1550), can be used, which contains 500–600 HPV 16 DNA copies per cell, or the HeLa cell line (ATCC number CRL-5844), which contains 20–50 HPV 18 DNA copies per cell.
- Fresh or cryopreserved TF1 cell line (ATCC number CRL-2003), negative for HPV DNA ▲ CRITICAL Alternatively, any other cell line known to be negative for HPV DNA can be used.
- Plasmid containing the complete HPV 16 sequence (Clonit-Alfa Wassermann, cat. no. 05 960479)
- KCl (Merck, cat. no. 109924)
- Tris–HCl (Sigma-Aldrich, cat. no. T1378)
- Tween-20 (Sigma-Aldrich, cat. no. P1379)
- Proteinase K (Roche Molecular Biochemicals, cat. no. 03115844001)
- Dig dNTPs (2 mM) (Roche Molecular Biochemicals, cat. no. 11585550901)
- Consensus primers MY11/09 (10 μM) to amplify a broad spectrum (~30) of HPV DNAs (see **Table 1** and ref. 31)
- Primers GH20/PC04 (1 μM) to amplify β globin DNA as housekeeping gene (see **Table 1** and ref. 34)
- Consensus primers MY11 and MY09 amplify a broad spectrum (~30) of HPV DNAs, while PC04 and GH20 primers amplify β globin DNA as housekeeping gene. **R**: A or G; **Y**: C or T; **M**: A or C; **W**: A or T
- FastStart *Taq* DNA polymerase (5 U μl^{-1}), including 10× amplification buffer and magnesium chloride (25 mM) (Roche Molecular Biochemicals, cat. no. 12032953001 (5,000 U))
- NaOH (Sigma-Aldrich, cat. no. S5881) ! CAUTION Toxic and caustic, should be handled with great care. Wear appropriate gloves and a face mask.
- Type-specific biotinylated probes (100 pmol μl^{-1} in 1 ml volume) for HPV 6 (MY12), 11 (MY13), 16 (MY95), 18 (R18C), 31 (WD128), 33 (MY59), 35 (MY117), 45 (MY70), 52 (MY82), 58 (MY179). See **Table 2** for sequences
- Biotinylated probe (100 pmol μl^{-1} in 1 ml volume) for human β globin gene sequence (PC03; see **Table 2**) *Note*: All primers and probes were synthesized by MWG Biotech's Oligonucleotide Synthesis Services
- Anti-Dig POD (poly) Fab fragments (Roche Molecular Biochemicals, cat. no. 11633716001)
- Conjugate buffer (Roche Molecular Biochemicals, cat. no. 11684825001)
- ABTS substrate solution (Roche Molecular Biochemicals, cat. no. 11684302001) ! CAUTION This product is a mild oxidizing agent. The toxicological properties of this product have not been thoroughly investigated.
- Agarose gel (Bio-Rad, cat. no. 1620126)
- Ethidium bromide (Merck, cat. no. 331564) ! CAUTION A powerful mutagen and is toxic. Avoid breathing the dust. Wear appropriate gloves when working with solution that contain this dye.
- Tris–acetate (Sigma-Aldrich, cat. no. T6025)

TABLE 1 | Primer sequences.

Primer	Bases	Sequence (5′→3′)
MY11 (human papillomavirus (HPV))	20	GCMCAGGGWCATAAYAATGG
MY09 (HPV)	20	CGTCCMARRGGAWACTGATC
PC04 (β globin)	20	CAACTTCATCCACGTTCACC
GH20 (β globin)	20	GAAGAGCCAAGGACAGGTAC

- Bromophenol blue (Merck, cat. no. 111746) (see REAGENT SETUP)
  ! CAUTION May be harmful by inhalation, ingestion or skin absorption. Wear appropriate gloves and safety glasses. Use in a chemical fume hood.
- Glycerol (Sigma-Aldrich, cat. no. 191612)
- DNA marker VI (Roche Molecular Biochemicals, cat. no. 11062590001)
- Anti-Dig peroxidase-conjugated solution
- Digestion buffer (see REAGENT SETUP)
- TE 1× (see REAGENT SETUP)
- Denaturing solution (see REAGENT SETUP)
- Hybridization buffer (see REAGENT SETUP)
- TBST (see REAGENT SETUP)
- TAE 1× (see REAGENT SETUP)

EQUIPMENT

- Microfuge
- Burker chamber
- Thermo mixer
- Thermal cycler
- Electrophoresis cell for agarose gels
- Streptavidinated 96-well microtiter plates (Roche Molecular Biochemicals, cat. no. 11664778001; NUNC, cat. no. 23604)
- Automated washing equipment for microtiter plates
- Spectrophotometer for microtiter plates equipped with 405- and 492-nm filters

REAGENT SETUP

Digestion buffer 50 mM KCl, 10 mM Tris–HCl pH 8.3, 0.05% (vol/vol) Tween-20 (store at +4 °C) and 400 μg ml^{-1} of proteinase K (can be stored at +4 °C for at least 2 months and add just before use).
TE 1× 10 mM Tris–HCl pH 7.5, 1 mM EDTA (can be stored at +4 °C for at least 2 months).
Denaturing solution 100 mM NaOH, 0.1% (vol/vol) Tween-20 (can be stored at +4 °C for at least 2 months).
Hybridization buffer 300 mM NaCl, 100 mM Tris–HCl pH 6.5, 10 mM EDTA, 0.1% (vol/vol) Tween-20 (can be stored at +4 °C for at least 2 months).
TBST 150 mM NaCl, 100 mM Tris–HCl pH 7.5, 0.1% Tween-20 (vol/vol) (can be stored at +4 °C for at least 2 months).
TAE 1× 40 mM Tris–acetate, 1 mM EDTA (can be stored at +4 °C for at least 2 months).
Bromophenol blue solution 25% (wt/vol) glycerol, TAE 5×, 0.5% (wt/vol) bromophenol blue (can be stored at room temperature (20–25 °C) for at least 2 months).

PROCEDURE

Preparation of samples ● TIMING ~3 h

1| Samples can be prepared for PCR amplification using the following options, for biopsies (option A) and cells (option B).

(A) Preparation of biopsy samples for PCR amplification

(i) Dewax paraffin-embedded sections (thickness 10 μm/5 μm) from biopsy specimens by adding 100 μl of sterile water and 100 μl of mineral oil for 15 min in Eppendorf screw-capped tubes[43,44].
▲ CRITICAL STEP Cross-contaminations due to microtome cut can occur in sections from different biopsies. Clean the equipment for each biopsy.
(ii) Centrifuge at 14,000*g* for 5 min.
(iii) Digest in 200 μl of digestion buffer by incubation at 55 °C for 2 h.
(iv) Heat inactivate at 95 °C for 5 min.

TABLE 2 | Probe sequences.

No	Probe	Bases	Sequence (5′→3′)
1	MY12 (HPV 06)	21	BIO-CAT CCG TAA CTA CAT CTT CCA
2	MY13 (HPV 11)	21	BIO-TCT GTG TCT AAA TCT GCT ACA
3	MY95 (HPV 16)	21	BIO-GAT ATG GCA GCA CAT AAT GAC
4	R18C (HPV 18)	24	BIO-CTT AAA TTT GGT AGC ATC ATA TTG
5	WD128 (HPV 31)	22	BIO-TTG CAA ACA GTG ATA CTA CATT
6	MY59 (HPV 33)	21	BIO-AAA AAC AGT ACC TCC AAA GGA
7	MY117 (HPV 35)	21	BIO-ATC ATC TTT AGG TTT TGG TGC
8	MY70 (HPV 45)	19	BIO-TAGTGGACACTACCCGCAG
9	MY82 (HPV 52)	21	BIO-ACA CAC CAC CTA AGG GAA AGG
10	MY179 (HPV 58)	25	BIO-GAC ATT ATG CAC TGA AGT AAC TAA G
11	PC03 (β globin)	20	BIO-ACACAACTGTGTTCACTAGC

Abbreviation: HPV, Human papillomavirus. Sequences of biotinylated (represented as BIO) HPV type-specific probes are given for 10 HPV types (in brackets after probe name). PC03 probe is for human globin housekeeping gene.

(v) Optional: Pellet the tissue debris (if visible by eye) by centrifuging at 14,000*g* for 1 min and transfer the cleared lysate to a new tube.

■ **PAUSE POINT** Samples can be stored at −20 °C until use.

(B) Preparation of cell samples for PCR amplification

(i) Collect cytological specimens from cervical scrapes, SiHa cells, TF1 cells in 10 ml of PBS in test tubes.

(ii) Centrifuge cells at 1,000*g* for 10 min, resuspend in 0.1–1 ml of PBS and count in a Burker chamber.

▲ **CRITICAL STEP** In order to standardize the clinical samples and to compare the data of different specimens, cells need to be counted so that aliquots of the same number of cells can be processed by PCR–ELISA[5].

(iii) Divide SiHa cells into aliquots (10 µl) of 2,000 cells each or cervical scrape cells/TF1 cells into aliquots (10 µl) of 20,000 cells each.

■ **PAUSE POINT** Cells can be stored at −20 °C until use.

(iv) Digest cell aliquots (cervical scrapes, SiHa and TF1) in 200 µl of digestion buffer by incubating at 55 °C for 90 min in thermo mixer.

(v) Heat inactivate at 95 °C for 10 min.

(vi) Optional: Pellet the cell debris (if visible by eye) by centrifuging at 14,000*g* for 1 min and transfer the cleared lysate to a new tube.

■ **PAUSE POINT** Samples can be stored at −20 °C until use.

PCR amplification and direct labeling timing ● TIMING ~3.5 h

2| For each sample, prepare the following reaction mix (which contains primer set MY11/09 to amplify HPV DNAs and primer set PC04/GH20 to amplify β globin DNA):

Reagents	Volume for each sample (µl)	Final concentration
Amplification buffer (10×)	10	1×
Magnesium chloride (25 mM)	12	3 mM
Digoxigenin (Dig) dNTPs (2 mM)	5.0	0.1 mM
Primers MY11/09 (10 µM)	1.0	0.1 µM
Primers PC04/GH20 (1 µM)	2.0	0.02 µM
FastStart *Taq* (5 U μl^{-1})	0.8	4.0 U
H_2O	59.2	Up to 90 µl final volume

▲ **CRITICAL STEP** Reaction mixtures for ten samples (892 µl) without FastStart *Taq* polymerase enzyme can be pre-prepared and aliquots kept at −20 °C. FastStart *Taq* is then added for ten reactions (8 µl) before use.

▲ **CRITICAL STEP** The direct incorporation of a labeled nucleotide during amplification reaction increases the sensitivity of the assay in comparison to the use of labeled primers, giving a more efficient labeling. Dig dNTP-labeled PCR products can be detected with high sensitivity as Dig is incorporated at a ratio of 1:19 (dUTP:dTTP) instead of only one or few Dig molecules being linked to a PCR primer.

3| Add 90 µl of the reaction mix (from Step 2) to 10 µl of each test sample (from Step 1) in a separate Eppendorf tube.

4| Set up control reactions as follows: positive control 1: add 90 µl of the reaction mix to 10 µl of lysed SiHa cells in an Eppendorf tube; positive control 2: add 90 µl of the reaction mix to 10 µl of TE buffer containing 100 copies of the plasmid with the complete HPV 16 sequence in an Eppendorf tube; negative control: add 90 µl of the reaction mix to 10 µl of lysed TF1 cells in an Eppendorf tube; blank samples: add 90 µl of the reaction mix to 10 µl of TE buffer to Eppendorf tube.

▲ **CRITICAL STEP** If the thermal cycler is not equipped with a heated lid, use either mineral oil or paraffin wax to prevent evaporation of liquid from the reaction mixture during PCR and aerosol cross-contamination.

5| Amplify the nucleic acids using the following PCR-cycling conditions:

Step	Temperature (°C)	Time
Hot start	95	5 min
Amplification (40 cycles)		
Denaturation	95	30 s
Annealing	52	30 s
Extension	72	1 min
Final extension	72	5 min
Hold	+4	

■ **PAUSE POINT** Samples can be stored at +4 °C (overnight) or at −20 °C (for longer periods until use).

? TROUBLESHOOTING

BOX 1 | ELECTROPHORETIC CONTROL ● TIMING ~1.5 H

With electrophoresis, human papillomavirus (HPV)-positive samples can be recognized even if amplified products cannot be detected by the type-specific oligoprobes described in this protocol.

1. Load 10 μl samples (from Step 5) in 2.5 μl bromophenol blue solution onto a 2% (wt/vol) agarose gel containing 0.5 μg ml^{-1} ethidium bromide.
2. Also load 2 μl DNA marker VI in 8 μl H_2O and 2.5 μl bromophenol blue solution.
3. Run in TAE 1× for 1 h at 80 V.
4. Visualize ethidium bromide-stained DNA under UV light.

ANTICIPATED RESULTS

Experimental samples can be positive for HPV band (~450 bp), and must be positive for β globin band (268 bp), SiHa cells must be positive for HPV and for β globin bands. Plasmid containing the complete HPV 16 sequence must be positive for HPV and negative for β globin band. TF1 cells must be negative for HPV but positive for β globin band. Blank sample must be negative for HPV and for β globin bands. In case of samples that are positive for HPV band but are negative in ELISA, a positive band is a signal of the presence of a genotype that cannot be distinguished using the specific oligoprobes included in this protocol. In case of samples positive in ELISA for a specific genotype, a positive band is a confirmation of positivity.

Hybridization and detection reaction ● TIMING ~2 h

6| For each PCR-amplified sample, prepare a set of 11 tubes, each containing 5 μl of Dig-labeled PCR-amplified product (from Step 5) plus 10 μl of denaturing solution.
▲ CRITICAL STEP Instead of using tubes, this reaction can be performed also in wells of normal microplates.

7| Denature at room temperature for 10 min.

8| Hybridize by adding to each of the 11 tubes (or well), 2 μl (1 pmol μl^{-1}) of one of the biotinylated type-specific probes (HPV 6, 11, 16, 18, 31, 33, 35, 45, 52, 58 and globin) in 190 μl of hybridization buffer.

9| Incubate for 10 min at 55 °C in thermo mixer.

10| Transfer 200 μl to a streptavidin-coated microtiter plate well and incubate at 55 °C for 60 min in thermo mixer.

11| Wash plates four times with TBST at room temperature.

12| Add to each well 200 μl of anti-Dig peroxidase-conjugated solution (diluted 1/1,000 in conjugate buffer).
▲ CRITICAL STEP Must be freshly prepared and used within a few hours.

13| Incubate the plates at room temperature for 30 min.

14| Wash four times in TBST at room temperature.

15| Add to each well 200 μl of ABTS substrate solution, and develop for 10 min at room temperature.

16| Measure the colorimetric reaction by spectrophotometer at an OD of 405 nm (reference filter 492 nm).
▲ CRITICAL STEP Absorbance values are determined 10 min after the addition of substrate and are expressed as OD.
? TROUBLESHOOTING

17| Optional: An electrophoretic control step can also be undertaken to detect HPV-positive samples by electrophoresis, to detect amplified products that cannot be distinguished using one of the type-specific oligoprobes described in this protocol (see **Box 1**).

● TIMING
Step 1 (A or B), preparation of samples: ~3 h
Steps 2–5, PCR-amplification and direct labeling: ~3.5 h
Steps 6–16, hybridization and detection reaction: ~2 h

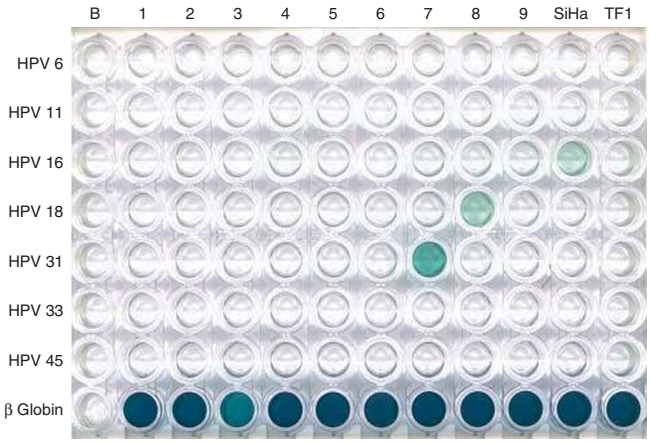

Figure 2 | PCR–ELISA microtiter plate. Rows: human papillomavirus (HPV) genotype-specific probes and β globin probe (amplification control); columns: B = blank, no. 1 – no. 9 = clinical samples, SiHa = HPV 16-positive control, TF1 = HPV negative control. The sample no. 7 is positive for HPV 31 and the sample no. 8 is positive for HPV 18. β globin is positive in negative (TF1 cell line) and positive (SiHa line cell) controls and in all clinical samples, but not in the blank. Dig, digoxigenin.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting for qualitative PCR–ELISA.

Step	Problem	Possible reason	Solution
PCR			
5	No amplification occurring (neither amplification controls nor positive controls)	A reagent is missing from the PCR	Prepare new reaction mix
		Too high annealing temperature	Decrease annealing temperature
		Insufficient extension times	Check the length of the amplicon and increase the extension time
		Too few cycles in the PCR	Increase cycle number; redesign more efficient assay
	No globin amplification occurring in cell control (TF1, SiHa, CaSki, HeLa)	The sample digestion is not efficient	Repeat digestion step (increase incubation time or proteinase K concentration)
		The target DNA is too fragmented	Redesign or choose a primer pair to amplify a smaller fragment
	Amplification occurring in blank or negative controls	PCR-carry-over contamination	Separate DNA extraction, pre-PCR setup and post-PCR examination (ELISA) facilities; do not move equipment like pipettes, racks, microfuges, etc. between facilities
			Reagents should be made up and stored in small aliquots that can be discarded if carryover contamination is suspected or observed
			Use filter tips to reduce the risk of transferring DNA between tubes
ELISA			
16	No signal detected when a signal is expected (positive control) or low signal	Probes are not well chosen	Redesign more efficient probes
		Probes were inefficiently labeled during synthesis or labels have been degraded by incorrect use or storage	Resynthesize probes and store them properly
		Degraded anti-digoxigenin (anti-Dig) POD conjugate or substrate	Check anti-Dig POD conjugate and substrate concentration. Make fresh solutions
	Signal detected when a signal is not expected	Probes are not type-specific	Redesign more specific probes
	Variation amongst replicates	Insufficient washing	If using an automatic plate washer, check that all ports are clean and free of obstruction
		Uneven streptavidin plate coating due to poor plate quality	Check plate used
	High background	Insufficient washing	Increase number of washes
		Contaminated substrate	Substrate should be colorless
		Uneven temperatures around work surface	Avoid incubating plates in areas where environmental condition vary

ANTICIPATED RESULTS

Figure 2 shows the appearance of a typical PCR–ELISA result. Experimental samples must be positive for β globin and can be positive for one or more HPV probes, which identifies the HPV genotype (HPV 6, 11, 16, 18, 31, 33, 35, 45, 52 and 58) of the sample. SiHa cells must be positive only for HPV 16 probe and for β globin and negative for the other HPV probes. Plasmid

containing the complete HPV 16 sequence must be positive only for HPV 16 probe and negative for the other HPV probes and for β globin. TF1 cells must be negative for all HPV probes and positive for β globin. Blank buffer sample must be negative for all HPV probes and for β globin. For each HPV probe, the CO of the reaction is determined as twice the OD value of negative TF1 control, tested in duplicate, as defined with ROC analysis. For β globin, the CO of the reaction is determined as twice the OD value the of plasmid containing HPV sequence. Results are expressed as net absorbance after the absorbance of the blank sample is subtracted, and an index value is calculated as OD sample/CO + 20%; thus an index value >1 is considered positive. Experimental samples are therefore considered negative when ODHPV $<$ CO – 20%. The sample is positive when ODHPV $>$ CO + 20%. Test samples whose values are in the range of ±20% of the CO value are retested for confirmation. Sensitivity of the PCR–ELISA with the probes tested varied between 50 and 100 target copies among the different viral types.

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1. Landgraf, A., Reckmann, B. & Pingoud, A. Direct analysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. *Anal. Biochem.* **198**, 86–91 (1991).
2. Gibellini, D. *et al.* Microplate capture hybridization of amplified parvovirus B19 DNA fragment labelled with digoxigenin. *Mol. Cell. Probes* **7**, 453–458 (1993).
3. Zerbini, M. *et al.* Automated detection of digoxigenin-labelled B19 parvovirus amplicons by a capture hybridization assay. *J. Virol. Methods* **55**, 1–9 (1995).
4. Venturoli, S. *et al.* Detection of adeno-associated virus DNA in female genital samples by PCR-ELISA. *J. Med. Virol.* **64**, 577–582 (2001).
5. Venturoli, S. *et al.* Human papillomavirus DNA testing by PCR-ELISA and hybrid capture II from a single cytological specimen: concordance and correlation with cytological results. *J. Clin. Virol.* **25**, 177–185 (2002).
6. Koskiniemi, M., Mannonen, L., Kallio, A. & Vaheri, A. Luminometric microplate hybridization for detection of varicella-zoster virus PCR product from cerebrospinal fluid. *J. Virol. Methods* **63**, 71–79 (1997).
7. Roda, A. *et al.* Microtiter format for simultaneous multianalyte detection and development of a PCR-chemiluminescent enzyme immunoassay for typing human papillomavirus DNAs. *Clin. Chem.* **48**, 1654–1660 (2002).
8. Ambretti, S. *et al.* High-throughput polymerase chain reaction chemiluminescent enzyme immunoassay for typing and quantifying human papillomavirus DNAs. *Anal. Biochem.* **332**, 349–357 (2004).
9. Watzinger, F., Ebner, K. & Lion, T. Detection and monitoring of virus infections by real-time PCR. *Mol. Aspects Med.* **27**, 254–298 (2006).
10. Kim, J.W. *et al.* Development of PCR-ELISA for the detection of hepatitis B virus x gene expression and clinical application. *J. Clin. Lab. Anal.* **19**, 139–145 (2005).
11. Metzger-Boddien, C. & Kehle, J. Development and evaluation of a sensitive PCR-ELISA for detection of adenoviruses in feces. *Intervirology* **48**, 297–300 (2005).
12. Adler, M., Schulz, S., Fischer, R. & Niemeyer, C.M. Detection of rotavirus from stool samples using a standardized immuno-PCR ("Imperacer") method with end-point and real-time detection. *Biochem. Biophys. Res. Commun.* **333**, 1289–1294 (2005).
13. Metzger-Boddien, C. *et al.* Automated high-throughput immunomagnetic separation-PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk. *Int. J. Food Microbiol.* **110**, 201–208 (2006).
14. Daeschlein, G., Assadian, O., Daxboeck, F. & Kramer, A. Multiplex PCR-ELISA for direct detection of MRSA in nasal swabs advantageous for rapid identification of non-MRSA carriers. *Eur. J. Clin. Microbiol. Infect. Dis.* **25**, 328–330 (2006).
15. Perelle, S. *et al.* Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting *Salmonella* spp. in milk and meat samples. *Mol. Cell. Probes.* **18**, 409–420 (2004).
16. Kurupati, P., Kumarasinghe, G. & Laa Poh, C. Direct identification of *Pseudomonas aeruginosa* from blood culture bottles by PCR-enzyme linked immunosorbent assay using oprI gene specific primers. *Mol. Cell. Probes* **19**, 417–421 (2005).
17. Waters, S.M., Doyle, S., Murphy, R.A. & Power, R.F. Development of solution phase hybridisation PCR-ELISA for the detection and quantification of *Enterococcus faecalis* and *Pediococcus pentosaceus* in Nurmi-type cultures. *J. Microbiol. Methods* **63**, 264–275 (2005).
18. Martín-Sánchez, J., Gállego, M., Barón, S., Castillejo, S. & Morillas-Marquez, F. Pool screen PCR for estimating the prevalence of *Leishmania infantum* infection in sandflies (Diptera: Nematocera, Phlebotomidae). *Trans. R. Soc. Trop. Med. Hyg.* **100**, 527–532 (2006).
19. Heidari, A. *et al.* Genotypes and *in vivo* resistance of *Plasmodium falciparum* isolates in an endemic region of Iran. *Parasitol. Res.* **100**, 589–592 (2007).
20. Sow, A., Sidibe, I., Desquesnes, M., Bengaly, Z. & Panqui, L.J. The application of PCR-ELISA to the detection of *Trypanosoma congolense* type savannah (TCS) in bovine blood samples. *Trop. Biomed.* **23**, 123–129 (2006).
21. Hassan, M. *et al.* Detection of DNA of *W. bancrofti* in blood samples by QC-PCR-ELISA-based. *J. Egypt Soc. Parasitol.* **35**, 963–970 (2005).
22. Florent, M. *et al.* Prospective evaluation of a polymerase chain reaction-ELISA targeted to *Aspergillus fumigatus* and *Aspergillus flavus* for the early diagnosis of invasive aspergillosis in patients with hematological malignancies. *J. Infect. Dis.* **193**, 741–747 (2006).
23. de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U. & zur Hausen, H. Classification of papillomaviruses. *Virology* **324**, 17–27 (2004).
24. Clifford, G.M., Smith, J.S., Plummer, M., Muñoz, N. & Franceschi, S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br. J. Cancer* **88**, 63–73 (2003).
25. Lungu, O., Wright, T.C. Jr. & Silverstein, S. Typing of human papillomaviruses by polymerase chain reaction amplification with L1 consensus primers and RFLP analysis. *Mol. Cell. Probes* **6**, 145–152 (1992).
26. Kay, P., Meehan, K. & Williamson, A.L. The use of nested polymerase chain reaction and restriction fragment length polymorphism for the detection and typing of mucosal human papillomaviruses in samples containing low copy numbers of viral DNA. *J. Virol. Methods* **105**, 159–170 (2002).
27. Feoli-Fonseca, J.C. *et al.* Human papillomavirus (HPV) study of 691 pathological specimens from Quebec by PCR-direct sequencing approach. *J. Med. Virol.* **63**, 284–292 (2001).
28. Speich, N., Schmitt, C., Bollmann, R. & Bollmann, M. Human papillomavirus (HPV) study of 2916 cytological samples by PCR and DNA sequencing: genotype spectrum of patients from the west German area. *J. Med. Microbiol.* **53**, 125–128 (2004).
29. van den Brule, A.J. *et al.* $GP^{5+/6+}$ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J. Clin. Microbiol.* **40**, 779–787 (2002).
30. de Roda Husman, A.M., Walboomers, J.M., van den Brule, A.J., Meijer, C.J. & Snijders, P.J. The use of general primers GP5 and GP6 elongated at their 3 ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J. Gen. Virol.* **76**, 1057–1062 (1995).
31. Manos, M.M. *et al.* Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* **7**, 209–214 (1989).
32. Gravitt, P.E. *et al.* Improved amplification of genital human papillomaviruses. *J. Clin. Microbiol.* **38**, 357–361 (2000).
33. Kleter, B. *et al.* Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J. Clin. Microbiol.* **37**, 2508–2517 (1999).
34. Resnick, R.M. *et al.* Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J. Natl. Cancer Inst.* **82**, 1477–1484 (1990).
35. Venturoli, S. *et al.* Evaluation of immunoassays for the detection and typing of PCR amplified human papillomavirus DNA. *J. Clin. Pathol.* **51**, 143–148 (1998).
36. Zerbini, M. *et al.* Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA. *J. Clin. Pathol.* **54**, 377–380 (2001).
37. Schneede, P. *et al.* Evaluation of HPV testing by Hybrid Capture II for routine gynaecologic screening. *Acta Obstet. Gynecol. Scand.* **80**, 750–752 (2001).
38. Söderlund-Strand, A., Rymark, P., Andersson, P., Dillner, J. & Dillner, L. Comparison between the Hybrid Capture II test and a PCR-based human papillomavirus detection method for diagnosis and posttreatment follow-up of cervical intraepithelial neoplasia. *J. Clin. Microbiol.* **43**, 3260–3266 (2005).
39. Ehardt, A., Schaefer, S., Athanassiou, N., Kann, M. & Gerlich, W.H. Quantitative assay of PCR-amplified hepatitis B virus DNA using a peroxidase-labelled DNA

probe and enhanced chemiluminescence. *J. Clin. Microbiol.* **34**, 1885–1891 (1996).
40. Laitinen, R., Malinen, E. & Palva, A. PCR-ELISA I: Application to simultaneous analysis of mixed bacterial samples composed of intestinal species. *Syst. Appl. Microbiol.* **25**, 241–248 (2002).
41. Swets, J.A. Measuring the accuracy of diagnostic systems. *Science* **240**, 1285–1293 (1988).
42. Moret, H. *et al.* New commercially available PCR and microplate hybridization assay for detection and differentiation of human polyomaviruses JC and BK in cerebrospinal fluid, serum, and urine samples. *J. Clin. Microbiol.* **44**, 1305–1309 (2006).
43. Bonvicini, F. *et al.* Presence and type of oncogenic human papillomavirus in classic and in differentiated vulvar intraepithelial neoplasia and keratinizing vulvar squamous cell carcinoma. *J. Med. Virol.* **77**, 102–106 (2005).
44. La Placa, M. *et al.* Presence of high-risk mucosal human papillomavirus genotypes in primary melanoma and in acquired dysplastic melanocytic naevi. *Br. J. Dermatol.* **152**, 909–914 (2005).