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Real-Time PCR-Based System for Simultaneous Quantification of Human Papillomavirus Types Associated with High Risk of Cervical Cancer

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We have previously shown that women with a high titer of human papillomavirus type 16 (HPV16) in cervical epithelial cells have an increased risk of developing cervical carcinoma in situ. In order to study the relationship between viral DNA amount and risk of cervical carcinoma for the HPV types most commonly found in cervical tumors, we developed a real-time PCR assay for the detection and quantification of HPV16, -18, -31, -33, -35, -39, -45, -52, -58, and -67. These HPV types are analyzed in two reaction tubes, allowing for independent quantification of three viral types, or groups of viral types, in each reaction. A separate reaction is used for estimating the number of a nuclear single-copy gene and is used to calculate the HPV copy number per genomic DNA equivalent in the sample. The system has a dynamic range from 10^2 to 10^7 HPV copies per assay and is applicable to both fresh clinical samples and DNA extracted from archival samples. Reconstitution experiments, made to mimic infections with several HPV types, shows that individual HPV types can be detected in a mixture as long as they represent 1 to 10% of the main type. The system was evaluated with respect to technical specificity and sensitivity, reproducibility, reagent stability, and sample preparation protocol and then used to analyze clinical samples. This homogeneous assay provides a fast and sensitive way for estimating the viral load of a series of the most frequent oncogenic HPV types in biopsies, as well as cervical smear samples.

Cervical carcinoma is considered to be the third most common cancer in women in the world. In 1994 an estimated 55,000 women in the United States were diagnosed with carcinoma in situ of the cervix, with an additional 15,000 cases of invasive cancer (15). Although in the United States and Europe major progress has been made in the control of cervical cancer, it remains a significant cause of morbidity and mortality in the developing world.

Infection by certain types of human papillomavirus (HPV) is the single most important risk factor for the development of cervical cancer (10, 17). More than 99% of cervical cancer biopsies have been found to contain HPV DNA, most commonly HPV type 16 (HPV16), followed by HPV18, -45, -31, and -33 (1, 22). Given the importance of HPV infection in the etiology of cervical cancer, a large number of methods have been developed for detecting of the virus or for identifying the cellular changes resulting from viral transformation (8).

We have previously described an assay based on real-time PCR for the detection and quantification of high-risk HPV DNA (7). The 5′ exonuclease assay, employed in Taqman, is based on the ability of the 5′-to-3′ exonuclease activity of *Taq* polymerase to cleave a dually labeled, nonextendable hybridization probe during the extension phase of the PCR (4, 5, 11, 13). Other groups have applied the 5′ exonuclease assay either for endpoint determination of the amount of HPV PCR product (18) or for real-time detection of HPV (19–21). Additional methods for the quantification or semiquantification of HPV have also been described, based on PCR and seven-color fluorescence hybridization (16), based on restriction immunoassay (6), or with scorpion probes in real-time PCR (3).

Using our previously described method we were able to demonstrate, in a retrospective case-control study, that the titer of HPV16 in cervical smears can be used to predict the risk of development of cervical cancer in situ (cervical intraepithelial neoplasia, stage III [CIN III]) (9, 23). These results indicate that determination of the HPV titer present an opportunity to assess whether an infection will progress into cervical cancer or be cleared. Subsequently, based on other study designs or methods for quantification of the amount of HPV, it has been confirmed that a high HPV16 titer is associated with an increased risk of developing more-severe dysplasia and that reduction of the viral load may predict regression of CIN to normalcy (21). In the present study we devised a novel typing system for HPV, one that employs the quantitative ability and dynamic range provided by real-time PCR. The system has been designed to detect and quantify HPV types commonly detected in cervical tumors while minimizing the number of parallel reactions performed for each sample, making the system suitable for use in routine screening of cervical swab samples.

MATERIALS AND METHODS

DNA extraction. Plasmids containing HPV16, -18, -31, -33, -35, -39, -45, -52, -58, and -67 were either kindly supplied by T. Matsukura, A. Lörincz, and G. Orth or else prepared by cloning from PCR products of clinical samples and used as positive controls and to estimate the sensitivity of the assay. The plasmids with

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TABLE 1. PCR primers used in reactions 1, 2, and 3[a]

Primer	Specificity[b]	Sequence (5′ to 3′)
F16E7	HPV16	AGCTCAGAGGAGGAGGATGAA
R16E7	HPV16	GGTTACAATATTGTAATGGGCTC
F18E1	HPV18 and −45	CATTTTGTGAACAGGCAGAGC
R18E1	HPV18	ACTTGTGCATCATTGTGGACC
R45E1	HPV45	CAACACCTGTGCATCATTCTGA
F31E6	HPV31	ACGATTCCACAACATAGGAGGA
R31E6	HPV31	TACACTTGGGTTTCAGTACGAGGT
F35E4	HPV35	GCCTGCTCCGTGGGC
R35E4	HPV35	GCACTGAGTCGCACTCGC
F33L1	HPV33, −52, and −67	CGTCGCAGGCGTAAACG
F58L1	HPV58	GCGTCGCAGACGTAAACG
R33/etcI.1	HPV33, −52, −58, and −67	ACAGGAGGCAGGTACAC
F39E7	HPV39	CGAGCAATTAGGAGAGTCAGAGG
R39E7	HPV39	TGTGTGACGCTGTGGTTCAT
HMBS F	HMBS	GCCTGCAGTTTGAAATCAGTG
HMBS R	HMBS	CGGGACGGGCTTTAGCTA

[a] In denoting the primers, "F" refers to forward, primers and "R" refers to reverse primers. The primers are located in the reading frames E7, E6, E1, E4 and L1, as indicated in the primer name and in the text. The HMBS primers span an exon-intron border (see the text).

[b] That is, what the indicated primer is specific for.

integrated HPV were transformed into TOP10F′ cells (Invitrogen, Groningen, The Netherlands). Positive transformants were isolated and grown in 100 ml of Luria-Bertani medium in 37°C overnight. Plasmid DNA was extracted by using the Qiagen Maxiprep kit (Qiagen, Inc., Valencia, Calif.). The copy numbers for individual plasmid preparations were estimated by spectrophotometry (GeneQuant; Pharma Biotech, Cambridge, England).

DNA from blood samples, used for development of the human nuclear gene assay, was extracted by using a standard protocol based on proteinase K treatment, followed by phenol-chloroform extraction and ethanol precipitation (14). DNA from formalin-fixed biopsies was extracted by using published protocols (8, 12). DNA purity and concentration was determined by spectrophotometry (GeneQuant; Pharma Biotech, Cambridge, England).

For studies of the DNA from archival smears, a modification of the protocol described previously was used (2, 7). Briefly, this protocol includes incubation in xylene to remove the coverslip, destaining, proteinase K treatment (60°C minimum 1 h), and then a transfer of cells into sterile Eppendorf tubes. Saturated ammonium acetate is then added to precipitate the protein. The DNA supernatant is recovered by using ethanol, and the pellet is washed with 70% ethanol, dried, and dissolved in 200 μl of TE-Low (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA).

Sample preparation from cervical swabs. For the study of extraction protocols, we used cervical swab samples and compared five different extraction protocols (A to E).

Protocol A is based on freezing and boiling of the samples. The cervical swab (or brush) is immersed in 1 ml of phosphate-buffered saline (PBS) and swirled to release the cells. Then, 250 μl of this suspension is used as described in the following protocol. The solution is spun at 3,000 × *g* for 10 min at room temperature (RT). The supernatant is collected, and 250 μl of 10 mM Tris-HCl (pH 7.4) is added. The sample is then vortexed to distribute the cells evenly. Next, 100 μl of the solution is transferred to a new Eppendorf tube, and both the 100-μl aliquot and the remaining volume (used as backup) are frozen at −20°C. The 100-μl aliquot is then thawed and boiled in a heating block at 100°C for 10 min, and a 2-μl portion of it is used for the real-time PCR.

In protocol B, a commercial kit for DNA extraction based on precipitation of proteins (Wizard; Promega, Madison, Wis.) is used. The cervical swab (or brush) is immersed in 1 ml of PBS and swirled to release the cells. Then, 250 μl of this suspension is subjected to the following protocol. The solution is spun at maximum speed in an Eppendorf centrifuge for 5 min. The supernatant is discarded, and 300 μl of Nuclei Lysis Solution (Wizard kit) is added. The solution is mixed by pipetting and incubated at 37°C for 1 h. The sample is cooled to RT, and 100 μl of protein precipitation solution (Wizard kit) is added. The solution is then vortexed for 10 to 20 s and centrifuged at 13,000 to 16,000 × *g* for 3 min. The supernatant is transferred to a new Eppendorf tube with 300 μl of isopropanol (at RT), and the solutions are mixed and centrifuged at 13,000 to 16,000 × *g* for 1 min. The supernatant is removed, and the pellet is washed with 70% ethanol and centrifuged again at 13,000 to 16,000 × *g* for 1 min. Finally, the ethanol is removed, and the pellet is air dried. The pellet is dissolved in 100 μl of rehydration solution (10 mM Tris-HCl, 1 mM EDTA; pH 7.4), followed by incubation at 65°C, and then 2 μl is used for each real-time PCR analysis.

Protocol C is based on proteinase K digestion of the samples. Briefly, the cervical swab (or brush) is immersed in 1 ml of PBS and swirled to release the cells. Then, 250 μl of this suspension is used in the protocol below. The solution is spun at maximum speed in an Eppendorf centrifuge for 5 min. The supernatant is removed, and a proteinase K solution (148 μl of digestion buffer [50 mM Tris base, 0.5% Tween 20, 1 mM EDTA] and 1.95 μl of proteinase K [20 mg/ml]) is added. The sample is incubated at 56°C for 2 h, and the proteinase K is inactivated at 95°C for 5 min. The sample is finally centrifuged for 5 min, and 2 μl of the top phase is used for each real-time PCR.

Protocol D includes organic extraction (phenol-chloroform) of the samples. Initially, protocol B above is used, including the addition of the Nuclei Lysis Solution (Wizard kit) and incubation at 37°C for 1 h. Then 300 μl of equilibrated phenol is added to the sample. The solution is mixed and spun, the water phase is extracted once more with phenol and then with chloroform, the DNA is collected by ethanol precipitation, and the pellet is washed, dried, and dissolved in 100 μl of TE-Low. Then, 2 μl of the dissolved DNA preparation is used for each real-time PCR analysis.

Finally, in protocol E, a commercial kit for DNA extraction based on binding of nucleic acid to glass beads (Nuclisens; Nasba Diagnostics, Organon-Teknica, Boxtel, The Netherlands) is used. The lysis buffer and wash buffer is heated to 37°C for 30 min with intermittent vortexing. The wash buffer and lysis buffer is subsequently cooled to RT. The sample (10 to 200 μl of cervical swab solution) is then added to 900 μl of lysis buffer, the mixture is vortexed, and the tube is spun at 10,000 × *g* for 30 s. The silica solution is vortexed until it becomes opaque, 50 μl is added to each sample, and the mixture is vortexed. The tube is incubated at RT for 10 min and vortexed every second minute. The silica beads are spun down at 10,000 × *g*, 30 s, the supernatant is removed, and 1 ml of wash buffer is added. The pellet is then vortexed until dissolved and washed first with 1 ml of 70% ethanol (twice) and then with 1 ml of acetone (once). Residual acetone is removed, and the pellet is dried at 56°C for 10 min. When the silica pellet is dry, the pellet is dissolved in 50 μl of elution buffer. The tube is incubated at 56°C for 10 min, with intermittent vortexing. The samples are centrifuged for 2 min at 10,000 × *g*, and the supernatant (30 to 35 μl) transferred to a new tube. Then, 2 to 5 μl of the supernatant is used for each real-time PCR analysis.

Primers. Oligonucleotide primers were designed by using Primer Express software (version 1.0; Applied Biosystems, Foster City, Calif.).

TABLE 2. Fluorescently labeled hybridization probes used in reaction 1 (Pb1.1, −1.2, and −1.3), reaction 2 (Pb2.1, −2.2, and −2.3), and reaction 3 (Pb3)

Probe	Specificity[a]	5′ Fluorophore	Sequence (5′ to 3′)	3′ Fluorophore
Pb1.1	HPV16	FAM	CCAGCTGGACAAGCAGAACCGG	TAMRA
Pb1.2	HPV18 and −45	VIC	AGAGACAGCACAGGCATTGTTCCATG	TAMRA
Pb1.3	HPV31	TET	CTCCAACATGCTATGCAACGTCC	TAMRA
Pb2.1	HPV33, −52, −58, and −67	FAM	AGATGTCCGTGTGGCGGCCTAG	TAMRA
Pb2.2	HPV35	VIC	CAGAAGACAAATCACAAACGACTTCGAGGG	TAMRA
Pb2.3	HPV39	TET	AACCCGACCATGCAGTTAATCACCAAC	TAMRA
Pb3	HMBS	VIC	TGGAAGCTAATGGGAAGCCCAGTACC	TAMRA

[a] That is, what the indicated probe is specific for.

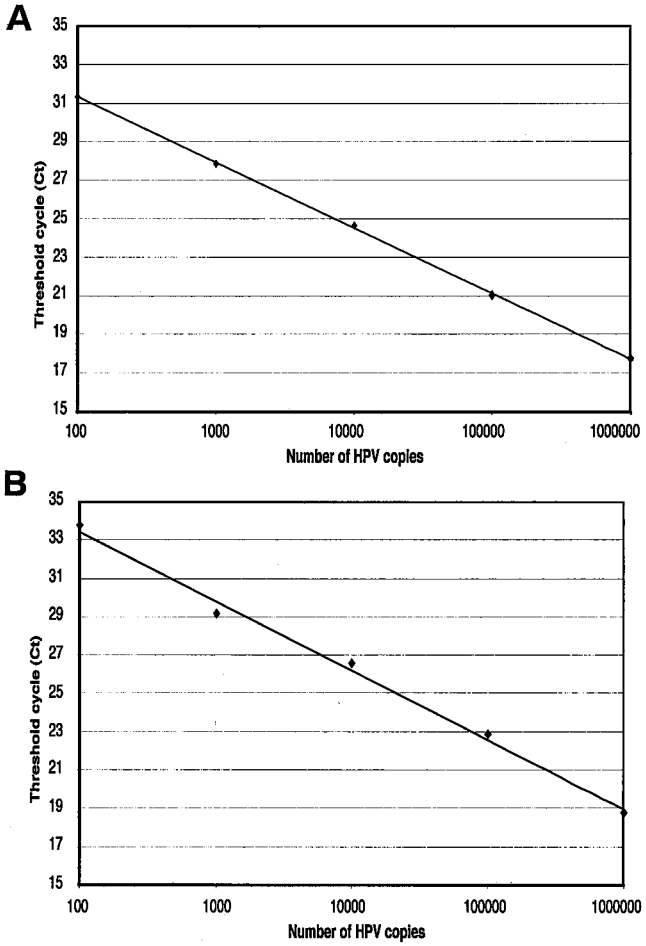

FIG. 1. Standard curves for the HPV16 (A) and HPV31 (B) assays. The threshold cycle (C_t) number is plotted against number of HPV genomes. The datum points represent the mean of 12 independent measurements. r^2 values: $r^2_{HPV16} = 0.984$; $r^2_{HPV31} = 0.972$.

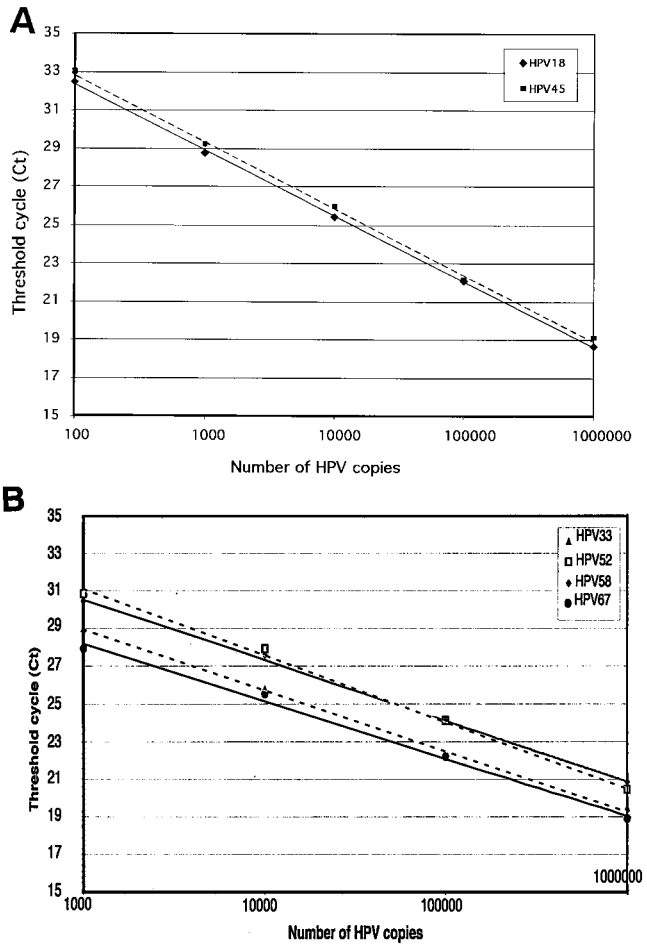

FIG. 2. Standard curves for HPV18 (solid line) and HPV45 (broken line) (A) and HPV33 group (i.e., HPV33, -52, -58, and -67) (B) assays. The threshold cycle (C_t) number is plotted against number of HPV genomes. The datum points represent the mean of 12 independent measurements. r^2 values: $r^2_{HPV18} = 0.883$; $r^2_{HPV45} = 0.981$; $r^2_{HPV33} = 0.968$; $r^2_{HPV52} = 0.971$; $r^2_{HPV58} = 0.960$; $r^2_{HPV67} = 0.944$.

Probes. The probes were designed to ensure a higher T_m than for the primers. Synthesis was performed by Applied Biosystems (Cheshire, United Kingdom) and Cybergene (Huddinge, Sweden).

Real-time PCR. The PCR amplification was performed in a 25-μl volume containing 1× buffer A (Applied Biosystems); 3.5 mM $MgCl_2$; 200 nM concentrations (each) of dATP, dCTP, and dGTP and 400 nM dUTP (Pharmacia Biotech, Uppsala, Sweden); 0.625 U of AmpliTaq Gold (Applied Biosystems); 3.1 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.); a 200 nM concentration of each primer and probe; and DNA (according to the extraction protocol).

Amplification and detection was performed by using a 7700 sequence detection system (Applied Biosystems). The amplification ramp included an initial hold step of 10 min at 95°C, followed by a two-step cycle consisting of 15 s at 95°C and 1 min at 57°C. In the development of the assay, we used 50 PCR cycles, whereas in the analysis of clinical samples only 40 cycles were used due to the high efficiency of the PCR. Tubes, including all PCR components but without template DNA (denoted NTC reactions), were used to ensure that the reagents mix were free of contamination.

Analysis of real-time PCR data. The sequence detection system software (version 1.6.3; Applied Biosystems) was used to produce a file with raw data. Since this software does not handle quantification of three different fluorophores in the same PCR, a software was developed and used for the calculation of threshold cycle number and conversion into HPV copy numbers per cell. The method used for analysis will be described separately (M. P. Moberg et al., unpublished data).

Statistics. Statistics and graphs were produced by using Microsoft Excel, Statview (version 4.5; SAS Institute, Inc., Cary, N.C.), and the SAS system (version 6.12).

RESULTS

Rationale and design of the typing system. The present typing system was designed to permit viral load estimates for the range of HPV types most frequently found in different grades of cervical intraepithelial neoplasia (CIN I to CIN III) and cervical tumors. Although the set of HPV types varies between studies, we focused on HPV16, -18, -31, -33, -35, -39, -45, -52, -58, and -67.

The assay is based on three parallel real-time PCRs from each patient sample: (i) reaction 1 detects and quantifies HPV16, -31, -18, and -45 (HPV18 and -45 were detected and quantified together) with three different fluorophores; (ii) reaction 2 detects and quantifies HPV33, -35, -39, -52, -58, and -67 (HPV33, -52, -58, and -67 were detected and quantified together), again with three different fluorophores; and (iii) reaction 3 detects and quantifies the amount of a human single

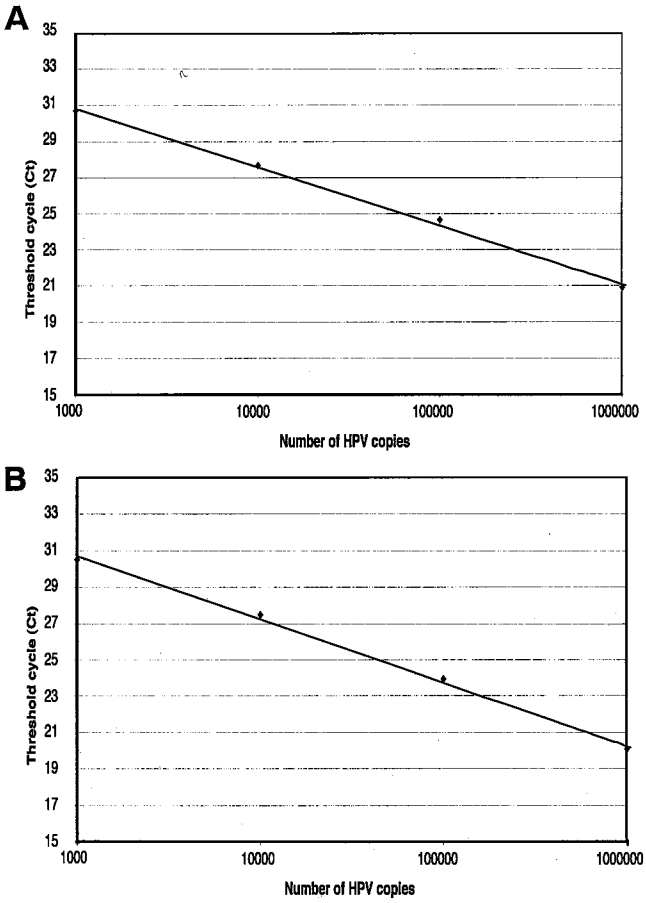

FIG. 3. Standard curves for HPV35 (A) and HPV39 (B) assays. The threshold cycle (C_t) number is plotted against the number of HPV genomes. The datum points represent the mean of 12 independent measurements. r^2 values: $r^2_{HPV35} = 0.963$; $r^2_{HPV39} = 0.869$.

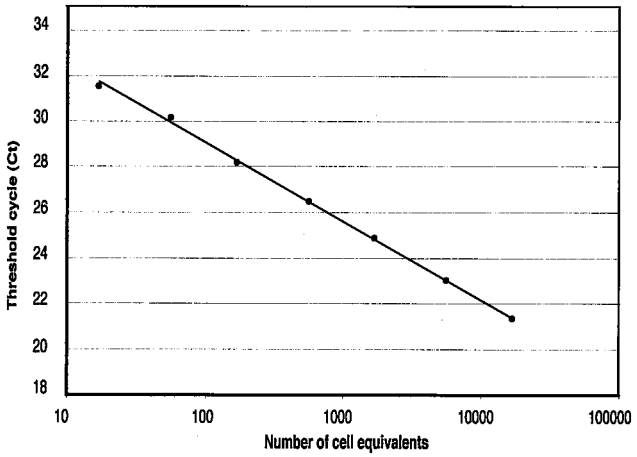

FIG. 4. Standard curves for the human gene (HMBS) assay. The threshold cycle (C_t) number is plotted against number of cell equivalents. The datum points represent the mean of 12 independent measurements. $r^2_{HMBS} = 0.996$.

copy gene (HMBS, *Homo sapiens* hydroxymethylbilane synthase; GenBank accession no. M95623.1). Reaction 1 includes a total of seven PCR primers and three probes, reaction 2 includes a total of seven PCR primers and three probes, and reaction 3 includes two PCR primers and a single probe (Tables 1 and 2).

Technical sensitivity and specificity. The sensitivity and specificity of the HPV assay was determined by using plasmids containing the entire genome of the different HPV types studied, together with 33 ng of human genomic DNA to mimic the complex nucleic acid environment present in an amplification from genomic DNA (such as cervical smear samples). Dilution series were made with 10^2 to 10^7 HPV copies, and high-molecular-weight human genomic DNA (lacking integrated HPV) was added. Standard curves ranging from 10^2 to 10^7 copies per sample were constructed for each of the HPV types, or groups of HPV types, based on 12 independent measurements for each HPV copy number (Fig. 1 to 3). A highly significant linear regression between HPV copy number and threshold cycle (C_t), representing the PCR cycle number at which the signal exceeds a given baseline, was seen for all of the HPV types tested. The curves for HPV18 and -45 have the same slope and the same intercept. Similarly, the HPV33, -52, -58, and -67 were detected together by using a single probe. Their standard curves have the same slope but differ somewhat with respect to the intercept. This may result in less precision when viral types within this group are quantified together. Finally, a significant linear regression was seen between the copy number of the human single-copy gene and the threshold cycle (Fig. 4). The variation seen in the HPV and human DNA quantification systems, expressed as the C_t, is shown in Tables 3 and 4. The mean standard deviation (SD) of the C_t values for the HPV assay was 0.89 (Table 3). The mean SD of the C_t values for the human gene system was found to be 0.85 (Table 4).

The specificity of the quantitative PCR system was tested by determining the ability of the reagents in reactions 1 and 2 to discriminate against plasmids with different HPV types. The specificity of the reagents in reaction 1 was tested against the HPV types detected in reaction 2 (i.e., HPV33, -35, -39, -52, -58, and -67). No signal was observed with any of these HPV types at 10^4 initial viral copies (data not shown). Similarly, the specificity of the reagents in reaction 2 against the HPV types detected in reaction 1 (i.e., HPV16, -18, -31, and -45) was tested. No signal was observed with HPV16, -18, -31, or -45 at a concentration of 10^4 initial viral copies (data not shown). Primers and probes were designed to be specific for the individual HPV types (using sequence alignments from a large number of HPV types) and reaction 3 for the nuclear gene (using GenBank and BLAST searches), respectively.

Analysis of mixed infections. An important aspect of a typing system is the ability to detect mixed infections between HPV16 and any of the other frequent types found to be associated with cervical cancer, such as HPV18, -31, and -45. We examined our real-time PCR assay's ability to correctly detect and quantify HPV titer in samples with multiple infections by producing synthetic mixtures of HPV-containing plasmids in a background of 33 ng of high-molecular-weight genomic DNA per reaction. First, we tested the detection of HPV31 in a background of HPV16. For the range of 10^2 to 10^5 copies of HPV16, we tested the ability to correctly quantify the amount of HPV31 over the same range of copy numbers. A measure-

TABLE 3. Variation in C_t for different copy numbers of HPV types in reactions 1 and 2[a]

Reaction and HPV type	Mean C_t ± SD of (no. of HPV genomes):				
	10^6	10^5	10^4	10^3	10^2
Reaction 1					
HPV16	17.75 ± 0.41	21.04 ± 0.50	24.65 ± 0.52	27.85 ± 0.81	31.35 ± 0.95
HPV31	18.78 ± 0.77	22.87 ± 1.64	26.55 ± 0.60	29.18 ± 0.84	33.73 ± 0.71
HPV18	18.69 ± 0.46	22.09 ± 0.82	25.47 ± 0.57	28.78 ± 0.5	32.53 ± 0.59
HPV45	19.11 ± 0.64	22.15 ± 0.75	25.96 ± 0.84	29.23 ± 0.67	33.02 ± 0.57
Reaction 2					
HPV33	19.47 ± 0.54	22.30 ± 0.55	25.78 ± 0.87	29.02 ± 0.64	
HPV35	20.88 ± 0.79	24.67 ± 0.94	27.69 ± 1.04	30.70 ± 1.00	
HPV39	20.07 ± 1.27	23.94 ± 0.77	26.87[b] ± 1.38	30.55 ± 1.02	
HPV52	20.48 ± 0.58	24.10 ± 0.60	27.90 ± 0.88	30.84 ± 0.53	
HPV58	20.85 ± 0.62	24.12 ± 0.82	27.58 ± 0.94	30.44 ± 0.59	
HPV67	18.89 ± 0.67	22.22 ± 0.57	25.55 ± 0.86	27.93 ± 1.07	

[a] Values are based on 12 independent measurements at each copy number.
[b] Based on 11 independent measurements.

ment was considered incorrect if the mean C_t value fell outside the2 95% confidence interval of the mean C_t value obtained when no additional HPV type was present in the reaction. The confidence interval is based on the general variance of the assay described below.

In the case of quantification of HP31 in a background of HPV16, the assay was able to correctly estimate the amount of HPV31 when the ratio of HPV31 to HPV16 was as low as 1:100 (Fig. 5A). Similarly, the reverse experiment (detection of HPV16 in a background of HPV31) demonstrates that the amount of HPV16 can be measured with precision in a background of HPV31, as long as the ratio of HPV16 to HPV31 is not below 1:10 (Fig. 5B).

Similar results were obtained when HPV16 was mixed with HPV18 or HPV45. To correctly quantify HPV18 (or HPV45) with a background of HPV16, the ratio of HPV18 (or HPV45) to HPV16 must not be lower than 1:100 (data not shown). When we quantified HPV16 in the presence of HPV18 or -45, the ratio of HPV16 to HPV18 or -45 must not be lower than 1:100 (data not shown).

Reproducibility. The reproducibility of the test was studied by repeated measurements of the HPV copy numbers and human DNA in a series of clinical samples. Variation due to test variable operator, reagent lot, and week were calculated by using the GLM procedure of SAS.

(i) Intralaboratory reproducibility. Three different technicians (operators) analyzed a set of clinical samples in the same laboratory with the same reagent lot. The sample DNA was extracted according to protocol D (see Materials and Methods). The correspondence between operators with respect to HPV positivity (+/−) was 98.6% (139 of 141), and the SD of the C_t due to operator was 0.62 U (Table 5).

(ii) Lot-to-lot reproducibility. A single technician analyzed a set of clinical samples by using three different reagents lots. The sample DNA was extracted according to protocol D (see Materials and Methods). The correspondence between different lots with respect to HPV positivity was 100% (141 of 141), and the SD of the C_t due to different reagent lots was 0.75 U (Table 6).

(iii) Reproducibility over time. A single technician analyzed a set of clinical samples once a week during a period of four weeks, by using the same lot of reagents. The sample DNA was extracted according to protocol D (see Materials and Methods). The correspondence between the different time periods with respect to HPV positivity was 98.9% (186 of 188), and the SD in C_t values due to different time points was 0.19 U (Table 7).

(iv) General variation in measurements of the test. The general variance of the C_t estimates (not explained by the test variables above) was calculated in the three experiments described above. The general variance was determined to be 1.57 in the intralaboratory experiment, 1.45 in the lot-to-lot experiment, and 0.76 in the variance-over-time experiment. The average SD was 1.1.

(v) Stability. The stability of the assay reagents was examined by studying the amount of HPV and human DNA in a series of clinical samples. The sample DNA was extracted according to protocol D (see Materials and Methods). The same lot of reagents was used, and the three different storage temperatures tested were −20, 4, and 30°C (accelerated RT stability test). No significant change in performance was seen over a test period of 30 days when the reagents were stored at −20°C or at 4°C. At 30°C, the reagents fail to function after 6 days (data not shown).

(vi) Sample preparation. Five different extraction protocols were compared by using two different experimental designs. In the first experiment a set of 17 fresh frozen cervical swab samples were each divided into four equal aliquots that were then subjected to the four extraction protocols—A (freezing-

TABLE 4. Variation in C_t values for different amounts of genomic DNA[a]

DNA amt (ng)	Mean human DNA C_t ± SD
100	21.33 ± 0.95
33	23.01 ± 0.80
10	24.88 ± 0.99
3.3	26.46 ± 0.80
1.0	28.21 ± 1.01
0.33	30.38 ± 0.67
0.10	31.44 ± 0.76

[a] The values are based on 12 independent measurements at each copy number.

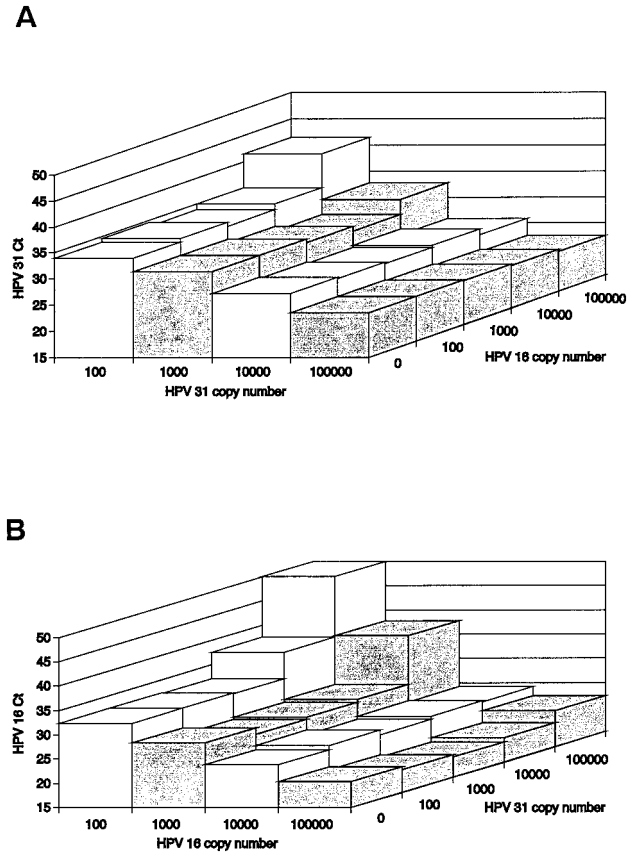

FIG. 5. Analysis of individual HPV types in synthetic mixtures made to mimic mixed infections. Each bar represents the mean threshold cycle (C_t) of three independent measures. (A) Quantification of HPV31 in a background of HPV16. The 95% confidence interval is based on the C_t values obtained when no HPV16 is added to the reaction. When the ratio of HPV31 to HPV16 is less than 1:100, the estimate of HPV31 falls outside of the 95% confidence interval. (B) Quantification of HPV16 in a background of HPV31. The 95% confidence interval is based on the C_t value obtained when no HPV31 is added to the reaction. When the ratio of HPV16 to HPV31 is less than 1:10 the estimate of HPV16 falls outside of the 95% confidence interval.

boiling), B (Wizard kit), C (digestion), and D (Wizard digestion followed by organic extraction)—as described in Materials and Methods. The results of the real-time PCR assay in the three first protocols were compared to that of protocol D (which was used as the "gold standard"). When we compared the yields of human DNA, the differences between the extraction protocols were pronounced (Fig. 6). The numbers of samples that tested negative for human DNA by the separate protocols were as follows: protocol A, four samples; protocol B, one sample; protocol C, four samples; and protocol D, no samples. After exclusion of the DNA-negative samples, protocol B show the highest correlation to method D ($r^2 = 0.90$, $P < 0.0001$). Protocols A ($r^2 = 0.88$, $P < 0.0001$ and C ($r^2 = 0.74$, $P = 0.0002$) display somewhat more variable results. Notably, the regression lines indicate trends of higher C_t for protocols A and C. These results indicate that, relative to the organic extraction protocol, which is usually considered unsuited for clinical use, the Wizard kit is to be preferred over the very simple freezing-boiling procedure or the quick protocol with proteinase K digestion.

Given the results of our comparison between extraction protocols A to D and the widespread use of the freezing-boiling method, we performed a second experiment with a set of cervical swab samples collected during routine gynecological health exams and used yet another commercial sample preparation method, the Nuclisens kit, which is frequently used in diagnostic virology laboratories. The samples were first extracted by using the freezing-boiling method (protocol A), and a real-time PCR assay was performed (Table 8). A number of the samples failed to show the presence of human DNA, although HPV typing proved successful. After application of the Nuclisens kit, the real-time PCR assay was again performed on these samples, and a much higher frequency of samples showed measurable amounts of human DNA (Table 8). Thus, the application of the Nuclisens protocol presumably resulted in the removal of inhibitory agents and a more reliable real-time PCR assay.

TABLE 5. Results of intralaboratory reproducibility test

HPV type or DNA	No. of positive samples/total no. of samples as tested by techician:		
	1	2	3
HPV16	5/5	5/5	5/5
HPV31	7/7	7/7	7/7
HPV18 and −45	7/7	7/7	7/7
HPV33 group	7/7	7/7	7/7
HPV39	6/7	6/7	7/7
Human DNA	14/14	14/14	14/14

TABLE 6. Results of lot-to-lot reproducibility test

HPV type or DNA	No. of positive samples/total no. of samples in lot:		
	1	2	3
HPV16	5/5	5/5	5/5
HPV31	7/7	7/7	7/7
HPV18 and −45	7/7	7/7	7/7
HPV33 group	7/7	7/7	7/7
HPV39	7/7	7/7	7/7
Human DNA	14/14	14/14	14/14

DISCUSSION

We developed a quantitative assay, one suitable for clinical use, for a range of HPV types. In light of the observation that a high HPV16 DNA titer is associated with a significant risk of

TABLE 7. Results of reproducibility-over-time test

HPV type or DNA	No. of positive samples/total no. of samples at wk:			
	1	2	3	4
HPV16	5/5	5/5	5/5	5/5
HPV31	7/7	7/7	7/7	7/7
HPV18 and −45	7/7	7/7	7/7	7/7
HPV33 group	6/7	7/7	7/7	7/7
HPV39	7/7	7/7	7/7	6/7
Human DNA	14/14	14/14	14/14	14/14

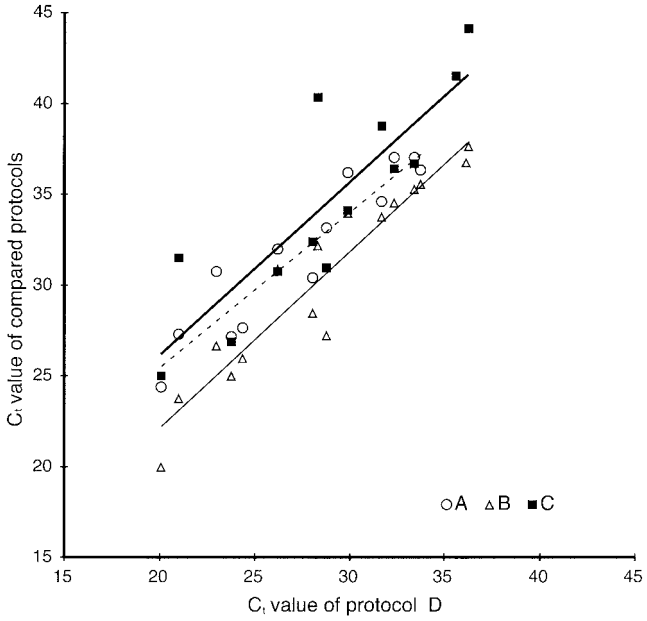

FIG. 6. DNA yields of three DNA-extraction protocols compared to that of organic extraction (protocol D). Protocols: A, freezing-boiling; B, Wizard kit; C, proteinase K digestion. The number of samples that did not result in measurable amounts of DNA by each protocol was as follows: protocol A, $n = 4$; protocol B, $n = 1$; protocol C, $n = 4$; and protocol D, $n = 0$. These datum points are excluded. The lines represent the linear regression of the comparisons (broken line, protocol D versus protocol A [$r^2 = 0.877$]; thin solid line, protocol D versus protocol B [$r^2 = 0.902$]; thick solid line, protocol D versus protocol C [$r^2 = 0.736$]).

developing cervical cancer in situ (9, 23), analyses of viral titer may have a diagnostic use. Suitable methods for such a titer test must have a wide dynamic range, must be easy to use, and must permit a range of the highly divergent HPV types associated with the development of cervical dysplasia to be assayed. We extended the usefulness of the real-time PCR method by quantifying three different fluorophores in each reaction tube, thereby limiting the number of parallel reaction that have to be performed. We chose to design our system with an external control to avoid any competition between the amplicons of the HPV and the human gene since such competition may lead to an erroneous estimate of the copy number per cell. Competition between an internal control and an HPV PCR product may result in an underestimate of the HPV copy number for samples with low viral load and an overestimate of HPV copy number in samples with a high viral load. A system with an internal control may therefore tend to reduce the range in HPV copy number among samples. The HPV copy number per cell measured in our samples span over a wide range. Using a system with an internal control over such a wide range of copy number would most likely limit the resolution of the data substantially.

Rather than using commercially available assays, we developed a separate assay for a single-copy nuclear gene. Using our human single-copy gene assay, the HPV copy numbers can be normalized for the amount of genomic DNA (equivalent to the number of cells included). Given the variation both in the amount of HPV copies between samples and the amount of genomic DNA between samples, normalization appears to be necessary in order to obtain comparable and meaningful HPV titer estimates. Of course, such a measure does not indicate the number of infected cells or the relative distribution of HPV genomes among cells.

An important aspect of any diagnostic technique is the ability to identify false-negative samples that result either from an insufficient amount of starting DNA or the presence of inhibitors to the PCR. The lack of a signal for the human gene assay either indicates the presence of inhibitors or insufficient amount of DNA in the assay. Indeed, in comparing the sample extraction protocols, we noted that with one of the faster protocols a number of samples failed to give a signal for the human single-copy gene. Most of these samples gave a positive result with the nuclear gene assay when the samples had been further purified.

An important aspect of the assay is the ability to quantify

TABLE 8. Comparison of the results obtained by using extraction method C and the Nuclisens kit[a]

Sample no	Copy number when DNA was extracted by:							
	Protocol C (freezing-boiling)				Nuclisens extraction protocol			
	HPV16	HPV18/45	HPV33 group	Human DNA	HPV16	HPV18/45	HPV33 group	Human DNA
1	0	0	0	0	0	0	0	0
2	0	390	2,500	0	270	1,400	11,000	100
3	0	0	30	30	0	0	3,000	150
4	58	0	0	0	140	0	0	1
5	0	0	19	0	0	0	612	12
6	0	0	0	0	0	0	0	40
7	0	0	372	3.1	0	0	1,365	6.5
8	0	0	0	0	0	0	0	6.6
9	0	0	0	0	0	0	0	9.1
10	0	0	0	0	0	0	0	250
11	0	800	0	0	0	16,490	0	97
12	0	0	0	0	0	0	1,430	1,100
13	0	0	360	0	0	0	1,836	5.1
14	0	22.5	0	3	0	21	0	14
15	0	0	14	0	0	0	2,760	690

[a] HPV titers are not normalized with respect to the sample amount.

individual HPV types in mixed infections. There are indications that the copy number in infected cervical cells differs not only between different stages of dysplasia (9, 19, 23) but also between HPV types (19). Therefore, a diagnostic assay must have the ability to identify and quantify individual HPV types in a mixed infection. For most combinations of HPV types in our synthetic mixes, the assay showed an ability to detect and quantify an HPV type, as long as it represents at least 1 to 10% of the amount of the major HPV type. When the ratio between HPV types is less than 1:100, the reduced sensitivity is likely to be due to a competition between the PCR products of different HPV types. For clinical samples infected with several HPV types, the ratios vary widely, emphasizing the need for an assay that can provide reliable quantification over a wide range of ratios.

In summary, the fluorescent 5′ exonuclease assay described here has a number of characteristics that make it suitable for quantification of HPV viral titers. Other assays exist that have a wide coverage of viral types but require complex post-PCR analysis, making them unable to compete with the rapidity, convenience, and flexibility of the PCR-based fluorescent 5′ exonuclease assay.

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REFERENCES

1. **Bosch, F. X., M. M. Manos, N. Munoz, M. Sherman, A. M. Jansen, J. Peto, M. H. Schiffman, V. Moreno, R. Kurman, K. V. Shah, et al.** 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Natl. Cancer Inst. **87:**796–802.
2. **Chua, K. L., and A. Hjerpe.** 1995. Polymerase chain reaction analysis of human papillomavirus in archival cervical cytologic smears. Anal. Quant. Cytol. Histol. **17:**221–229.
3. **Hart, K. W., O. M. Williams, N. Thelwell, A. N. Fiander, T. Brown, L. K. Borysiewicz, and C. M. Gelder.** 2001. Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples. J. Clin. Microbiol. **39:**3204–3212.
4. **Higuchi, R., C. Fockler, G. Dollinger, and R. Watson.** 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Bio/Technology **11:**1026–1030.
5. **Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand.** 1991. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA **88:**7276–7280.
6. **Jacobs, M. V., J. M. Walboomers, J. van Beek, F. J. Voorhorst, R. H. Verheijen, C. J. Meijer, A. J. van den Brule, T. J. Helmerhorst, and P. J. Snijders.** 1999. A quantitative polymerase chain reaction-enzyme immunoassay for accurate measurements of human papillomavirus type 16 DNA levels in cervical scrapings. Br. J. Cancer **81:**114–121.
7. **Josefsson, A., K. Livak, and U. Gyllensten.** 1999. Detection and quantitation of human papillomavirus by using the fluorescent 5′ exonuclease assay. J. Clin. Microbiol. **37:**490–496.
8. **Josefsson, A. M., P. K. Magnusson, and U. B. Gyllensten.** 1999. Human papillomavirus detection by PCR and typing by dot-blot, p. 171–193. *In* S. P. Peeling (ed.), Sexually transmitted diseases: methods and protocols, vol. 20. Humana Press, Inc., Totowa, N.J.
9. **Josefsson, A. M., P. K. Magnusson, N. Ylitalo, P. Sorensen, P. Qwarforth-Tubbin, P. K. Andersen, M. Melbye, H. O. Adami, and U. B. Gyllensten.** 2000. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet **355:**2189–2193.
10. **Kjaer, S. K., A. J. van den Brule, J. E. Bock, P. A. Poll, G. Engholm, M. E. Sherman, J. M. Walboomers, and C. J. Meijer.** 1996. Human papillomavirus: the most significant risk determinant of cervical intraepithelial neoplasia. Int. J. Cancer **65:**601–606.
11. **Lee, L. G., C. R. Connell, and W. Bloch.** 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. **21:**3761–3766.
12. **Lindqvist, A. K., P. K. Magnusson, J. Balciuniene, C. Wadelius, E. Lindholm, M. E. Alarcon-Riquelme, and U. B. Gyllensten.** 1996. Chromosome-specific panels of tri- and tetranucleotide microsatellite markers for multiplex fluorescent detection and automated genotyping: evaluation of their utility in pathology and forensics. Genome Res. **6:**1170–1176.
13. **Livak, K. J., J. Marmaro, and J. A. Todd.** 1995. Towards fully automated genome-wide polymorphism screening. Nat. Genet. **9:**341–342.
14. **Maniatis, T. E., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Palefsky, J. M., and E. A. Holly.** 1995. Molecular virology and epidemiology of human papillomavirus and cervical cancer. Cancer Epidemiol. Biomarkers Prev. **4:**415–428.
16. **Samiotaki, M., M. Kwiatkowski, N. Ylitalo, and U. Landegren.** 1997. Seven-color time-resolved fluorescence hybridization analysis of human papilloma virus types. Anal. Biochem. **253:**156–161.
17. **Schiffman, M. H., H. M. Bauer, R. N. Hoover, A. G. Glass, D. M. Cadell, B. B. Rush, D. R. Scott, M. E. Sherman, R. J. Kurman, S. Wacholder, et al.** 1993. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. J. Natl. Cancer Inst. **85:**958–964.
18. **Swan, D. C., R. A. Tucker, B. P. Holloway, and J. P. Icenogle.** 1997. A sensitive, type-specific, fluorogenic probe assay for detection of human papillomavirus DNA. J. Clin. Microbiol. **35:**886–891.
19. **Swan, D. C., R. A. Tucker, G. Tortolero-Luna, M. F. Mitchell, L. Wideroff, E. R. Unger, R. A. Nisenbaum, W. C. Reeves, and J. P. Icenogle.** 1999. Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type. J. Clin. Microbiol. **37:**1030–1034.
20. **Tucker, R. A., E. R. Unger, B. P. Holloway, and D. C. Swan.** 2001. Real-time PCR-based fluorescent assay for quantitation of human papillomavirus types 6, 11, 16, and 18. Mol. Diagn. **6:**39–47.
21. **van Duin, M., P. J. Snijders, H. F. Schrijnemakers, F. J. Voorhorst, L. Rozendaal, M. A. Nobbenhuis, A. J. van den Brule, R. H. Verheijen, T. J. Helmerhorst, and C. J. Meijer.** 2002. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int. J. Cancer **98:**590–595.
22. **Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. **189:**12–19.
23. **Ylitalo, N., P. Sorensen, A. M. Josefsson, P. K. Magnusson, P. K. Andersen, J. Ponten, H. O. Adami, U. B. Gyllensten, and M. Melbye.** 2000. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet **355:**2194–2198.