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A Sensitive, Type-Specific, Fluorogenic Probe Assay for Detection of Human Papillomavirus DNA

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A simple method for the detection of a number of human papillomavirus (HPV) genotypes associated with cervical cancer has been developed. The assay exploits the 5′→3′ exonucleolytic activity of *Taq* DNA polymerase to increase the signal from fluorescent dyes by releasing them from genotype-specific probes during PCR. The probes are oligonucleotides with a 5′ reporter dye (6-carboxyfluorescein), a quencher dye (6-carboxy-tetramethyl-rhodamine), and a phosphate-blocked 3′ end. In the intact probe, the proximity of the reporter and the quencher results in suppression of reporter fluorescence by Förster-type energy transfer (V. T. Förster. Ann. Phys. 2:55–75, 1948). If the probe is bound downstream of either primer during PCR, the 5′→3′ exonucleolytic activity of *Taq* polymerase degrades it, allowing the reporter to diffuse away from the quencher, which results in an increase in reporter fluorescence. The increased fluorescence is directly related to the amount of target DNA and can be detected with an automated fluorometer. Probes for the L1 region of the cervical-cancer-associated HPV types 16, 18, 31, 33, and 35 were synthesized and the assays were optimized. The most sensitive assay can detect as few as two copies of HPV DNA in human cervical specimens.

Almost all carcinomas of the cervix are found to contain specific genotypes of human papillomavirus (HPV) DNA, the high-risk types. Common high-risk types of HPV in cervical cancer in the United States are HPV type 16 (HPV-16), HPV-18, HPV-31, HPV-33, and HPV-35 (3). Testing for high-risk HPV in conjunction with cytology may improve detection of precancerous cells in cervical samples, but the protocol for such testing has not been determined. Although some studies have indicated that the quantity of detectable HPV DNA is predictive of cervical disease (4, 5, 15), it remains to be determined if more specific tests, such as assaying for specific open reading frames, integrated DNA, or specific mRNAs, would be more predictive. Nevertheless, some properties of assays for high-risk HPV DNAs that would be necessary for the assays to be useful in screening programs are clear. At some stages of cervical disease, the amount of HPV DNA may be very low (<1 HPV genome per infected cell); thus, useful HPV tests must be sensitive as well as type specific. A number of PCR assays that are both type specific and sensitive have been described, but most of them are not practical for screening purposes since they are not easily used for testing large numbers of specimens (2, 3, 9, 11, 17).

The fluorogenic probe assays for HPV DNAs described here are type specific, quantitative, sensitive, and useful for testing large numbers of specimens. They are based on the PCR amplification of a portion of the L1 open reading frames of HPV-16, -18, -31, -33, and -35 DNAs by using genotype-specific primers in the presence of fluorescent, genotype-specific probes that bind to the amplified DNA. The probes are blocked at their 3′ termini and hence cannot be extended by the polymerase (Fig. 1). If, during the course of primer extension, *Taq* polymerase encounters a bound probe, its 5′→3′ exonuclease activity degrades the probe, releasing the 5′ fluor from the proximity of the 3′ quencher. This causes an increase in the fluorescence emitted by the reporter (6) which, in the presence of an excess amount of the probe, is directly related to the amount of HPV DNA present in the sample before amplification. This type of assay has been described previously and has been used to detect other pathogens (1, 13).

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MATERIALS AND METHODS

Probes. The probe sequences for each of the high-risk HPVs were selected based on the following criteria: (i) a predicted lack of cross-hybridization to the other common high- and low-risk HPVs (HPV-6, -11, -16, -18, -31, -33, -35, -45, -51, -52, -56, and -58), (ii) a melting temperature of approximately 70°C, (iii) a lack of predicted dimer formation with corresponding primers, (iv) a lack of self-annealing, and (v) no runs of identical nucleotides longer than four, especially Gs. Fluorescent probes for globin and for HPV-16, -18, -31, -33, and -35 were synthesized in the Biotechnology Core Facility at the Centers for Disease Control and Prevention (Table 1). The fluorescent reporter dye on the 5′ ends of the HPV probes was FAM (6-carboxyfluorescein). The globin probe contained HEX (hexachloro-fluorescein) on its 5′ end. The rhodamine quencher dye (TAMRA; 6-carboxy-tetramethyl-rhodamine) was incorporated into a linker arm thymidine (amino modifier C6 dT; Glen Research, Sterling, Va.) at or close to the 3′ end. Extension from the 3′ end of the probe was blocked by the addition of a 3′ phosphate. Synthesis employed standard DNA phosphoramidites in addition to 6-carboxyfluorescein phosphoramidite, 5′-hexachloro-fluorescein-cyano-ethyl phosphoramidite, 6-carboxy-tetramethyl-rhodamine succinamidyl ester, and amino modifier C6 dT. The 3′-blocking phosphate was attached by using 3′ phosphate control pore glass (Glen Research).

Primers. The primer sequences were selected by using the Oligo 5.0 primer analysis program (National Biosciences, Inc., Plymouth, Minn.). The primer pairs for each of the HPV types were selected based on having a melting temperature of approximately 65°C, a predicted lack of cross-hybridization to other common HPV types, and no loop or dimer formation with the other primer. Primers were synthesized in the Biotechnology Core Facility at the Centers for Disease Control and Prevention by standard techniques.

DNA samples. Cervicovaginal lavage samples from 20 patients were thawed and 100 μl was removed from each. Samples were transferred to Phase-Lock gel tubes (5 Prime→3 Prime, Boulder, Colo.) containing 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), mixed by inversion, and centrifuged at 12,000 × *g* for 2 min. An additional 100 μl of chloroform was added to each tube, and the tube contents were mixed and centrifuged as described above. Each supernatant from the Phase-Lock procedure was diluted to 2 ml with water and centrifuged in a Centricon 100 microconcentrator (Amicon, Inc., Beverly, Mass.) at 1,000 × *g* for 30 min. An additional 2 ml of water was added to each retentate, and the centrifugation was repeated. Retentates were then collected and diluted to 250 μl with water.

Control templates. Control templates for HPV-16, -18, -31, -33, -35, -45, and -52 were prepared by PCR amplification of cloned DNA with the L1-, type-specific primers (Table 1) and purified in Centricon 100 microconcentrators. The

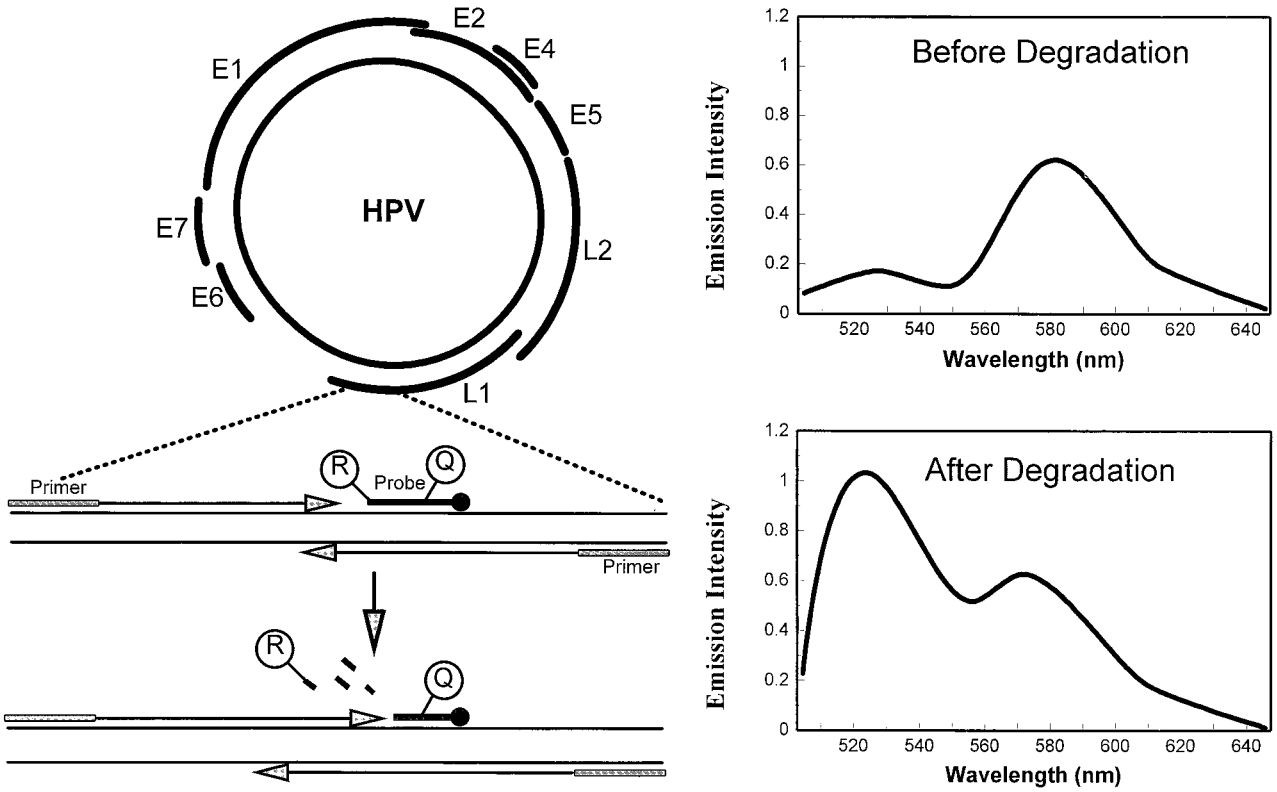

FIG. 1. Schematic of HPV detection by fluorogenic probe assay. The circular HPV genome and the open reading frames are shown on the left. An approximately 470-bp region near the 3′ end of the L1 gene of HPV is amplified with type-specific primers, and the amplified product is detected with a fluorogenic probe that binds between the primers. Each HPV-type-specific probe is blocked at the 3′ end, contains a reporter (R) and quencher (Q) dye, and was selected to have only one binding site in the specific amplified product. During PCR amplification of HPV DNA, the 5′→3′ exonuclease activity of *Taq* polymerase degrades any probe bound to the template between the primers. Prior to degradation, the fluorescent spectrum of the probe (right) shows almost no emission in the 520-nm range, whereas after degradation, there is a large peak in this range. The intensity of the reporter fluorescence increases as the amount of specific product increases.

DNA concentrations were determined by fluorometry (DyNA Quant 200; Hoefer-Pharmacia Biotech, San Francisco, Calif.) after 2 μl of DNA solution was added to 2 ml of freshly prepared Hoechst 33258 dye solution (1 μg/ml in 10 mM Tris [pH 7.4]–1 mM EDTA–200 mM NaCl) (Pharmacia Biotech Inc., Piscataway, N.J.). A 100-μg/ml DNA standard was used.

Controls, consisting of a dilution series of the homologous template and a set of nine heterologous templates (HPV-16, -18, -31, -33, -35, -45, -51, -52, and -56), were included in each run. By comparing the results obtained with patient DNA to those obtained with the control samples, we estimated the amount of HPV DNA in the patient samples. Each control sample contained 50 ng of human placental DNA.

Fluorogenic PCR. The 50-μl PCR mixtures contained 10 mM Tris (pH 8.3), 50 mM KCl, 4.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.3 μM each primer, 50 nM each fluorogenic probe, 0.025 U of *Taq* polymerase/μl and 10 μl of template DNA. Following template denaturation for 2 min at 95°C, amplification conditions were as follows: 40 cycles (each) of 30 s at 94°C, 10 s at 60°C, and 2 min at 65°C. Amplification was carried out in a 9600 thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.), after which the samples were transferred to a MicroFLUOR W 96-well, white microtiter plate (Dynatech Industries, Inc., McLean, Va.). Fluorescence was measured with a Perkin-Elmer LS-50B luminescence spectrometer equipped with a microtiter plate reader. Excitation was at 480 nm, and fluorescence was measured at 518 nm (FAM emission maximum), 556 nm (HEX emission maximum), and 582 nm (TAMRA emission maximum). Data acquisition and analysis were performed with the TaqMan fluorescence data manager (Perkin-Elmer Corp.) and Excel 5.0 (Microsoft Corporation, Redmond, Wash.).

The ratios of fluorescence intensity (RQ^+) (FAM/TAMRA or HEX/TAMRA) were calculated for each sample. The average ratio for three no-template control samples was used to calculate RQ^-. ΔRQ was then calculated for each sample by the formula $\Delta RQ = RQ^+ - RQ^-$. Threshold ΔRQ, used to establish a baseline for positive samples, was calculated for a 99% confidence level by using the standard deviation of the results from the three no-template control samples (TaqMan PCR reagent kit protocol; Perkin-Elmer).

PCR product sequencing. Products from the PCR were purified in Centricon 100 microconcentrators and sequenced by standard dye terminator methodology (cycle sequencing kits from Applied Biotechnology Division, Perkin-Elmer Corp., Emeryville, Calif.). Sequences were analyzed by the Genetics Computer Group (University of Wisconsin) sequence analysis package.

RESULTS

Assay characterization. The fluorogenic assays were characterized with a dilution series of the homologous template (1×10^4 to 3×10^1 copies) and a series of heterologous templates (2×10^3 copies). In Fig. 2, the results with the homologous HPV-16 template dilution series show the approximately semilogarithmic relationship between the number of template copies and the signal intensity and a sensitivity limit of approximately 30 copies of HPV-16. Each sample contained 50 ng of human placental DNA (equivalent to 10,000 cells); thus, the limit of detectability was 30 HPV copies in a total of 10,000 cells, or approximately 1 HPV copy in 300 cells. These sensitivities are approximately equivalent to those obtained by PCR amplification and ethidium bromide detection of the products on agarose gels (16). Results obtained with the heterologous templates indicated good specificity. The HPV-16 probe gave a detectable signal with HPV-18, -35, and -56 templates, but despite the fact that 2,000 copies of these templates were present, the signal was less than 35% of the level of the 30-copy homologous template. Thus, heterologous templates would not give a positive signal equivalent to 30 copies of HPV-16 unless they were present in more than about 6,000 copies.

The sensitivities and specificities for all the fluorogenic assays are shown in Fig. 3. Each had a sensitivity limit of less than 100 copies of the homologous template per sample. The HPV-

TABLE 1. Primers and probes used in HPV fluorogenic assays

Gene	Primer or probe[a]	Sequence
HPV-16 L1	U primer 6564	CCT TAT TGG TTA CAA CGA GCA C
	L primer 7012	GCG TCC TAA AGG AAA CTG ATC TA
	U probe 6862	CCC CAG GAG GCA CAC TAG AAG A(T)[b]
HPV-18 L1	U primer 6548	GTT ACA TAA GGC ACA GGG TCA T
	L primer 6993	CGT CCA AGG GGA TAT TGA TC
	U probe 6902	AAA GGA TGC TGC ACC GGC T[b]
HPV-31 L1	U primer 6490	GAT GCA ACG TGC TCA GGG A
	L primer 6930	GCG ACC CAG TGG AAA CTG ATC TA
	U probe 6852	CCC AAA AGC CCA AGG AAG AT[b]C
HPV-33 L1	U primer 6490	GGT TAC TTC CGA ATC TCA GTT ATT T
	L primer 6964	TCC CAA AGG AAA CTG ATC TAA A
	L probe 6787	TGT TAA ACC AAA TTG CCA ATC TTC T[b]
HPV-35 L1	U primer 6518	ACG TGC ACA AGG CCA TAA TA
	L primer 6947	CCA ACG GAA ACT GAT CTA AGT CT
	U probe 6757	TGA ACC CGT CCA TTT TAG AGG AT[b]
Globin	U primer 61992	GAA GAG CCA AGG ACA GGT AC
	L primer 62240	CAA CTT CAT CCA CGT TCA CC
	U probe 62049	CCC TAG GGT TGG CCA ATC TAC T[b]C

[a] Strand sense and end nucleotide position (5′ terminus in U primers and probes and 3′ terminus in L primers and probes) as numbered in the GenBank reference sequences. Accession numbers are as follows: HPV-16, K02718; HPV-18, X05015; HPV-31, J04353; HPV-33, A12360; HPV-35, M74117; and globin, U01317.
[b] T to which the TAMRA is coupled. Parentheses indicate that T was not present in the target DNA sequence.

16, -31, and -35 assays had sensitivities of less than 30 copies per assay sample. The degree of cross-reactivity was low for all the assays. The HPV-33 assay showed the greatest degree of cross-reactivity: a signal equivalent to between 30 and 100 copies of HPV-33 was seen with 2,000 copies of HPV-16 or HPV-51.

Analysis of patient samples. To evaluate the performance of the assay with actual patient specimens, 20 cervicovaginal lavage samples (12 from patients with known cervical disease and 8 from women without disease) were tested in an HPV-16–globin multiplex assay (Fig. 4). Patients 9, 10, 12, 15, and 20 were positive for HPV-16; the rest were negative. Comparison of patient ΔRQ signals with the HPV-16 dilution series showed that the positive patient samples contained between <30 copies (patient 12) and about 1,000 copies (patient 20) per 10-μl aliquot.

The PCR products from the assay shown in Fig. 4A were

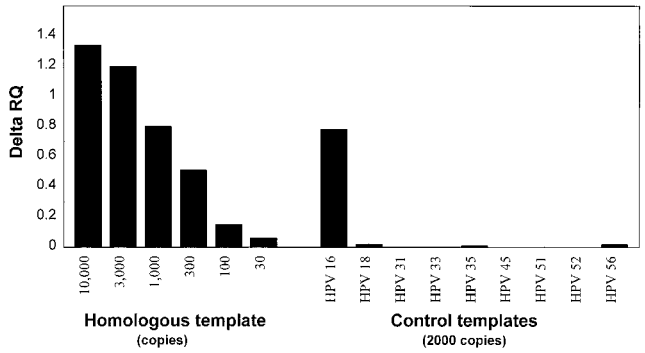

FIG. 2. Sensitivity and specificity of the HPV-16 fluorogenic probe assay. The homologous template was present in the amounts shown; the heterologous templates were each present at 2,000 copies. Primers, probes, and amplification conditions were as described in Table 1 and Materials and Methods.

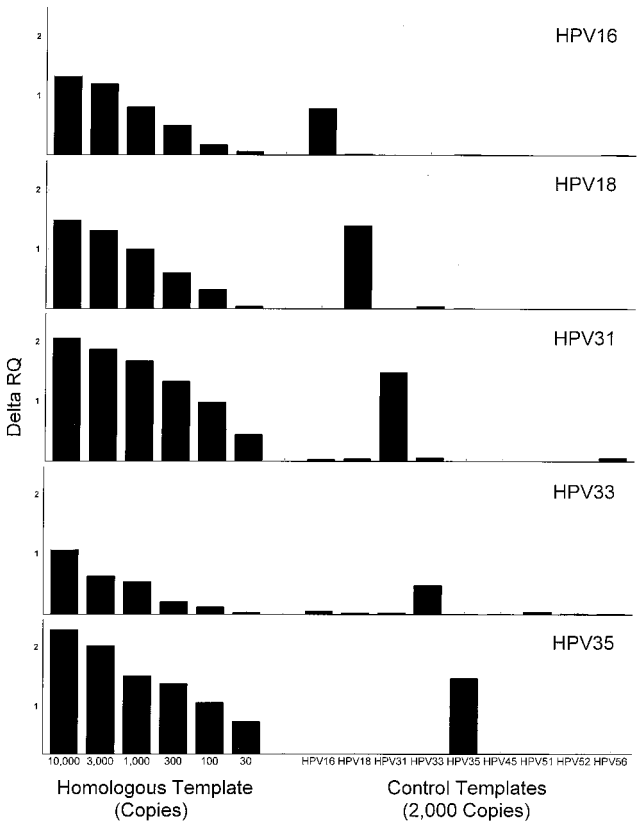

FIG. 3. Sensitivities and specificities of various HPV fluorogenic probe assays. Each assay included the indicated amounts of a homologous template and a set of heterologous templates (2,000 copies each). Other reaction conditions were as described in Table 1 and Materials and Methods.

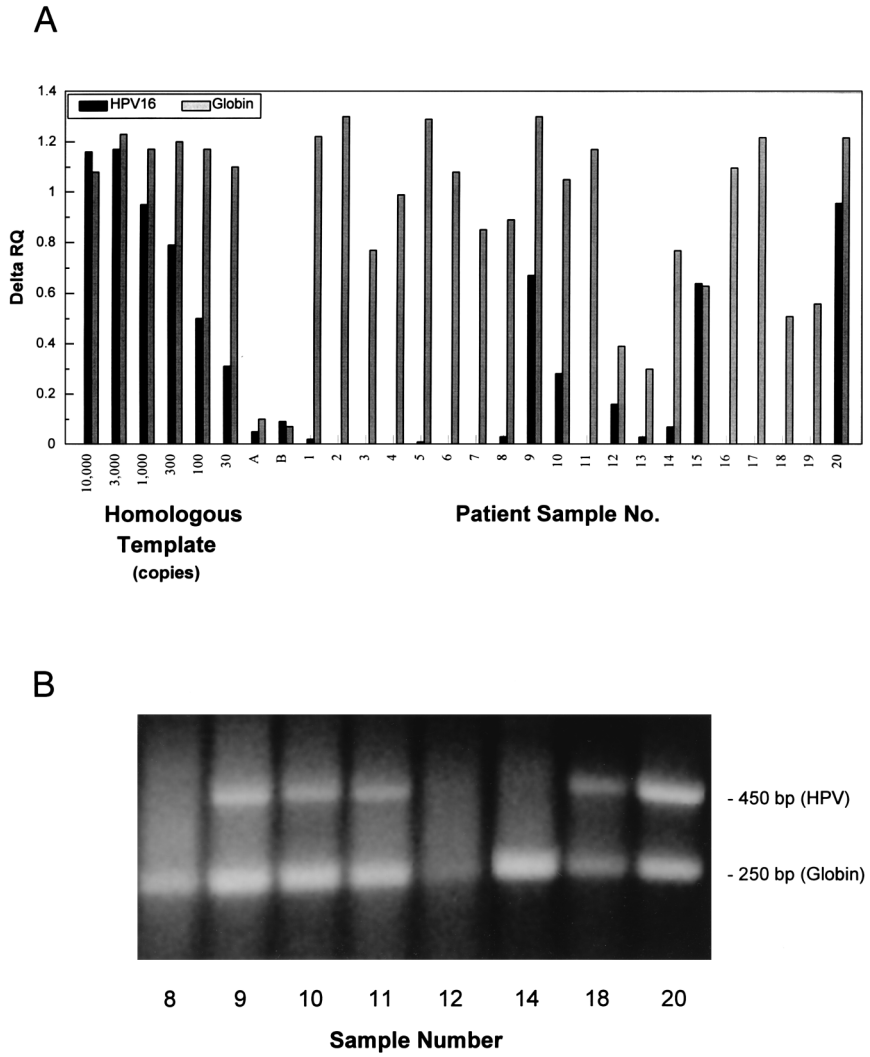

FIG. 4. (A) Analysis of patient samples by HPV-16–globin multiplex assay. Patient samples were amplified in a multiplex assay that contained the primers and probes for both HPV-16 and globin. Samples 1 through 8 were from women without cervical disease, and 9 through 20 were from patients with various levels of disease as indicated in Table 2. It was possible to detect each product independently since the HPV-16 and the globin probes were labeled with FAM and HEX, respectively. The sensitivity controls contained HPV-16 DNA in the amounts shown and a constant amount of human placental DNA (2,000 genome copies). Samples A and B were water blanks that were carried through the entire extraction procedure as contamination controls. The assay conditions are given in Materials and Methods. (B) Analysis of PCR products by agarose gel electrophoresis. Selected samples from panel A were analyzed by agarose gel electrophoresis and ethidium bromide staining. From the standard TaqMan reaction mixture, 40 μl was applied to a 1% agarose gel containing 1× TAE (10 mM Tris acetate [pH 7.4], 1 mM EDTA) and 0.25 μg of ethidium bromide/ml. Gels were electrophoresed for 2 h at 80 V. The products were visualized on a UV light box and photographed.

analyzed by agarose gel electrophoresis (Fig. 4B). Some samples contained an HPV-16-sized band but did not give a positive signal in the fluorogenic assay. For example, the sample from patient 11 showed a strong ethidium bromide band but was negative for HPV-16 (and also for HPV-18, -31, -33, and -35; see below) by the fluorogenic assay. The sequence of this product, determined by using consensus primers my09 and my11, matched that of HPV-66. It was unexpected that an HPV-66 template would be amplifiable with the HPV-16-specific primers since HPV-66 has several bases mismatched with both the upper and lower primers. However, the fluorogenic assay was still specific, since the probe was not degraded despite the presence of a large amount of HPV-66 product. The ethidium bromide staining band from sample 18 did not react with the HPV-16 probe; HPV-31 is the predominant type in this sample (see below). Sample 12 gave an appreciable fluorogenic signal for HPV-16 despite the fact that the product was undetectable by ethidium bromide staining. The homologous template dilution series all contained 50 ng of human placental DNA and showed a reproducible signal with the globin probe. The differences in globin signal in the patient samples could be due to differences in input DNA amount and to inhibition by the extract. Samples 12 and 13 gave a low globin signal in the multiplex assay but, when spiked with HPV-16 DNA, showed a good HPV-16 signal (Fig. 5). Thus, the original low globin signals in these samples presumably reflected low DNA levels. There is, however, evidence of inhibition in some patient samples. For example, sample 4, when spiked with 2,000 copies of HPV-16, showed a signal that corresponded to only approximately 500 copies. Inhibitors in patient samples have been reported previously (12). The range of signals observed for samples 1 through 8 after addition of 2,000 HPV-16 DNA copies is not likely to be a problem since the differences in amounts of DNA between normal and dis-

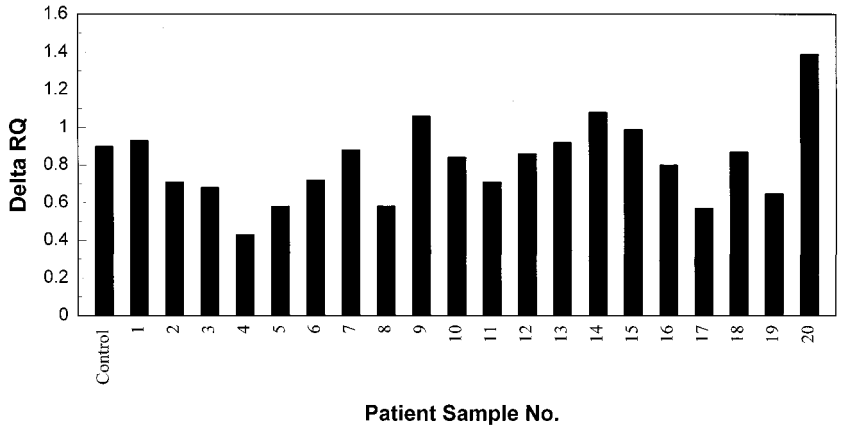

FIG. 5. Test for inhibitors in patient samples. Each of the patient samples used for Fig. 3 was retested for HPV-16 after the addition of 2,000 copies of an HPV-16 template.

eased specimens have been reported to be fivefold or greater (20).

The patient samples that had been assayed for HPV-16 DNA were also assayed for HPV-18, -31, -33, and -35 DNAs. Table 2 summarizes the results from these assays in relation to pathological diagnosis. Eight of the 12 samples from women with cervical disease were shown by fluorogenic assays to contain detectable HPV. While it is possible that the four negative samples contained no HPV DNA, they may have contained HPV types for which we did not test (e.g., HPV-66; sample 11). None of the samples from women without cervical disease was positive for HPV-16, -18, -31, -33, or -35.

DISCUSSION

The HPV fluorogenic assay described here is a fast, simple method for detecting and typing HPV DNA. It is a convenient, relatively inexpensive assay for use in studies of large numbers of specimens (fluorescent probes can be purchased for approximately $0.10 per PCR test). The speed and simplicity of the test result from the fact that the probe is present in the reaction mixture during PCR and that after amplification the fluorescence is measured without any further manipulations.

The combination of type-specific primers and probes was used to make the assay both highly sensitive and specific (Fig. 3). The sensitivities of the assays for HPV-16, -18, -31, -33, and -35 DNAs varied between approximately one copy of HPV in 100 cells and one copy in 1,000 cells for 50 ng (10,000 cells) of input DNA. The sensitivity was approximately the same as that obtained with some HPV PCR assays (14, 21) but was greater than that of some primer-probe systems (9, 18, 19). For example, Snijders et al. constructed primers and probes that were capable of detecting almost all HPVs, but because of the degeneracy of the oligonucleotides, the sensitivity was only about 7,000 copies of HPV DNA, even after overnight hybridization with probes (19). The assays for HPV-16, -18, -31, and -35 gave signals with 2,000 copies of heterologous templates that were almost undetectable (equivalent to approximately 5 to 10 copies of homologous templates). The HPV-33 assay is the least specific, but even in this case, 2,000 copies of HPV-16 gave a signal strength equivalent to only about 60 copies of the homologous template.

TABLE 2. HPV types in lavage samples from patients with cervical disease

Sample	Diagnosis[a]	HPV type[b]
1–8	No disease	None
9	CIN I, CIS	16
10	CIN I, CIN III	16, 31
11	CIN III	None[c]
12	CIN I	16
13	CIV	None
14	CIN II, CIN III	31
15	CIN III	16
16	CIV	None
17	CIS	31
18	CIS	31
19	CIV	None
20	CIV	16

[a] CIS, carcinoma in situ; CIV, invasive carcinoma. For those patients in which more than one biopsy sample was analyzed, the result for each is given.
[b] Detected by fluorogenic assay.
[c] HPV-66 identified by sequencing of the PCR product.

Development of protocols for HPV testing in cervical disease screening programs may require quantitative HPV DNA detection assays followed by systematic evaluation of the predictive values for various levels of HPV DNA; this fluorogenic assay would work well for that purpose (5, 7, 8, 10, 15). The fluorogenic assay is not superior to a PCR dot blot hybridization assay for quantitation (9), but it has the advantage of being simpler and being compatible with automatic data collection techniques.

Inhibitors of amplification have been reported for some patient samples (16, 22). An assay with a β-globin probe would allow the detection of low β-globin signals indicative of potentially inhibitory specimens, and spiking such samples with standard templates should separate inhibitory specimens from those with a low DNA content. Normalizing the HPV signal to the β-globin signal should result in a better estimate of the amount of HPV DNA present, independent of the cause of the low β-globin signal.

All the HPV fluorogenic probes described here should be useful with the commonly used my09-my11 consensus primer system. For example, the HPV-16 fluorogenic probe gave sensitivities and specificities approximately equal to those described here when used with primers my09 and my11 (data not shown). Fluorogenic screening for HPV-positive samples with a consensus system followed by typing of the positive samples with a type-specific assay would require fewer assays than would a series of type-specific assays alone, but a good "pan-

HPV" fluorogenic probe is difficult to design (18). For example, such a consensus probe for the region of the L1 gene amplified by the L1-type-specific primers was found to require nine degenerate positions, resulting in a mixture of 512 different probes. The alternative of screening for HPV positivity by assays that involve electrophoretic separation of products defeats the purpose of having a simple HPV test.

The following protocol is suggested to determine DNA amplifiability by using the β-globin assay and to minimize the number of reactions by using multiplexing. Sufficiently pure templates for the fluorogenic assay would be obtained by simple, standard methods such as those described here. The first multiplex PCR would include primers and probes for any HPV type of interest and for β-globin. Subsequently, β-globin-positive samples could be screened simultaneously for two HPV types in a multiplex assay with two HPV probes, one labeled with FAM and the other with HEX.

The simplicity of the fluorogenic assay makes it useful for screening large numbers of samples for the detection of rare events. For example, it is currently being adapted as a specific test for integrated HPV, which may occur rarely in women with low-grade cervical lesions (cervical intraepithelial neoplasia [CIN] grades I and II).

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