

Simultaneous Amplification and Identification of 25 Human Papillomavirus Types with Templex Technology[∇]

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The majority of existing human papillomavirus (HPV) genotyping assays are based on multiplex PCR using consensus or degenerate primers. We developed a Templex HPV assay that simultaneously detects and identifies 25 common HPV genotypes in a single-tube reaction using type-specific primers for the HPV-specific E6 and E7 genes. The analytical sensitivities of the Templex assay for HPV type 16 (HPV-16), -18, and -56 were 20, 100, and 20 copies per reaction mixture, respectively. The Templex assay provides semiquantitative information on each type when multiple HPV types coexist in one reaction. We tested 109 clinical cervical specimens previously evaluated with the Digene HC2 high-risk HPV DNA test and found 95.4% concordance between the assay results. The Templex assay provided type-specific results and found multiple types in 29.2% (14 of 48) of high-risk HPV-positive samples. The entire Templex procedure, including DNA extraction, can be completed within 5 hours, providing a rapid and reliable diagnostic tool for HPV detection and typing that is amenable to automation.

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Human papillomavirus (HPV) infection is linked with cervical cancer (1, 12, 24). HPV can be divided into “high-risk (HR)” and “low-risk” groups on the basis of their association with cervical lesions (7, 11, 17). The HR group includes HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 67, 68, 69, 82, 26, 53, 66, 70, and 73, while the low-risk group includes types 6, 11, 40, 42, 43, 44, 54, 61, 72, 81, and 89 (11, 15, 19). Recently, quadrivalent (HPV-6, -11, -16, and -18) and bivalent (HPV-16 and -18) vaccines have been demonstrated to effectively prevent type-specific persistent infection and disease (6, 23). To monitor the impact of vaccine implementation strategies, determine type-specific persistence, and evaluate the clinical significance of coinfection with multiple HPV types, HPV testing will require type-specific results. A high-throughput, sensitive, specific, and reproducible HPV detection and typing assay is therefore highly desirable.

Most established HPV typing assays used in epidemiologic studies are based on consensus PCR to amplify the relatively conserved L1 gene region with hybridization, restriction enzyme digestion, or sequencing of the amplicon to determine type(s) (2, 22). Widely used L1 consensus primer PCR systems include the GP5+/6+, PGM09/11, and SPF systems (4, 5, 13, 21). However, in all of these methods, variations in the efficiency of type-specific priming, primer competition, and limitations on the reagent concentrations in the assay may affect the observed type distribution, particularly when large numbers of types at greatly different copy numbers are present. In addition, typing requires a variable number of additional post-

PCR steps, such as amplicon purification, gel electrophoresis, hybridization, and washing. These additional steps increase the time and labor required.

We report the development of a novel HPV genotyping assay that is based on proprietary Templex technology. Templex is a unique multiplex PCR platform that uses a target-enriched multiplex PCR method (●●●●●●●●, patent pending, international publication number WO 2005/038039 A2). Templex uses nested gene-specific primers at extremely low concentrations to enrich the specific targets during initial PCR cycles and relies on universal forward and reverse SuperPrimers at high but unequal concentrations to achieve exponential but asymmetric amplification. The biotin-labeled reverse SuperPrimer, present at a concentration four times higher than that of the forward SuperPrimer, results in an excess of single-stranded reverse products that can be easily detected (Fig. 1). This assay was used successfully to evaluate a Digene-recommended algorithm for HPV low positive results present in a “retest zone” (10).

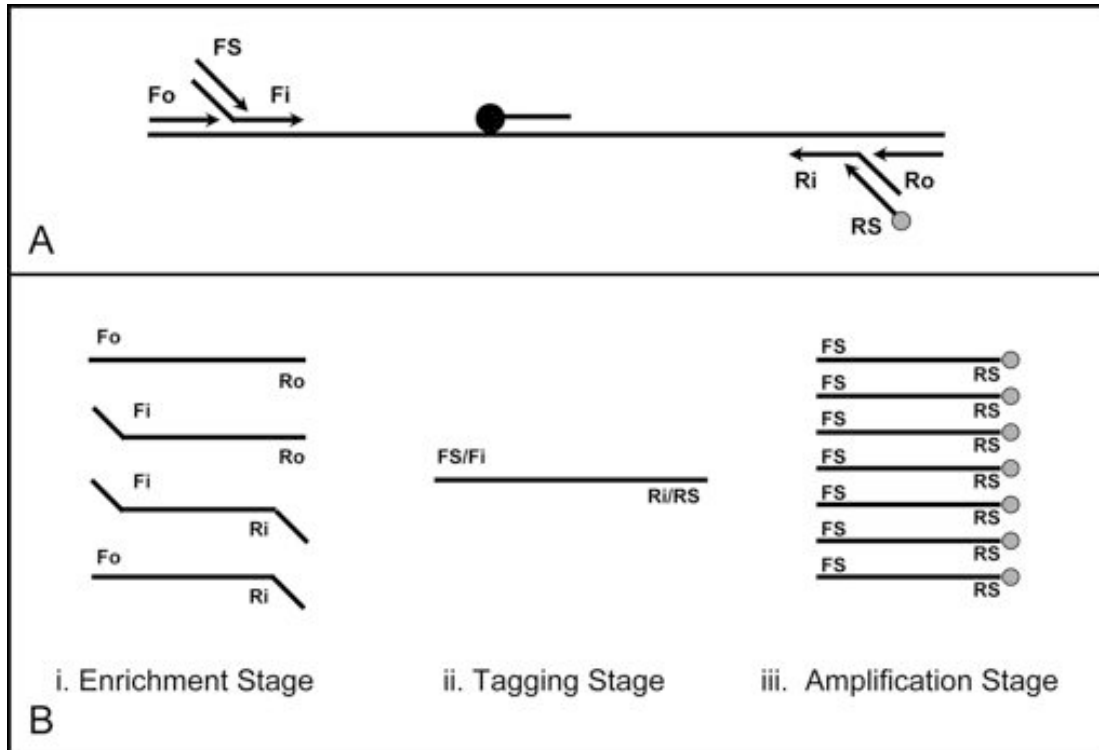
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We describe the Templex HPV assay that targets the highly variable E6 and E7 gene regions and includes type-specific primers for 25 commonly encountered HPV types, including 21 HR HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, and 82) and 4 HPV low-risk types (types 11, 6, 42, and 44). We determined the analytical sensitivity of the Templex assay for HPV types 16, 18, and 56 on the basis of pooled HPV-negative cervical specimens spiked with HPV clones. We evaluated the HR HPV detection on 109 cervical specimens previously tested with the Digene HC2 high-risk HPV DNA test assay. The Templex HPV typing I assay system requires only 2 hours of hands-on time and yields semiquantitative results for each type detected. The assay format is amenable to automation and clinical implementation and merits further evaluation and validation.

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AQ: J FIG. 1. Templex HPV amplification. In the Templex HPV assay, nested gene-specific primers are designed to enrich the targets during initial PCR cycling. Later, universal SuperPrimers are used to amplify all targets. Primer abbreviations: Fo, forward out; Fi, forward in; Ri, reverse in; Ro, reverse out; FS, forward SuperPrimer; RS, reverse SuperPrimer. The gene-specific primers (Fo, Fi, Ri, and Ro) are used at extremely low concentrations. Panel B shows that different primers are involved in the Templex process at each of the three major stages. First, at the “enrichment” stage, low-concentration gene-specific primers are given enough time to find the templates. For each intended target, depending on which primers are used, four possible products may be generated: Fo/Ro, Fi/Ro, Fi/Ri, and Fo/Ri. The enrichment stage is typically carried out for 10 cycles. In the second, “tagging” stage, by raising the annealing temperature to 72°C, only the long, 40-nucleotide inside primers (Fi and Ri) will work. After 10 cycles and at the end of this tagging stage, all PCR products are “tagged” with the universal SuperPrimer sequences. Then, at the third, “amplification” stage, high-concentration SuperPrimers work efficiently to amplify all targets and label the PCR products with biotin during the process.

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MATERIALS AND METHODS

HPV plasmid clones and cervical specimens. HPV DNA-containing plasmids for HPV-11, -16, -18, and -56 were purchased from American Tissue Culture Collection (Manassas, VA). To increase the number of HPV types evaluated, we selected residual anonymized DNA extracts from a variety of ongoing studies that were previously determined to be positive for single and multiple types included in the Templex HPV assay on the basis of L1 consensus PCR as previously described (14). We also retested excess anonymized material from 109 consecutive PreservCyt (Cytoc Corp., Marlborough, MA) cervical specimens submitted to the Molecular Infectious Diseases Laboratory at Vanderbilt University Medical Center for reflex HPV DNA testing using the Digene HC2 high-risk HPV DNA test. Three PreservCyt cervical specimens negative for HPV were pooled and included as negative controls in each run in triplicate.

Nucleic acid extraction. DNA from a 100- μ l aliquot of each residual resuspended PreservCyt cervical specimen was extracted using a spin column-based kit (E.Z.N.A. total RNA kit II; Omega-Biotek, Doraville, GA) as directed by the manufacturer. The final volume of DNA eluate in water was 50 μ l. Extracts were stored at -80°C until tested.

Templex HPV multiplex amplification. The Templex HPV typing I assay system (catalog number 018-01-S; Genaco Biomedical Products, Inc., Huntsville, AL) was used in a 50- μ l reaction mix composed of 6 μ l Templex HPV SuperPrimers (mixture of type-specific nested primers for the E6/E7 region of 25 HPV types, endogenous positive-control gene iduronate 2-sulfatase, and Templex

SuperPrimers), 25 μ l of QIAGEN multiplex master mix (QIAGEN Inc., Valencia, CA), 5 μ l of extracted DNA, and 14 μ l of water. Amplification was carried out by using the five-stage Templex cycling program: (i) hot start (15 min at 95°C), (ii) enrichment stage (10 cycles, with 1 cycle consisting of 30 seconds at 94°C, 1 min 30 seconds at 60°C, and 1 min at 72°C), (iii) tagging stage (10 cycles, with 1 cycle consisting of 30 seconds at 94°C and 1 min 30 seconds at 72°C), (iv) amplification stage (35 cycles, with 1 cycle consisting of 20 seconds at 94°C, 20 seconds at 55°C, and 20 seconds at 72°C), and (v) extension stage (3 min at 72°C).

Amplification product identification. The amplified products were further characterized using a suspension array for multiplex detection on a Luminex 100 instrument (Luminex, Austin, TX). In brief, 35 μ l hybridization buffer was mixed with 10 μ l bead mix (sequence-tagged beads specific for each HPV type and control gene [iduronate 2-sulfatase] in assay; Genaco Biomedical Products, Inc.), and 5 μ l PCR product was then added. The mixture was denatured at 98°C for 3 min and then hybridized at 52°C for 10 min. Ten microliters of streptavidin-PE was added into the mixture. After an additional incubation for 5 min, 120 μ l of prewarmed stopping buffer was added into the mixture, and results were read immediately using the Luminex instrument. A red laser identifies each bead (or target) by its color coding, while a green laser detects the hybridization signal associated with each bead. Results for each channel are expressed as the median fluorescence intensity (MFI) value. The cutoff value for a positive result in each channel was set at four times the mean MFI values of the negative controls.

Analytic sensitivity and semiquantitation. To determine the analytic sensitivity of the assay for HPV-16, -18, and -56, purified plasmid DNA for each type was diluted with water. The copy numbers for each HPV type were determined on the basis of the molecular weight of each of the plasmid. For example, the total molecular weight for the plasmid containing HPV type 16 insert is 7.06E + 0.6 g, which is about 85 copies per fg. Serial 10-fold dilutions ranging from 10⁵ to 2

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Sample	Known Type	146	148	156	151	133	135	139	145	151	152	153	156	158	159	166	167	168	169	170	173	182	18	191	142	144	105	
1	ALCOLO111D	147	56	60	48	54	60	48	50	76	89	59	64	57	82	85	61	68	55	60	63	61	63	63	60	50	54	
2	ALCOLO111D	67	1192	63	63	50	62	54	44	67	67	61	65	68	60	62	43	46	72	67	69	74	64	57	67	60	62	
3	ALCOLO111D	60	57	70	46	61	60	47	54	70	60	61	1385	63	56	39	58	53	54	69	52	57	71	64	48	66	61	
4		18	261	40	32	38	50	43	35	34	28	29	30	35	40	32	35	44	48	27	50	31	50	38	44	32	1807	
5		18	33	1536	20	31	33	28	30	60	16	28	23	31	35	22	32	27	37	28	22	25	39	21	27	1466		
6	18.26.51.20.82	33	928	3441	31	41	1024	30	28	883	34	28	72	34	34	27	33	33	18	22	20	4104	43	40	68	37	1267	
7		31	26	17	178	38	41	36	37	38	37	31	32	25	34	26	27	26	35	240	24	37	52	38	30	32	1157	
8	31.39	53	46	34	45	1144	42	283	29	46	38	28	45	40	40	39	39	40	41	38	29	35	27	38	33	38	1125	
9		35	38	22	25	30	35	515	31	24	30	26	27	29	28	28	19	32	27	33	44	32	30	28	37	24	794	
10		29	32	30	21	37	51	33	857	35	33	28	29	36	43	34	37	29	32	31	29	21	31	41	27	32	762	
11		45	29	76	31	37	33	31	41	2247	32	32	34	30	31	23	42	29	28	27	18	32	31	37	30	28	1445	
12		51	37	41	23	102	40	44	30	30	1293	37	32	32	27	20	27	21	29	31	41	29	28	34	40	38	843	
13		62	30	28	689	20	38	32	32	34	30	29	28	22	21	19	27	26	23	40	40	17	37	33	31	23	1179	
14		63	31	40	37	27	47	40	30	25	46	38	34	25	33	26	25	30	36	34	25	40	49	42	43	30	819	
15		64	42	57	25	29	48	38	50	29	59	59	31	2856	35	20	27	29	34	23	37	34	37	42	36	50	1713	
16		64.58	41	35	22	30	36	29	27	278	27	26	32	28	23	22	23	22	31	29	33	20	36	101	38	27	870	
17		69.44	39	34	28	46	42	39	39	30	29	28	26	33	1202	25	28	20	31	29	37	35	30	40	35	321	1438	
18	59.06	39	64	24	40	28	41	25	32	36	41	27	1330	45	20	2348	22	24	17	31	49	42	39	47	42	30	1628	
19		67	22	43	28	31	47	33	44	37	38	27	30	33	42	30	30	43	29	35	38	38	30	37	30	660		
20		68	42	30	23	30	31	38	42	28	27	24	34	29	18	21	37	1385	33	40	18	33	36	32	35	20	1280	
21	18.60.69	38	1277	21	30	172	32	28	42	23	35	37	2418	35	34	32	28	28	30	2988	44	50	25	24	40	28	30	1332
22		70	19	37	17	19	29	32	22	19	19	17	35	14	20	13	17	11	17	12	1626	23	28	32	29	18	82	1736
23		73	30	35	19	33	34	32	27	25	23	21	29	24	25	23	24	28	27	38	1168	27	30	25	29	29	832	
24		82	43	31	31	23	43	35	20	28	32	174	18	25	32	24	26	28	32	21	34	40	4025	25	26	28	27	1356
25		8	25	42	35	30	41	33	33	31	30	20	30	30	29	40	35	32	30	14	40	33	28	3450	34	40	39	1357
26		11	20	23	22	24	47	20	27	20	29	34	23	24	24	27	29	25	19	37	11	29	29	486	32	29	1366	
27	56.62	30	31	28	27	44	40	37	34	31	30	16	766	26	29	24	21	30	30	27	16	21	177	24	1463	22	1411	
28		44	29	31	23	24	36	31	26	27	29	30	32	35	32	30	29	31	17	42	41	38	189	43	40	2970	1830	
29	PCR Blank	29	28	23	15	47	31	24	20	21	20	27	24	33	27	24	23	22	23	21	17	16	20	20	34	31	25	92
	Cutoff	116	112	92	60	148	124	96	89	84	108	128	96	133	88	72	100	100	92	112	72	80	136	124	100	92	92	

FIG. 2. Representative data from the Tempex HPV assay on control plasmids and known HPV samples. Values are expressed as the median fluorescence intensity value from the Luminesx 100 instrument. Each row represents a sample, and each column represents a specific HPV type. The IDS target is an X-chromosome-coded gene (Iduronate 2-sulfatase gene) that serves as an internal positive control. The cutoff value for a positive result was set at four times the mean MFI values of the negative controls. The positive results are indicated in bold type on a pink background.

TABLE 1. Analytic sensitivity of the Templex assay for detecting HPV genotypes 16, 18, and 56

HPV genotype	No. of positive samples/no. of samples tested with the following no. of copies of HPV per reaction mixture:								Analytical sensitivity (no. of copies/reaction mixture)
	0	2	20	100	200	10 ³	10 ⁴	10 ⁵	
16	0/5	0/5	9/9	3/3	3/3	3/3	3/3	3/3	20
18	0/5	0/5	0/9	5/5	3/3	3/3	3/3	3/3	100
56	0/5	5/6	5/5	3/3	3/3	3/3	3/3	3/3	20

copies per reaction mixture were prepared. Three to nine replicates of each dilution were assayed independently. The limiting dilution was the one with the fewest copies of HPV that gave a positive result for all replicates. To demonstrate HPV type-independent amplification and relative quantitation of results, various amounts of HPV plasmid DNA were mixed to mimic coinfection with different HPV types at 10- to 100-fold differences in concentration.

Digene HC2 testing. Cervical specimen preparation and the Digene HC2 high-risk HPV DNA test using only the HR probe set were performed according to the manufacturer's instructions as described previously (17). For each specimen, relative light unit/cutoff (RLU/CO) values were calculated as the ratio of the specimen luminescence relative to the luminescence of the 1.0 pg/ml HPV-16 cutoff standard and reflect a semiquantitative value of the cumulative viral burden from one or more of 13 HR HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). A RLU/CO value of ≥ 2.5 was considered a HR HPV-positive result. Any RLU/CO values from 1.0 to 2.5 were retested. Specimens with a RLU/CO value of < 1.0 were considered negative (17).

L1 consensus PCR. Excess DNA previously evaluated for HPV was typed using the prototype linear array assay (reagents provided by Roche Molecular Diagnostics, Alameda, CA) as previously described (14). For evaluation of samples giving discordant results by the Digene and Templex HPV assays, we tested 5 μ l extract in the Roche linear array HPV genotyping test (Roche Molecular Systems, Pleasanton, CA) according to the manufacturer's instructions.

Statistical analysis. Statistical comparisons were performed with EpiInfo software (version 6; Centers for Disease Control and Prevention, Atlanta, GA). *P* values of ≤ 0.05 were considered statistically significant. Sensitivity, specificity,

and positive and negative predictive values were calculated using standard definitions (3).

RESULTS

Three ATCC HPV plasmids and 25 specimens with known HPV types were tested by the Templex HPV assay, and representative results are shown in Fig. 2. The HPV plasmid controls are positive for the expected HPV type but negative for the endogenous positive-control gene (no human DNA in samples). The 25 previously typed HPV samples gave the expected results and were positive for the control gene.

Using the HPV DNA-containing plasmids, we determined the analytical sensitivity of the Templex assay for detecting HPV types 16, 18, and 56. As shown in Table 1, the analytical sensitivities of the Templex assay for HPV types 16, 18, and 56 were 20, 100, and 20 copies/reaction mixture, respectively.

The Templex HPV assay relies on end-point PCR detection. It is recognized that end-point PCR has limited ability to achieve quantitative results, but relative quantitation is possible. In experiments using mixtures of input plasmid DNA templates with 10- to 100-fold differences in concentration, the resulting MFI values for each HPV type in the Templex reaction mixture faithfully reflected the relative concentrations of targets included in the reaction mixture. An examples of this is shown in Fig. 3 where different concentrations of HPV type 11, 16, and 18 plasmid DNA were mixed to mimic coinfections of multiple HPV types.

For the 109 samples evaluated for HR HPV with reflex Digene HC2 high-risk HPV DNA test, results of the Templex HPV assay were concordant in 104 samples (95.4%; 46 of 48 HC2-positive samples and 58 of 61 HC2-negative samples). The most frequent types detected in these samples were HPV-

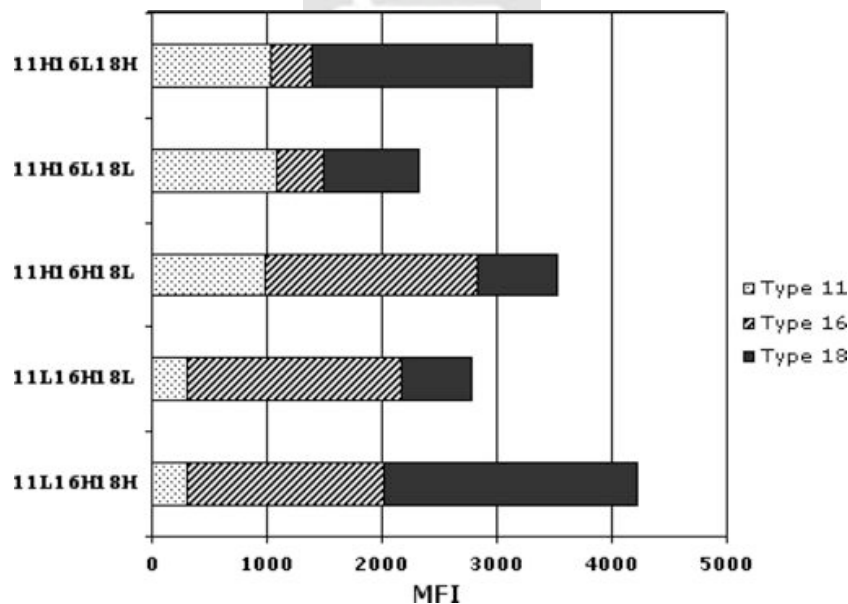


FIG. 3. Semiquantitative analysis with the Templex HPV assay. Relatively high and low concentrations of HPV types 11, 16, and 18 were mixed in different combinations to mimic coinfections of multiple HPV types. The high and low concentrations for HPV type 11 represent about 83,000 and 8,300 copies, respectively. For HPV type 16, the high and low concentrations represent 8,300 and 83 copies, respectively. For HPV type 18, the high and low concentrations represent 83,000 and 830 copies, respectively. The letter "H" or "L" indicates high or low concentrations, and the number "11," "16," or "18" stands for HPV type 11, 16, or 18.

TABLE 2. Roche linear array results for specimens with discrepant results for HR HPV by the Digene HC2 high-risk HPV DNA and Templex assays

Specimen no.	Assay result		
	H2C HR HPV ^a	Templex	Linear array
V16	Positive (112.8)	Negative	HPV-72 (low risk)
V46	Negative (0.2)	HPV-70	Negative
V57	Negative (0.3)	HPV-73	HPV-73
V76	Negative (0.2)	HPV-35	HPV-35
V103	Positive (14.8)	Negative	HPV-31 and -71 (low risk)

^a Numbers in parentheses are RLU/CO values.

16, -56, -39, -35, -66, -18, and -45, detected in 18.8%, 18.8%, 14.6%, 10.4%, 10.4%, 8.3%, and 8.3%, respectively. In addition, 29.2% of the positive samples were positive for more than two HPV types. The five specimens with discrepant HPV results were tested further with the Roche linear array assay (Table 2). For the HR HPV detection, the Templex assay result was concordant with the linear array result in three of five samples, whereas the Digene HC2 test result was concordant in two of five samples.

DISCUSSION

The Templex HPV assay simultaneously amplifies and identifies 25 HPV types in one reaction. Using HPV plasmid standards and previously typed clinical materials as controls, the assay shows excellent type specificity. The analytical sensitivity of the Templex assay was between 20 and 100 copies HPV per reaction mixture for each of the three types for which plasmid controls were available. Given the ability to use type-specific primers for each type, there is no reason to anticipate the efficiency of the amplification reaction to vary by type, so the analytic sensitivity should be comparable for all 25 types included in the assay. As demonstrated by the concordance (95.4%) with Digene HC2 high-risk HPV DNA test results in 109 cervical samples, the Templex assay can be used on clinical material.

The Templex HPV assay overcomes the most difficult problems associated with conventional multiplex PCR: incompatible primer sets and high background amplification/detection. Nested primers increase compatibility among loci. The primary problem with conventional multiplex PCR is the incompatibility among primer sets. A standard multiplex PCR is difficult to optimize because each amplification target has only one pair of primers that dictates one optimal annealing condition. If a particular target has PCR conditions that conflict with other targets, it will not amplify efficiently. Therefore, the range for "optimal" conditions, such as annealing temperature and salt concentration, is relatively narrow. In the Templex assay, given that nested primers are present for each target, four alternative products are generated to serve as templates for more efficient "super primer" amplification. Thus, the "optimal" range of temperature and salt concentration is wider, and a common amplification condition for all the intended targets is more attainable.

Low concentrations of unlabeled, gene-specific primers reduce background. High-concentration primers are required only at a much later stage of a PCR. During the initial cycles,

a much lower concentration is needed to give an adequate primer/target ratio. Despite the fact that more primers are used in the Templex HPV assay, the absolute primer amount is actually less than that in a regular multiplex PCR. The only primer that is labeled is the reverse super primer. Thus, signals caused by primer dimers and background amplification are less of a concern.

Use of low concentration, unlabeled primers may also improve reproducibility. The performance of conventional multiplex PCR varies from lot to lot and from different laboratories. Quality control for multiple sets of primers, especially those with biotin label, is difficult. With the Templex HPV assay, gene-specific primers are used at extremely low concentrations, and only one biotin-labeled primer is used; therefore, quality control for large-scale manufacturing is a more manageable task.

The Templex HPV technology does not require post-PCR cleanup, the kinetics of liquid hybridization are quite rapid, and results of the hybridization assay are read directly. These features result in an assay that requires only about 2 hours of hands-on time. The entire procedure is amenable to automation because only reagent addition is required. The rapid Luminex analysis platform can handle hundreds to thousands of samples per day. Luminex results are highly reproducible because the suspension arrays are manufactured and used in homogeneous environment. One potential problem with the Luminex platform are false-positive results caused by contamination of carryover PCR products. Careful control of the experimental environment is essential to prevent false-positive results. The Templex technology is an open platform that could theoretically be integrated with other high-throughput platforms for amplicon detection and identification.

The Templex HPV method also provides semiquantitative results for each type identified. With conventional multiplex PCR, the amplification efficiency may vary between types because of the complexity of the primer mix and sharing of primers between types. In the Templex format, only one pair of primers, the SuperPrimer, is used in the exponential phase of PCR amplification. Therefore, the end-point analysis of the signals generated from multiple targets reflects the ratio among the original targets. The clinical importance of infection with multiple types is increasingly recognized (20) and the ability to determine the type-specific profile of HPV-positive women will be important in evaluating the efficacy of HPV vaccine implementation.

The Templex HPV assay targets the E6/E7 region of the HPV genome. As expression of the E6 and E7 genes is directly associated with carcinogenesis, detection of these targets may provide more relevant biological and pathological information. E6 or E7 genes have been selected as amplification targets for HPV typing (16, 18), and studies have shown that in cervical cancer samples that were positive for E6, the L1 gene could not be detected in about 30% of the cases (8, 9). One potential disadvantage of targeting the E6/E7 region is the number of base pair variants in this region that could impact detection. Variants have been best studied in HPV-16, but to date, all HPV types studied have base pair changes in this region. Further work will be required to verify the independence of this assay from the impact of HPV variants.

This study evaluated only the Templex HPV system in com-

parison to detection of HR HPV in the Digene HC2 high-risk HPV DNA assay. Additional testing will be required to fully evaluate the type-specific sensitivity, specificity, and reproducibility in samples from a wide range of clinical settings.

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