Currently, human papillomavirus (HPV) DNA tests validated in large trials and epidemiological studies are the hybrid capture second-generation (HC2) HPV DNA assay and a variety of polymerase chain reaction (PCR) protocols employing degenerate or consensus primers. This article describes the currently available technology for HPV detection and discusses novel technologies and their potential for large-scale screening. Ideally, an HPV test should allow detection of multiple HPV types, identify individual types, and provide quantitative information about the viral load of each individual type found. Moreover, it should be easy to perform, be highly reproducible, with a high specificity and sensitivity, and amenable for high throughput analysis and automation. Because we do not yet fully understand the true value of viral load and the biological relevance of the different HPV types, any HPV test should be able to detect the clinically relevant high-risk types with a sufficient sensitivity of at least 10,000 genome copies per sample. To validate the different current and future test systems and to compare inter-laboratory performance we urgently need reference samples, validated reagents, and standardized protocols. [J Natl Cancer Inst Monogr 2003;31:80–8]

CURRENT TECHNOLOGY FOR HUMAN PAPILLOMAVIRUS DNA DETECTION OF GENITAL INFECTIONS

Currently, human papillomavirus (HPV) DNA tests validated in large trials and epidemiological studies are the hybrid capture second-generation (HC2) and polymerase chain reaction (PCR)-based methods employing either MY09/11 or GP5/6 consensus primers (Table 1). Current testing for the 13 high-risk types, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, which are represented by the high-risk hybridization mix (B) of HC2, is performed for cervical cancer screening, because they are the most prevalent types found in cervical cancer worldwide [(12); see chapters 13 and 14]. In addition, more recent studies have found types 66, 73, and MM4 (a novel type related to HPV82) to be present in squamous cell carcinomas of the cervix (13,13a), which might have to be included in future HPV test systems. It also should be noted, however, that the adaptation of the high-risk-type panel to geographically different HPV type prevalences might enhance the specificity of the test. It might be a proper solution for developing countries with bad or no surveillance programs in place to limit the number of high-risk HPV types screened for to the geographically most prevalent types found in cervical cancer instead of using the complete HC2 high-risk probe.

Hybrid Capture HPV DNA Assay

HC2 is based on hybridization in solution of long synthetic RNA probes complementary to the genomic sequence of 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and five low-risk (6, 11, 42, 43, 44) HPV types, which are used to prepare high (B) and low (A) probe cocktails that are used in two separate reactions. DNA present in the biological specimen is then hybridized in solution with each of the probe cocktails allowing the formation of specific HPV DNA-RNA hybrids. These hybrids are then captured by antibodies bound to the wells of a microtiter plate that recognize specifically RNA-DNA hybrids. After removal of excess antibodies and nonhybridized probes, the immobilized hybrids are detected by a series of reactions that give rise to a luminescent product that can be measured in a luminometer. The intensity of emitted light, expressed as relative light units, is proportional to the amount of target DNA present in the specimen, providing a semiquantitative measure of the viral load. The HC2 is currently available in a 96-well microplate format, is easy to perform in clinical settings, and is suitable for automation. Furthermore, HC2 does not require special facilities to avoid cross-contamination, because it does not rely on target amplification to achieve high sensitivity, as do PCR protocols. Often only the high-risk cocktail is used; this reduces time and cost of the test. The FDA-recommended cut-off value for test-positive results is 1.0 relative light units (equivalent to 1 pg HPV DNA per 1 ml of sampling buffer). Several studies have noted that the HC2’s high-risk probe cocktail cross-reacts with HPV types that are not represented in the probe mix (3,14,15). Peyton and colleagues (3) found that HC2 using the high-risk probe at a 1.0-pg/ml cut-off detected HPV types 53, 66, 67, 73, as well as other undefined types, and raising the cut-off to 10.0 pg/ml did not eliminate the cross-reactivity to types 53 and 67. Cross-reactivity of HC2 high-risk probe to HPV types that have a significant risk for cervical cancer may be considered beneficial, but cross-reaction with low-risk types causes false positive results and may decrease the specificity of the test (16).

PCR-Based Assays

HPV DNA can be selectively amplified by a series of reactions that lead to an exponential and reproducible increase in the viral sequences present in the biological specimen. Analysis of the amplified products can be done in different ways including gel electrophoresis, dot blot or line strip hybridization, and ultimately can be coupled to direct DNA sequencing. The sensitivity and specificity of PCR-based methods can vary, depending mainly on the primer sets, the size of the PCR product, reaction conditions and performance of the DNA polymerase used in the reaction, the spectrum of HPV DNA amplified and ability to detect multiple types. PCR can theoretically produce one million copies from a single double stranded DNA molecule after 30 cycles of amplification. Therefore, care must be taken to avoid false-positive results derived from cross-contaminated speci-
mens or reagents. Several procedures are available to avoid this potential problem in using PCR protocols for HPV DNA detection. Because of its versatility and very high sensitivity many PCR systems are available, but care must be taken for the lack of validation and comparison with established protocols like those described below.

The most widely used protocols employ consensus primers that are directed to a highly conserved region of the L1 gene, and are potentially capable of detecting all mucosal HPV types (17). Among these are the single pair of consensus primers GP5/6 (6,18) and its extended version GP5+/6+ (8) and the MY09/11 degenerate primers (2) and its modified version, PGMY09/11 (4,5). Full distinction of roughly 40 types can be achieved by hybridization with type-specific probes (4–8,10), that can be performed in different formats, including line strip assays and microtiter plates which are amenable to automation. Another pair of consensus primers is available that amplifies a smaller fragment (65 bp compared with 150 bp for the GP primers and 450 bp for MY09/11) of the L1 gene therefore potentially increasing the sensitivity of the assay. This SPF-PCR (10,11) is designed to discriminate a broad spectrum of HPVs by reverse line blot hybridization. However, because of the small size of the amplified fragment, one can anticipate reduced discrimination ability as compared with the other systems described, although the authors reported discrimination between 43 different HPV genotypes (10). The performance characteristics of these assays are presented in Table 1.

### Performance and Reproducibility of HPV DNA Tests

Several studies have carefully compared the performance of the available assays (Tables 2 and 3). In general, there is an excellent concordance with regard to sensitivities and specificities obtained in a large series of screening trials performed all over the world with the HC2 assay (Table 2). This underlines the importance of the availability of a commercial manufactured quality-controlled robust assay. Good agreement rates were also observed between PCR-based tests as MY09/11 and GP5+/6+ systems (Table 3). However, there are several conditions, such as the DNA extraction procedures, differences in sampling methods and sample transport/storage, and especially the use of different polymerases for the PCR reactions that can affect test performance, as recently reported by Castle et al. (27). Therefore, validated protocols, reagents, and reference samples need to be further developed and more generally used. Recently, a collaborative study has been launched by the World Health Organization to provide a tool for standardization of HPV DNA assays both for epidemiological studies and vaccine efficacy trials in different regions of the world. The goal is to prepare and characterize a panel of HPV DNA reference samples that would enable different laboratories to assess and compare the analytical performance of different HPV DNA tests.

### Table 1. Established HPV test technologies and performance characteristics*

<table>
<thead>
<tr>
<th>Hybridization</th>
<th>Designation</th>
<th>Reference</th>
<th>Probes/primers</th>
<th>Test reaction product</th>
<th>Analytical sensitivity, fg</th>
<th>No. of HPV types detectable</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>MY09/11 Dot blot</td>
<td>(2.3)</td>
<td>Degenerate primers</td>
<td>450 bp</td>
<td>0.1–100</td>
<td>39</td>
</tr>
<tr>
<td>PCR</td>
<td>PGMY09/11 reverse LBA</td>
<td>(4,5)</td>
<td>Mixture of consensus primers</td>
<td>150 bp</td>
<td>0.1–10</td>
<td>27</td>
</tr>
<tr>
<td>PCR</td>
<td>GP5+/GP6+ EIA</td>
<td>(6,7,8)</td>
<td>Consensus primers</td>
<td>450 bp</td>
<td>0.5–10</td>
<td>20</td>
</tr>
<tr>
<td>PCR</td>
<td>SPF-PCR reverse LiPA</td>
<td>(9)</td>
<td>Consensus primers</td>
<td>150 bp</td>
<td>0.5–10</td>
<td>37</td>
</tr>
<tr>
<td>PCR</td>
<td>SPF-PCR reverse LiPA</td>
<td>(10,11)</td>
<td>Mixture of consensus primers</td>
<td>65 bp</td>
<td>0.1–10</td>
<td>43</td>
</tr>
</tbody>
</table>

*HC2 = hybrid capture second-generation, HPV = human papillomavirus, LBA = line blot assay, LiPA = reverse hybridization line probe assay, PCR = polymerase chain reaction, SPF-PCR = short PCR fragment amplification and detection system.

### Table 2. Clinical performance data for HC2 HPV DNA assay compared with cervical cytology*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Objective</th>
<th>Country</th>
<th>Population</th>
<th>Disease threshold</th>
<th>HC2</th>
<th>Cervical cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td>(19)</td>
<td>ASCUS triage United States</td>
<td>957 women with ASCUS cytology</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>76.2</td>
<td>N/A</td>
</tr>
<tr>
<td>(20)</td>
<td>ASCUS triage United States</td>
<td>3488 women with ASCUS cytology</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>85.3</td>
<td>N/A</td>
</tr>
<tr>
<td>(21)</td>
<td>Screening France</td>
<td>1518 women—routine screening</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>85.3</td>
<td>94.9</td>
</tr>
<tr>
<td>(22)</td>
<td>Screening United Kingdom</td>
<td>1703 women aged &gt;35—routine screening</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>85.3</td>
<td>94.9</td>
</tr>
<tr>
<td>(23)</td>
<td>Screening South Africa</td>
<td>1365 previously unscreened women</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>85.3</td>
<td>94.9</td>
</tr>
<tr>
<td>(24)</td>
<td>Screening Costa Rica</td>
<td>1119 specimens</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>77.7</td>
<td>94.2</td>
</tr>
<tr>
<td>(24a)</td>
<td>Screening Germany</td>
<td>7908 women—routine screening</td>
<td>&gt;CIN2</td>
<td>&gt;=ASCUS</td>
<td>43.5</td>
<td>98.0</td>
</tr>
</tbody>
</table>

*ASCUS = atypical squamous cell of undetermined significance, CIN2 = cervical intra-epithelial lesion grade 2, HC2 = hybrid capture second-generation, HPV = human papillomavirus.
sensitivity and specificity of the assay in use. The results are still not available.

**Samples with multiple HPV types.** Studying the role of multiple infections in the development of high-grade cervical disease or cervical cancer has been hampered by the fact that the most frequently used HPV DNA test system, HC2, does not discriminate between different types in a multiple infection. Moreover, not all PCR-based methods perform equally well in detecting multiple infections because of limitations in the number of HPV types detectable and assay performance. It has been shown for example that the GP5+/6+ system detects only 47% of samples with multiple HPV types in comparison to 90% detected by MY09/11 PCR (26).

Another problem is the difference in sensitivity for distinctive HPV types between different test systems used and the reproducibility of different HPV tests for determining the exact HPV type in the sample (28,29).

In general, it seems that PCR systems using multiple primers such as PGMY09/11 and SPF-PCR are more robust to detect multiple infections than systems using single consensus primers such as GP5+/6+. This may especially be true in cases of mixed infections where one type is present in large amounts. The kinetics of the PCR reaction when using single primer pairs is then unfavorable for types present in smaller amounts.

Given that more accurate tools have now been developed for identifying multiple infections such as the reverse line blot assays, it would be worth establishing whether the presence of multiple infections/lesions would be a useful marker for persistent infection and disease onset or progression. Interestingly, Ho et al. (30), investigating the natural history of cervicovaginal papillomavirus infections in young women, defined an odds ratio of 4.1 (2.7–6.3 95% CI) associated with the presence of multiple types over a 6-month period for persistent HPV infections. Two other studies show, on the contrary, that persistence of HPV infection was independent of co-infection with other HPV types (31, 32).

**Viral load determination.** Viral load determination became a methodological challenge since it has been suggested that high copy numbers are correlated with increased risk of development of HPV-associated cervical lesions. HPV DNA quantification in the biological sample can be achieved by PCR-based methods or by HC2 assay in a rather semiquantitative way (33–38). Estimates of viral copy numbers depend directly on the total input of cells, and ultimately of DNA, in the test. Therefore, adjustment for cell load is an absolute requirement that is frequently not fulfilled, as is the case for HC2 and some PCR-based protocols. Currently, there is no consensus about the best method to quantify HPV in biological specimens. One of the most accurate and controlled assay available is Real-Time PCR (see below); but like any PCR-based methodology, it is subject to variations according to primer sequences, target DNA, detection method, and so on. Moreover, it requires expensive equipment and reagents. Nevertheless, its use in epidemiological studies seems to be warranted by the information provided. An alternative is a labor-intensive, Low Stringent-PCR with consensus primers (39) that has been shown to provide reliable information in epidemiological studies (40). It has been discussed that such methods with lower analytical sensitivities, including HC2, could be able to detect only “high” enough viral loads, which would be those clinically relevant (41,42). Together with the studies performed with Real-Time PCR, these studies provide an indication that high viral loads, at least for HPV 16, may provide a mechanism to distinguish between clinically relevant infections and those that are unlikely to progress. However, these results need to be extended and confirmed in further studies, including the dissection of differences in viral load by genotype. Unfortunately, the true clinical relevance of viral load measurement may continue to be clouded by sampling error in the collection of cervical specimens. Even with normalization to a cellular control, the lesion size and proportion of infected to normal cells will be extremely difficult to control for.
Real-time PCR. Recently, PCR protocols based on a 5′-exo-nuclease assay and real-time detection of the accumulation of fluorescence were developed. The release of fluorescence at each amplification cycle is directly proportional to the amount of amplicon generated and, therefore, it is considered to be an accurate method of estimating viral load. Moreover, the assay is designed to keep contamination to a minimum (43,44). The Taqman quantitative PCR system has been reported for assessing HPV viral load, while controlling for variation in the cellular content of the sample by quantification of the nuclear gene for beta-actin. Ylitalo and colleagues (45), using a nested case–control design, found that cases had consistently higher viral loads for HPV 16 than controls and that high viral loads could be detected up to 13 years before the diagnosis of cervical cancer. As such, women with high viral loads for HPV 16 had a 30 times greater relative risk compared with women who were HPV negative, and this increased risk was consistent over time. Importantly, this also applies to women under the age of 25, an age group that has a particularly high prevalence of HPV infection and in which a method to distinguish clinically relevant infections would be particularly valuable. A second study performed in the same population showed that 20% of the women with the highest viral loads for HPV 16 had a 60 times higher risk of developing carcinoma in situ than women who were HPV negative (46). In addition, a recent publication by Woodman et al. (47) investigating the natural history of cervical HPV infection in young women found that abnormal smears were significantly less likely to be associated with low-viral-load samples.

Recent developments exploit a multiplex format, including more than one HPV type as well as targeting a cellular gene, which controls for the amount of input DNA (47). Recently, a variation of real-time PCR that uses self-probing amplicons known as Scorpions has been suggested for detection, typing and quantification of more than 40 different HPV types in clinical samples (48). Although promising, the clinical application of methods based on real-time remains to be demonstrated.

Biological Specimen: Collection, Storage, and Processing

Sample quantity, quality and storage conditions, as well as DNA preparation procedures can affect the performance of different HPV test systems. Generally, tests that use no primary amplification step, like HC2, are less affected by most of these variables, whereas PCR-based procedures tolerate less well impurities because of their enzymatic nature. Therefore, it is desirable to use sample devices that allow the collection of a large cell sample, like a cytobrush, and storage/transport media that not only preserve cell morphology but also stabilize DNA as well as RNA.

Collection devices. Although a large variety of sample instruments for taking cervical swabs is available, preference should be given to devices that sample ecto- and endocervix at the same time, resulting in sufficient numbers of cells that are not sequestered by the instrument itself, as is the case for the cotton-tipped applicator.

Further development of devices for the self-collection of vaginal samples is warranted and field studies, especially in older women that do not comply with existing screening programs, in developed countries have to be performed (49,50). In addition, we need the development of special devices for sample taking on men. Studies have to be performed to define the sensitivity threshold when testing samples from males for HPV DNA.

Buffers and other transport media. Currently, there is a great interest in the development of collection media that could preserve cells and macromolecules including DNA, RNA, and proteins. The main reason is to be able to perform both morphological analysis and molecular tests from the very same specimen. Several methanol-based collection solutions are available with the advantage that each collection bottle has approximately 10–15 ml residual fluid after monolayer processing for cytological diagnosis. This amount of residual fluid should be sufficient for several molecular tests involving DNA, RNA, or immunohistochemical analysis of proteins performed in the residual cells. In fact, in some settings, reflex DNA test (HPV test from specimens obtained for cytology) from liquid cytology cervical specimens is already in use.

Lin et al. (51) have shown that the ThinPrep methanol fixation fluid-based collection of cervical cytological specimens preserves cellular contents that can be used shortly after collection for detailed molecular analysis of DNA (HPV detection by PCR and typing by DNA sequencing), RNA (expression of p53 and GAPDH by RT-PCR), and proteins (immunohistochemical analysis of CEA antigen). Most of the assays were conducted with samples stored for up to 30 days at room temperature, but it is possible that longer storage periods will still preserve the cellular contents. Tarkowski et al. (52) evaluated the recovery and detection of limiting amounts of high-risk HPV RNA from cells fixed in liquid-based cytology media (PreservCyt-fixed). Even after 1 year of storage at -20°C, RNA extracted from these cells was suitable in RT–PCR assay for HPV-16 E6-E7 oncogenic transcripts.

The new Universal collection medium (UCM) from Digene (Gaithersburg, MD) is a medium that is capable of preserving DNA, RNA, protein, as well as cell morphology. It is formulated to be directly compatible with molecular testing with minimal or no processing. It is not inhibitory for hybrid capture when placed directly into the reaction. For PCR there is a need to purify DNA in a spin column before the thermocycling. Preliminary date indicate that it is capable of preserving RNA for several weeks at room temperature, but further studies are required to demonstrate its utility. Ambion (Austin, TX) has a product named RNAlater Tissue collection: RNA stabilization solution that is an aqueous, nontoxic collection reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples. Cells or tissues can be collected into RNAlater and stored at room temperature for up to 1 week, at 4°C for up to 1 month, or at −20°C indefinitely. In our experience, it works very well for tissues, not only for RNA studies but also in histopathological diagnosis (better tissue preservation than formalin fixed). However, there are no reports of the use of this medium to extract RNA from smears or tissue culture cells. Moreover, this medium is not suitable for protein studies.

Importantly, there is a clear need for intensified public funding to support validation studies of different transport mediums and collection devices instead of relying on information obtained and delivered by industry as it is mostly the case at present.

Archival specimens. Archival specimens, including fixed smears and paraffin-embedded tissues, constitute an important source of materials for epidemiological retrospective studies. However, it is well known that time of fixation and the type of fixative used can considerably affect the quality of the extracted nucleic acids. Degradation of DNA and RNA is the most com-
mon type of damage. Formation of adducts and crosslinks with proteins can also occur, which directly interfere with any enzymatic or hybridization reaction. Two HPV detection methods can be considered suitable for archival specimens: in situ hybridization and PCR targeting a short region of the genes of interest. The sensitivities of these methods differ widely but, in general, PCR is more sensitive and reproducible. It is very difficult to compare the results obtained in different series, because of the diversity of protocols employed, both for DNA recovery and amplification reaction, and specimen preservation. The method of recovering DNA from the smear is an important determinant of successful amplification of HPV DNA (53). In addition, procedures such as laser microdissection of target cells from archival smears and tissues are currently widely used and strongly recommended to increase the test sensitivity.

A few studies have detected HPV in archival smears from epidemiological studies, mostly by using a consensus PCR. Detection of high-risk HPV in archival smears was suggested as a means to reduce the rate of false negatives by cytology (54). McGoogan et al. (55) compared the performance of high-risk-HC2 assay with a consensus PCR in archival smears, and concluded that both methods could be used, but that PCR was more sensitive. The utility of archival material for longitudinal studies of HPV presence was further corroborated by studies performed in Greenland and Denmark (56) and in a prospective study conducted in Sweden (45). The findings show the value of using archival specimens in epidemiological studies provided that optimal conditions for morphological classification, DNA extraction, and gene amplification are established. In our experience this is not an easy task to accomplish.

Novel HPV Technologies for Genital Samples

PGMY09/11 System

The PGMY09/11 primer system was developed by Gravitt et al. (4) to address some limitations in the traditional MY09/11 degenerate primer system. The demonstrated sensitivity for the PGMY09/11 primer system is 10 HPV genomes per PCR amplification for all representative genotypes. PGMY09/11 is comprised of two non-degenerate pools of oligonucleotide primers designed to amplify the same 450 bp region of the L1 gene as the original MY09/11 primers. Members of the primer pools were chosen using sequence alignments of all known genital HPV types and minimizing any potential mismatches while simultaneously minimizing the number of oligonucleotides in each pool. The upstream PGMY11 primer pool is composed of five oligonucleotides, whereas the PGMY09 pool contains 13. The PGMY primer system was evaluated using a reverse line-blot assay (5), which includes probes for 27 different HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, MM4 [Novel HPV types: MM4 related to HPV62; MM7 to HPV83; MM8 to HPV84; MM9 to HPV73], MM7, MM9 and 6, 11, 40, 42, 53, 54, 57, 56, MM8) along with two human beta-globin control lines. This reverse line-blot system is currently being expanded to include 12 additional genital HPV genotypes (61, 62, 64, 67, 69, 70, 71, 72, CP6108 [Novel HPV types: CP6108 related to HPV91; CP8304 related to HPV62; IS39 related to HPV82] CP8304, IS39) described by Peyton et al. (15). In the preliminary comparison of PGMY09/11 with the MY09/11 primer system, there was an overall agreement of 91.5%; however, the PGMY system picked up significantly more HPV positives. Among the 247 samples examined, there was an HPV prevalence of 62.8% using the PGMY09/11 compared with a prevalence of 55.1% with the MY09/11 primer pair. There was also a profound increase in the detection of multiple infections using the PGMY09/11 primers, and in the amplification of certain HPV types that are inefficiently detected using MY09/11 primers. This phenomenon has been observed in other studies where certain HPV genotypes are amplified with higher efficiency using PGMY09/11 compared to the MY09/11 (57). HPV typing is performed using a reverse hybridization line blot assay, which is based on the hybridization of the ampiclon to specific DNA probes that have been immobilized on nitrocellulose or nylon strips. To provide type discrimination, probes for specific HPV types are bound to the strip in individual parallel lines at defined positions and ampiclon hybridization at a particular position thereby identifies the type. To detect hybridization, PCR amplification must be undertaken with primers having an attached biotin molecule, which becomes incorporated into the ampiclon. The ampiclon is applied to the strips under conditions that allow for specific hybridization with the immobilized probe. Once this has occurred, the presence of the ampiclon is detected with an alkaline phosphatase-labeled streptavidin conjugate, whereby the streptavidin binds to the biotin on the ampiclon and immobilizes the alkaline phosphatase in the specific region where hybridization has occurred. The alkaline phosphatase catalyzes color formation upon addition of a substrate and a colored line develops where ampiclon has hybridized to a specific probe. Measuring the position of the colored line relative to an established base line allows the type to be identified.

GP5+/GP6+ System

Recently, a reverse line blot typing assay for the GP5+/6+ system capable of typing 37 mucosotropic HPVs has been developed, allowing for high-throughput testing both in epidemiological and clinical research (8).

SPF-PCR System (Innogenetics)

Kleter and colleagues (10) developed a short PCR fragment amplification and detection system (SPF-PCR). A novel set of primers (SPF1/2) was designed to target highly conserved regions of the HPV L1 gene and thereby allow for amplification of a broad spectrum of HPV types. Given that PCR amplification efficiency is, in general, inversely related to the size of the region amplified, the SPF1/2 primers target a small region of only 65 bp, which should enhance sensitivity. However, because of the small size of the fragment, type discrimination is more complex.

Amplicor MWP (Roche Molecular Diagnostics)

This assay is based on a nondegenerate pool of primers to amplify a short fragment of the L1 gene of 13 high-risk genotypes (170 bp, compared with the 450 bp obtained with PGMY09/11). The ampiclon is immobilized using a pool of capture molecules bound to the wells of a microtitre well plate (MWP) and visualized by colorimetric detection by Roche Amplicor chemistry. Moreover, the new test was developed to employ the TaqGold DNA polymerase, which minimizes the amount of nonspecific amplification and increases the sensitivity of the test. Because it amplifies a shorter fragment it is considered to be more sensitive and amenable for less-preserved specimens; it was reported that these primers detect about 13% more
HPV in cervical smears than the PGMY09/11 primers (Janet Kormegay, personal communication). However, because the new primers were designed for high risk types, this test is not truly generic. An automated system, completely hands-off probes, is under development with a capacity of performing 96 samples a day.

Hybrid Capture-3 (Digene Company)

The newly developed HC-3 assay (58) uses RNA probes as in HC-2, but in combination with biotinylated capture oligonucleotides that are directed to unique sequence regions within the desired target. These oligonucleotides are only used for capturing the desired target sequence into streptavidine-coated wells of a microtiter plate. Signals are generated by RNA probes that hybridize to other regions of the captured sequences as the DNA capture oligonucleotides. Moreover, the assay has been further developed to reduce unspecific hybridization by using “blocker oligonucleotides” (unlabeled DNA molecules that are complementary to the biotinylated capture oligonucleotides), aiming at eliminate cross-reactivity while maintaining specificity. The same assay can in principle be used either for DNA or for RNA targets. Using this technique will also make it possible to test for molecular variants of certain HPV types because even targets with single nucleotide exchanges can specifically be detected.

Rapid Capture System (Digene Company)

For high-volume laboratory testing, Digene has described the development of an automated robotic platform for hybrid capture called the Rapid Capture System (RCS; 58) that allows robotic handling of 96-well microplates. This robot station performs incubations, shakings, and washings. However, the denaturation of specimens in the sample device tubes still has to be performed by hand. This automatic station shall allow a single user to test 450 specimens in an 8-hour shift.

HPV Genotyping Chips (BioMedlab Company)

BioMedlab Company (Seoul, Korea) has developed an HPV Oligonucleotide Microarray-based detection system of HPV types that currently allows the detection of 22 HPV types, by immobilizing HPV type-specific oligonucleotide probes and a control (beta-globin probe) on an aldehyde-derivatized slide glass. Target DNA is submitted to a standard PCR in the presence of fluoresceinated nucleotides (Cy5 or Cy3) employing primers for both the beta-globin (PC03/04) and for the L1 region (modified Gp5/6 primers) of several HPV types. Randomly labeled PCR products are then hybridized onto the chip, which is then scanned by laser fluorescence. In the case of multiple infections, multiple hybridization signals can be seen. The utility and practicability of this method remains to be demonstrated, although preliminary data (T. Ifter, personal communication) demonstrated high sensitivity and a high detection rate for samples with multiple HPV types.

Ideally, a larger number of HPV type-specific oligonucleotides could be spotted easily on the Chip. In principle, such a system also allows semiquantitative analysis enabling at the same time viral load analysis and HPV typing. However, signal detection in microarrays is subject to variation, requires some sort of input DNA normalization, and in its present format requires expensive equipment for signal detection, but it certainly has a large potential for further development.

HPV Serological Assay

Overall, frequency and titer of several types of serum antibodies generated against HPV show a great variability that is dependent on the HPV type specificity, on the recognized epitopes, on the type of samples, and on the sensitivity of the assay. Anti-HPV humoral immune responses are generally measured by enzyme-linked immunosorbent assay (ELISA) with HPV type-specific virus-like particles (VLPs) adsorbed in the plates. Recombinant HPV proteins and peptides are also used in ELISA tests (59). Currently, several assays are available that exploit different formats including ‘sandwich’ ELISA and radioimmunnoasays (60,61). These assays have been developed to detect with high specificity antibodies against the early proteins E6 and E7. Assay conditions may vary considerably and most published studies do not provide information about standardization and quality control. The mere definition of cut-off values can hinder comparison between different epidemiological surveys. However, even an optimized HPV VLP-specific ELISA (62,63) will still rely on the availability of an expensive and unstable antigen (the native recombinant virus-like particle or VLP). Therefore, it is highly desirable to develop alternative antigens for HPV serology as well as to validate positive and negative sera controls.

Several studies have shown that serological diagnosis of HPV infection using genetically engineered HPV capsids (also known as virus-like particles or VLP) correlate well with HPV DNA presence in cervical smears. The antibodies invoked recognize type-specific conformational epitopes present on VLPs, and the humoral response (IgG) against HPV is stable over time. Moreover, HPV VLP ELISAs show sensitivities between 50 and 60%, very high specificities (>90%), and good interlaboratory agreement (64). Therefore, VLP serology has been used as a marker of cumulative exposure to HPV and of sexual behavior. However, it has been shown that seroconversion may be delayed or never occur in a subset of women testing positive for HPV DNA. These data originate from several studies including epidemiological surveys [reviewed in (59)].

The modest antibody responses measured in several studies may reflect lack of sensitivity of the assay or a deficient immune response to HPV, particularly in the case of cancer series where integration of HPV genomes impairs the expression of capsid antigens. Moreover, a weak immune response is often observed in HPV infections because these viruses do not cause viremia. As a consequence, one would expect misclassification as an important problem when considering VLP serology to estimate the cumulative prevalence of HPV infection. Nevertheless, stronger positive associations have been described for tumors of the anogenital region when compared with seropositivity in patients with epithelial cancers in other anatomical locations, which is consistent with the HPV DNA evidence (65,66). Moreover, high titers of antibodies have been measured in women who have received HPV VLP vaccines (67,68). Another source of misclassification could originate from different viral loads present in lesions (66) or a differential immune response according to anatomical site (69). In patients with head and neck tumors, it was found that among those seropositive all but one had antibodies against HPV16 early proteins (70).

Concerning type specificity, it has been demonstrated that VLPs from each HPV type induce serum antibody response that is genotypest-specific, with the exception of HPV types 6 and 11, which are cross-reactive, and HPV 31 and 45, which show low levels of cross-reactive antibodies against HPV 33 and 18, re-
spectively (71). Likewise, variants of HPV 16 have been demonstrated to belong to the same serotype (72). Most HPV sero-prevalence studies have measured serum antibodies against a single HPV type, mostly against HPV-16 VLPs. Others have tested sera from both healthy women and cancer patients for anti-VLP antibodies for HPV types 16, 18, 31, and 58 (73) or 16, 18, 31, 33, 39, 58, and 59 (74). Cross-reactivity was observed between HPV responses against 16 and 31, and between 58 and 18, 45 and 59.

It has been suggested that a search of immunoglobulins in genital secretions would provide a better indicator of HPV infection, but reproducibility and standardization of the detection methods are unresolved (75,76).

**References**


