Human Papillomavirus Testing Methods

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• Testing for human papillomavirus (HPV) relies exclusively on techniques of molecular biology using nucleic acid probes. Tests for HPV using nucleic acid probes have been commercially available since the late 1980s, but early tests were cumbersome, involving the use of nucleic acid probes labeled with radioactive phosphorus (^{32}P). These early HPV tests did not achieve widespread use because they did not detect all oncogenic HPV genotypes. The current commercial HPV detection kit, Digene's Hybrid Capture 2 kit, detects virtually all high-risk oncogenic HPV types, as well as most low-risk nononcogenic HPV genotypes. The Hybrid Capture 2 test format is a proprietary nucleic acid hybridization signal amplification system owned by Digene Corporation. Virtually all test formats for DNA sequence analysis are amenable to applications intended to detect and perhaps quantify the various HPV genotypes. These methods can involve direct hybridization with complementary DNA probes, such as Southern blotting or in situ hybridization, signal amplification, such as the Hybrid Capture 2 method or target nucleic acid amplification, most notably the polymerase chain reaction (PCR). Polymerase chain reaction has been used for HPV detection, genotyping, and viral load determination. General or consensus primer–mediated PCR assays have enabled screening for a broad spectrum of HPV types in clinical specimens using a single PCR reaction. Following amplification using consensus primers, individual HPV genotypes are identified using a variety of methods. Using consensus primers in a test format known as real-time quantitative PCR (RQ-PCR), it is possible to generate viral load (concentration) data from reaction curves generated by monitoring PCR reaction kinetics in real time.

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Members of the human papillomavirus (HPV) family do not lend themselves to culture in vitro; as such, detection of HPV relies strictly on molecular analyses of HPV DNA sequence. A variety of molecular methods exist for use in detection and quantitation of HPV. One of these, the Hybrid Capture 2 (HC2) test manufactured by Digene Corporation, Inc (Gaithersburg, Md), is the only HPV test cleared by the Food and Drug Administration for in vitro diagnostic use. The Digene HPV HC2 test is approved in the United States as an adjunct to the Papanicolaou test for cervical cancer screening and is being marketed in selected countries as a primary cervical cancer screen either in conjunction with or separate from the Papanicolaou test.

All molecular methods used in analysis of HPV in clinical specimens draw on the same principles and techniques of molecular biology used in the discovery of the extended HPV gene family, and while most of these techniques were developed in a research setting, there is a good possibility that they will eventually make their way into the molecular diagnostic laboratory.

Most HPV genotypes have nucleic acid sequences that exist within the public domain, but a few of the HPV types are patented. As such, commercial use of these proprietary sequences in a diagnostic test is prohibited unless the patent owner grants a license. Until recently, Digene's exclusive rights to many of the high-risk HPV sequences have limited commercialization of HPV tests intended for in vitro diagnostic use. However, the beginnings of what may prove to be a major change in the HPV testing landscape began in June 2002 when l'Institut Pasteur in France, the owner of many HPV sequences, transferred to F. Hoffmann-La Roche Ltd (Roche) the HPV intellectual property estate of l'Institut Pasteur. This exchange of HPV intellectual property to the sister company of Roche Molecular Systems, the owner of polymerase chain reaction (PCR) technology, may portend the future debut of PCR-based tests intended for in vitro diagnostic use.

HISTORY OF HPV TESTING

Tests for HPV using nucleic acid probes have been commercially available since the late 1980s, but early tests were quite cumbersome, in most cases involving the use of nucleic acid probes labeled with radioactive phosphorus (^{32}P) in a slot-blot hybridization format. These early HPV tests (ViraPap and ViraType), developed and marketed first by Life Technologies, Inc, then sold to Digene Corporation, did not achieve widespread use, due in large part to the reality that the tests did not detect all oncogenic HPV genotypes and the test format used lacked sensitivity.

In addition to the methods involving the use of ^{32}P-labeled nucleic acid probes, a variety of tests involving nucleic acid in situ hybridization (ISH) were commercially available in the late 1980s with kits manufactured by Enzo Diagnostics and Life Technologies, Inc. There has since been a recent resurgence of interest in ISH with the advent of automated platforms offered by Ventana Medical Systems, Inc; Dako Corporation; and others.

The current commercial HPV detection kit, Digene's HC2 kit, detects virtually all high-risk oncogenic HPV types, as well as most low-risk nononcogenic HPV genotypes. Within the HC2 test system, genotype-specific probes are mixed in high-risk and low-risk cocktail for-

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Attributes of Nucleic Acid Analysis Methods		
Method	**Strengths**	**Weaknesses**
Direct probe	Southern blot is gold standard for HPV genomic analysis Presence of HPV in association with morphology	Low sensitivity, time-consuming, may require large amounts of highly purified DNA Southern blot hybridization cannot use fixed tissue where DNA degradation has occurred
Signal amplification	Commercial kits Quantitative FDA-approved test (HC2)	Licensed and patented technologies Cocktail approach to genotyping
Target amplification	Flexible technology (viral load and genotype) Very high sensitivity Multiplex analysis	RUO testing only Home brew, no standardization Licensed by Roche for PCR Contamination with previously amplified material can lead to false positives

* HPV indicates human papillomavirus; FDA, Food and Drug Administration; RUO, research use only; and PCR, polymerase chain reaction.

mats. The HC2 is a nonradioactive, chemiluminescence method that can be performed in most clinical laboratories capable of high-complexity testing.

HPV TESTING METHODOLOGIES

There are essentially 3 types of nucleic acid hybridization method formats used to detect HPV, these being the direct nucleic acid probe methods, hybridization signal amplification, and target amplification methods. Individual attributes of these nucleic hybridization methods are given in the Table.

Direct Probe Methods

The gold standard technique for HPV genomic analysis is the Southern blot, a technique that was used in early studies of HPV.[1–3] Another direct probe method, ISH, uses technology akin to immunohistochemistry, in which one assesses antigenic expression within the context of histopathology.[3–8] With ISH, one assesses the presence of a target nucleic acid or gene expression within the context of histopathology. The nucleic acid probes used in ISH are derivatized, typically with biotin, in multiple sites. Detection is usually achieved using a sandwich approach involving streptavidin-chromogen complexes.[6,8]

The disadvantages of direct probe assays, however, include low sensitivity, time-consuming techniques, and the need for possibly large amounts of highly purified DNA. In fixed tissue, formalin-catalyzed DNA cross-linking resulting in DNA degradation makes Southern blot hybridization or restriction fragment length polymorphism (RFLP) impossible to perform.[6,9] Of the direct probe methods, ISH affords the lowest specificity for the detection of HPV sequences in clinical specimens, with an average specificity of 72% for condylomatous lesions and 30% for invasive cancer cells.[4]

Signal Amplification

Signal amplification methods are proprietary technologies that are not in the public domain. Digene's HC2 test is included in this category. Signal amplification methods are really an extension of direct probe techniques that have achieved a sensitivity boost by innovations in detection methods. These methods increase sensitivity by multimeric layering of reporter molecules on DNA probes.[6,9] Another example of signal amplification is found in the branched DNA (bDNA) assay, which is a proprietary technology owned by Bayer Corporation.

In the HC2 assay, specific RNA probes are used that are directed toward individual DNA sequences comprising the HPV genotypes to be detected. Digene owns a proprietary antibody that is directed toward DNA-RNA hybrids. The antibody is used both for the capture step and the detection step in which the antibody is labeled with a reporter molecule that is developed using a chemiluminescence detection system; in essence, the HC2 assay is an immunoassay.

Based on the strength of their association with cervical cancer, HPV genotypes detected by the HC2 assay can be grouped into the following risk strata: low-risk HPV types 6, 11, 42, 43, and 44; and high/intermediate-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The cocktail approach of the HPV HC2 test provides an excellent tool for the triage of patients with minor cytologic abnormalities on Papanicolaou tests, but the test cannot determine the specific HPV type present.

Target Amplification

Target nucleic acid amplifications, most notably PCR, are technologies that hold great promise for addressing all aspects of HPV testing.[2,10–12] Polymerase chain reaction is a valuable tool because it allows in vitro multiplication of unique regions of DNA so they can be detected within a large background, as is the case with most viral infections.

Target amplification is the most flexible and sensitive of all DNA analysis techniques. This technology can be used for detection, viral load quantitation, DNA sequencing, and mutation analysis.[13–21] These assays can also be performed in multiplex, whereby multiple target DNA sequences can be analyzed simultaneously. Target amplification methods increase sensitivity by specific, in vitro synthesis of target DNA sequences. Most PCR methods, while generally available for research use only testing, often involve the use of patented HPV sequences that limits their applicability as in vitro diagnostic tests due to legal or proprietary restrictions. Additionally, homebrew PCR tests are, by their very nature, lacking in interlaboratory standardization. Polymerase chain reaction assays by their nature are subject to environmental contamination because previously amplified material (amplicons) can potentially contaminate negative specimens such that false-positive results could be obtained. In almost all laboratories, this possibility is obviated by the use of stringent amplicon-containment procedures coupled with enzymatic amplicon elimination systems involving the uracil-*N*-glycosylase (AMPerase) enzyme.[6]

SENSITIVITY AND SPECIFICITY

The sensitivity level of an assay is usually defined as the detection limit or the lowest possible quantity of DNA

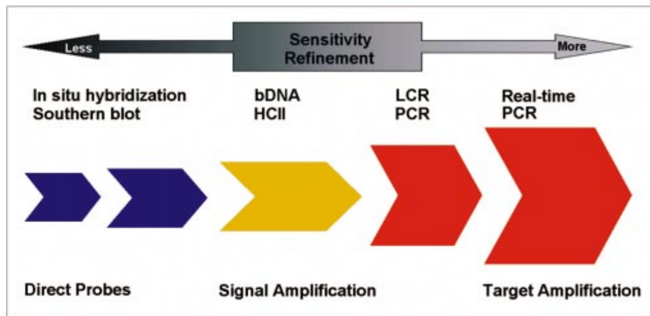

Figure 1. *Relative sensitivities of nucleic acid analysis techniques.*

available that can be detected. Specificity determines the level of accuracy of an assay. Ideally, achieving minimal false positives and false negatives increases the accuracy of an assay. Positive predictive value is the probability that the test method will give a true positive result. Likewise, negative predictive value is the probability that the test method will give a true-negative result. Any diagnostic assay should aspire to having the highest positive and negative predictive values. The sensitivity and specificity of an individual assay determine these values.

A test is said to have a particular sensitivity and specificity; however, there is not a single sensitivity or specificity for a test, rather a continuum of sensitivities and specificities. By varying the decision threshold, any sensitivity can be obtained from 0% to 100%, and each one will have a corresponding specificity. The ideal can be based on the clinical expectations for the test.

Figure 1 illustrates the relative hierarchy of sensitivities of direct probe methods and signal and target amplification methods. Direct probe techniques offer the least sensitivity for detecting a specific DNA sequence, whereas the highest sensitivity is achieved with target amplification.[2,4,22] Real-time quantitative PCR (RQ-PCR) is the most sensitive target amplification method.[14,23] To put sensitivity levels into perspective, the HC2 assay has a lower detection limit of 1.0 pg of target/mL; this concentration translates to approximately 120 000 virus particles.[24] The theoretical lower detection limit of RQ-PCR is 1 target nucleic acid sequence, but the practical lower limit is probably somewhat higher.

The ideal molecular diagnostic test should be able to differentiate a target sequence at the single nucleotide level, an attribute that is very important if one wishes to analyze mutations, for example. Target amplification techniques, RQ-PCR and direct PCR cycle sequencing, are also the most selective, being capable of discriminating minor sequence differences within a target DNA. Lower-selectivity test formats have difficulty differentiating between similar target sequences that have a high degree of sequence homology, as is the case with members of the HPV family. Direct probe tests and signal amplification tests are therefore prone to cross-reactivity or cross-hybridization.

Owing to varying degrees of sequence homology, probe cross-reactivity has been observed with the HC2 assay. pBR322 is an engineered cloning vector that would not be present in any clinical setting, but this example points to the possibility that a naturally occurring bacterial plasmid in high concentration can cause a cross-hybridization. The documented cross-reactivity between HPV-6 and HPV-42 could lead to the mistaken categorization of a specimen as positive for a high-risk type in situations of high concentration of the low-risk genotype HPV.[24]

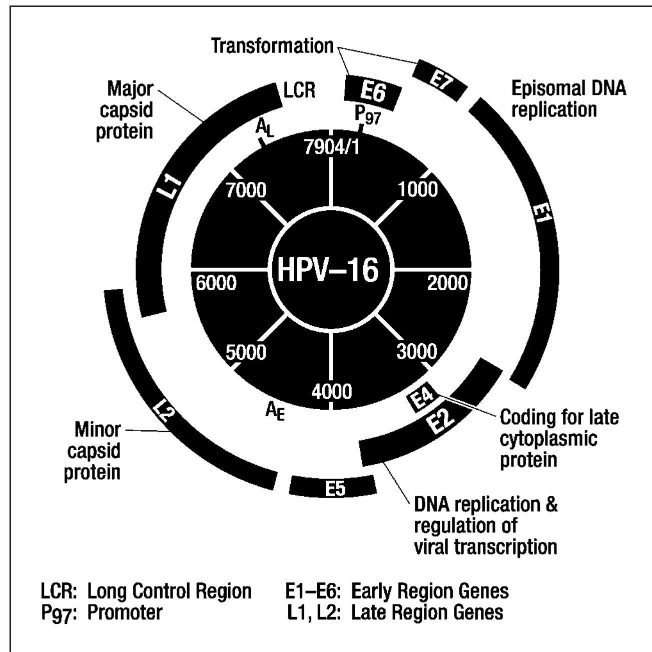

Figure 2. *Genomic map of human papillomavirus 16.*

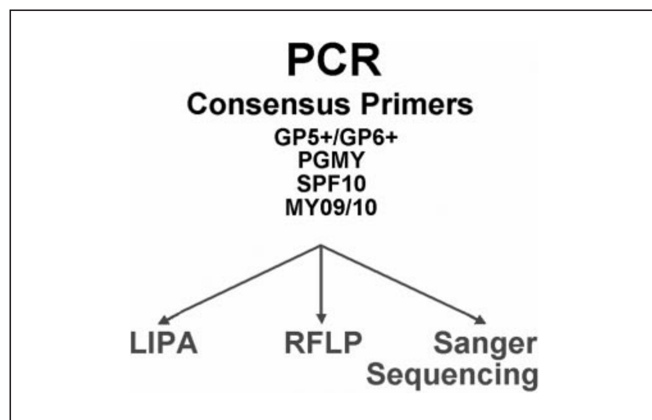

Figure 3. *Human papillomavirus genotyping strategy.*

LABORATORY METHODS USED TO DETERMINE HPV GENOTYPES

The vast majority of clinical data derived from HPV testing have been acquired using the commercially available HC2 assay, which categorizes patients with HPV infection into low-risk and high-risk groups.[24–30] Although these test results have been shown to be extremely useful triage tools, they do not provide specific information regarding which HPV genotypes are present within a specimen. Since the relative risk of most phylogenetically high-risk types for cervical disease outcomes is unknown, and given the finding that some HPV genotypes have been shown to differ in oncogenic potential, it is reasonable to assume that detection of individual HPV genotypes in cervical specimens could assist in more precise risk stratification.

Figure 2 is a genomic map of HPV-16, which could be considered a prototypical genome of all HPV genotypes. Of interest for genotype analysis are those areas that display the type-specific polymorphism. Several regions of

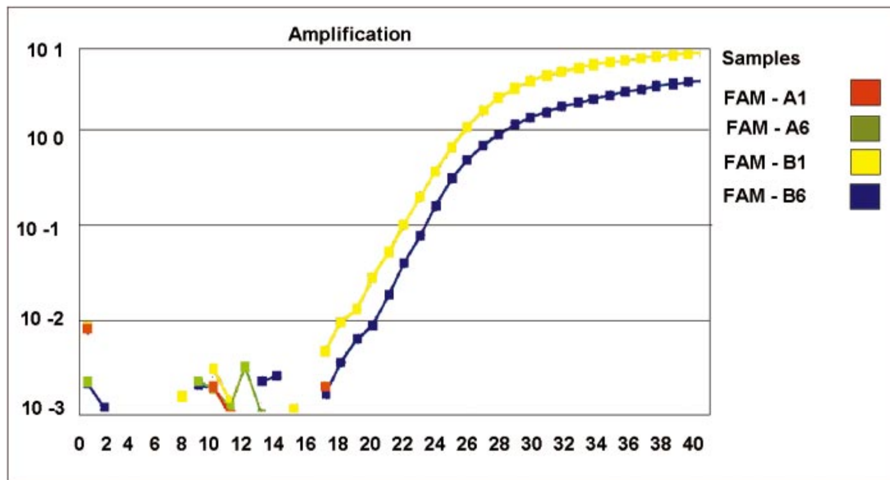

Figure 4. *Real-time quantitative polymerase chain reaction.*

type-specific sequence variability exist, but the late region gene *L1* that encodes a major capsid protein appears to be the most polymorphic.[13,15,20,31–34] In addition, sequence polymorphism exists in the early region genes *E6* and *E7*.[21,33,35–37] These sequences' differences endow individual HPV types with varying degrees of oncogenic transformation potential, and they can also be exploited for developing type-specific molecular tests. The *L1* gene segment is used most often in genotyping assays based on PCR.[13,20,34] Primers are selected from conserved or consensus sequences that flank polymorphic, type-specific sequences.

Polymerase chain reaction is the gateway technology to all genotyping methods. Once PCR using consensus primers has been performed, detection of individual genotypes can be accomplished by several methods, including RFLP,[13,34,38,39] reverse hybridization using what is known as a line probe assay,[40–42] or cycle sequencing and assignment of genotypes by sequence comparison.[35,43–45] An alternative, however, is to use genotype-specific PCR primers that are used to identify individual HPV types based on *E6* or *E7* polymorphisms.[32,36,37,46,47]

General or consensus primer-mediated PCR assays have been developed to screen for a broad spectrum of HPV types in clinical specimens using a single PCR reaction (Figure 3). These tests use primer pairs that are common to most, if not all, anogenital HPV types. The sequences of a variety of HPV consensus primers have been published. These primers, GP5+/GP6+,[41] SPF10,[48] MY09/11,[42,49] and PGMY,[20,31] to name a few, possess different attributes that enable them to detect and genotype a broad spectrum of HPV types.

Line probe assays detect amplified genotype-specific DNA by selective hybridization with oligonucleotides immobilized on nylon membranes. The technique and results are very similar to Western blotting. Using line probe assay techniques, individual HPV genotypes can be classified with a very high level of sensitivity, down to the 100-attogram level. This level of discrimination is useful in resolving individual genotypes in mixed infections.[5,42,48,50]

Restriction fragment length polymorphism methods are used to identify HPV genotype-specific restriction patterns derived from post-PCR consensus primer amplified DNA. The restriction enzymes used for most analyses are typically *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, and *Pst*I. Human papillomavirus RFLP data are sometimes difficult to interpret, especially when mixed infections are encountered. Furthermore, since restriction fragments are not, in practice, positively identified by specific hybridization (eg, Southern blot), identification of spurious bands can lead to major uncertainty in assigning genotypes.[13,32,34,38,41,51]

Direct nucleic acid sequencing of the products of PCR reactions using consensus primers is another approach that can be used to distinguish the HPV genotypes present in clinical specimens. This technology shows promise in yielding sequence information on as yet uncharacterized HPV genotypes, as well as information regarding mutations within known HPV genotypes. These research-based systems, however, appear to have limited utility as in vitro diagnostic tools.[32,35,43,45,51,52]

LABORATORY METHODS USED TO DETERMINE HPV VIRAL LOAD

Several studies have shown that HPV-infected patients with a high viral load (VL) may be at increased risk for developing cervical cancer.[19,27,30,53–58] Most of these studies involve RQ-PCR, although a few reports use the HC2 assay as an indicator of HPV VL.[27,59–61] All real-time PCR systems rely on the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. Using this technology, it is possible to mathematically extrapolate VL (concentration) data from reaction curves generated by monitoring PCR in real time.[14,23]

Assays based on RQ-PCR represent the best approach to target nucleic acid quantitation (Figure 4). Techniques that use RQ-PCR technology allow continuous monitoring of PCR products, since dual-labeled florigenic probes emit fluorescence as the PCR reaction proceeds.[14,23] Reactions are performed in 96-well plates without the need to analyze PCR products on gels, making it a useful tool for simultaneous testing of a large number of samples. Quantitation of target DNA, such as a viral pathogen, using real-time PCR has the advantage of being reproducible, rapid, and applicable in a clinical setting. It provides a real quantification by determination of standard curves, which then can be used to extrapolate the amount of starting DNA target in a patient's specimen.[14,23]

Real-time quantitative PCR is capable of quantitation over a 7-log dynamic range. Additionally, reactions can be run in multiplex with the use of different fluorochromes, such that the starting concentrations of several target DNAs can be analyzed at once.[14,23]

HPV VIRAL LOAD AND GENOTYPING—WHY?

There may be a direct correlation between the number of copies of an oncogenic HPV type within a cell or in an anatomic site and the risk of developing HPV-related disease. This idea may have been borrowed from experience with laboratory testing involving other viral infections, such as human immunodeficiency virus or hepatitis C virus infections.

In surveying the literature, there appears to be no clear utility for determining HPV VL at this time. The literature is inconsistent in reconciling HPV VL with a quantifiable standard; some report VL as a function of total input cells, whereas others report VL as a function of abnormal input cells or as a function of abnormal input cells with suitable DNA quality.[30,53,55,57,58,62] The best approach appears to relate HPV VL to the amount of input human DNA, as judged by the concentration of a housekeeping gene such as beta-globin.[53] According to Hart and colleagues,[53] high VLs may be produced in severe disease, rather than being the cause of severe disease. This suggestion is based on the fact that VL values are an average summed over many infected and uninfected cells; also, the viral DNA may be integrated, disrupted, or deleted from the probe target site.

The spectrum of disease caused by members of the extended HPV family is extremely diverse, ranging from benign warts[63] to esophageal,[64] laryngeal,[65,66] and cervical carcinoma,[63] as well as many carcinomas of the head and neck.[67] Many studies of cervical disease, both cross-sectional and prospective, have shown that specific HPV types predict the risk of progression to high-grade cervical intraepithelial neoplasia (CIN).[63] Most genital HPV types are associated with CIN 1, and the types associated with CIN 2 and 3 are more frequently those classified as high-risk or cancer-associated HPVs, including HPV-16, -18, -31, and -45 and several others.[28,63]

Although the HC2 test in common use today broadly classifies HPV genotypes into low-risk or high-risk categories, HPV genotyping technology also exists to classify individual HPV genotypes within a particular lesion. Genotyping could potentially provide information on individual risk stratification, therapeutic decisions, epidemiological studies, and vaccine development.[63,68,69] However, as is the case with HPV VL testing, there is no clear useful role for HPV genotyping at this time for the management of patients with atypical cervical cytology. Nevertheless, using HPV genotyping techniques, one cannot only determine which types are present, but, depending on the test employed, can also uncover novel HPV types or test for mutations within known genotypes.[10,21,35,69]

Most HPV infections are mixed, and there may be collateral effects of multiple HPV types present in a lesion. Infection with multiple HPV types appears to be associated with an increased risk of CIN[70,71]; however, it is not known whether specific HPV types can promote or exclude infection with specific other HPV types, or whether superinfection with multiple HPV types can potentiate malignant transformation. Many high-grade cervical lesions are associated with HPV-16 and/or HPV-18, which possess mutations within *E6* and *E7* loci that may increase their oncogenic potential.[21,35,69] Generalized HPV genotyping using consensus primers could possibly miss this subcategory of mutant HPVs.

The defining abnormality or abnormalities that lead to malignant transformation are likely not addressed by HPV genotyping alone. Intrinsic and extrinsic host-related issues, such as a variety of risk factors, possible mutations within tumor suppressor genes,[72,73] or HLA haplotype,[10,47,68] for example, have to be considered.

EMERGING TECHNOLOGIES

Methods for testing HPV that will appear on the horizon will probably consist of a variety of PCR methods. Additionally, DNA microarrays, also known as biochip, gene chip, genome chip, or gene array–based tests, such as those developed by Affymetrix, Nanogen, and others, may someday enter the realm of HPV testing.[74] These technologies possess tremendous nucleic acid analytical power, with an inherent ability to simultaneously analyze up to thousands of sequences. As such, within a single specimen it may someday be possible to concurrently test for all HPV genotypes and all known HPV mutations within the context of genetic factors, such as HLA haplotype or mutations within tumor suppressor genes.

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