MOLECULAR DETECTION OF HUMAN VIRAL PATHOGENS

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78 Human Papilloma Virus (HPV)

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78.1 INTRODUCTION

78.1.1 Classification, Morphology, and Biology

Human papilloma viruses (HPV) are double-stranded DNA viruses that comprise a remarkably heterogeneous family of more than 130 types [1,2]. Different HPV types vary in tissue distribution, oncogenic potential, and association with anatomically and histologically distinct diseases. HPVs are classified into cutaneous and mucosal types [3].

Cutaneous types infect the squamous epithelium of the skin and produce common, plantar and flat warts, which occur commonly on the hands, face, and feet. Specific cutaneous types are also detected in *Epidermodysplasia verruciformis*, a rare familial disorder that is related to the development of large cutaneous warts that can progress to skin cancer [4]. Mucosal types infect the mucous membranes and can cause cervical neoplasia in adults as well as anogenital warts in both children and adults.

Mucosal HPVs are classified into high-risk and low-risk types. High-risk HPV types have been implicated in the development of squamous intraepithelial lesions (SILs) and its progression to cervical cancer [1,5]. To date, 15 HPV types have been classified as high risk and these include HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 [6,7]. HPV-16 and -18 are considered to be the most frequent HPV types worldwide and are responsible for approximately 70% of cervical cancer cases [7,8]. Low-risk HPVs have been associated with benign warts of oral and urogenital epithelium in both adults and children, and are only rarely found in malignant tumors.

HPV is a small virus of 55 nm in diameter that consists of its viral genomic DNA and its coat. Its viral genome is double-stranded circular DNA of nearly 8000 base pairs. The HPV genome is divided into the eight open reading frames of E6, E7, E1, E2, E4, E5, L2, and L1, with E or L coding for early or late functions, respectively. Taxonomically, the DNA genome of different HPV types differs by at least 10% of the nucleotide sequence of the three open reading frames E6, E7, and L1 from that of any other known type [9]. The HPV genome encodes eight proteins: E1, E2, E4, E5, E6, E7, L1, and L2. The early proteins E5, E6, and E7 are involved in cell proliferation and survival, with E6 and E7 playing a key role in HPV-associated carcinogenesis. The early proteins E1, E2, and E4 are involved in the control of viral gene transcription and viral DNA replication. The coat of the virus is made up of two proteins: the major one being L1 and a minor component L2. The coat proteins assemble into structures known as capsomeres and 72 of these come together to form the spherical coat.

HPV's target host cell is the epithelial cell [1]. The virus enters the basal layer of the epithelium via microlesions of skin or mucosa. Its genome is transferred to the cell's nucleus, where it exists as a nonintegrated circular episome. As these infected basal cells undergo cell division, the viral genome replicates and becomes equally segregated between the two daughter cells, enabling maintenance of the HPV genome in this cell layer. Some of the progeny migrate into the suprabasal differentiating cell layers, where viral genes are activated, viral DNA is replicated, and capsid proteins are formed. Viral particles are formed and released at the surface.

HPV infection has a global distribution. HPV-16 represents the most commonly identified HPV type in low-grade and high-grade SILs as well as cervical cancer worldwide [8,10,11]. The prevalence of HPV-16 ranges from 9% in Africa to 21% in Asia, while in Europe the prevalence is

19%. A similar meta-analysis of high-grade SILs has shown 32% of HPV-16 in Africa, 37% in South America, 46% in North America, and 53% in Europe. The predominance of HPV-16 has also been demonstrated in cases with squamous cervical cancer (SCC). The prevalence of HPV-16 varies consistently with the majority of cases being found in Europe and the lowest in Africa. Among low-grade SILs, the prevalence of HPV-18 has been 5.3% in Africa, 7.1% in Asia, 9.2% in North America, 3.6% in South/Central America, and 5.2% in Europe. Among high-grade SILs, the respective prevalences range from 6.5% in Europe to 10% in North America, while the pattern is consistent among cases presenting with SCC. Although HPV-16 and -18 are the dominant HPV type detected in women with cervical cancer and its precursors worldwide, other HPV types have been detected more frequently than HPV-16 and -18 in certain areas. Among low-grade SILs other high-risk HPV types, such as HPV-31, 51, 52, 56, and 58 are detected more frequently than HPV-18. In Asia, a high prevalence of HPV-58 and in Europe HPV-31 have been demonstrated in low-grade and high-grade SILs. Among women with SILs the frequency of non-16/-18 HPV types ranges from 34 to 68%. A high prevalence of non-16/-18 HPV types is of great importance since no vaccines are currently available for these types.

78.1.2 Clinical Features

HPV has been identified as the principal etiologic agent for cervical cancer and its precursors in adulthood [8]. Different HPV types can cause a wide range of infections, including common warts, genital warts, recurrent respiratory papillomatosis (RRP), low-grade and high-grade SILs, and cervical cancer.

Recurrent respiratory papillomatosis (RRP) in childhood occurs at an incidence of 0.3–3.9/100,000 and is considered to be the most common benign tumor that affects the larynx in children [12,13]. It is characterized by the recurrent growth of benign papillomas along the epithelium of the upper respiratory tract including the larynx, vocal cords, arytenoids, subglottis, and the trachea. The most commonly affected area is the mucocutaneous margin of the true vocal cords where the squamous epithelium of the vocal cord contacts the respiratory epithelium of the larynx. RRP is a potentially life-threatening benign tumor as it has the tendency to grow in size and number causing complete airway obstruction. The etiology of RRP is the infection of the upper airway with HPV types 6 and 11. HPV infection is generally the result of perinatal transmission, implying that consideration of sexual abuse is unnecessary in RRP cases. Perinatal infection may occur transplacentally via amniotic fluid during gestation and delivery, as well as through direct exposure to cervical and genital lesions during birth. RRP rates are higher in first-born children and those delivered vaginally than subsequent children or those delivered by Caesarian section. Maternal history of anogenital warts, cytological, or histological lesions of HPV infection in the genital tract and maternal age of less than 20 years are also associated with higher rates of RRP in children. However, it is still unclear how frequently perinatal infection progresses to clinical lesions, whether genital, laryngeal, or oral. RRP is characterized by a relatively low HPV viral load, and HPV 11 is considered to be the most common cause of RRP. RRP infected children with HPV 11 are prone to develop more aggressive disease than those with HPV 6 and thus require more frequent surgical intervention. HPV 11 infection is also related to a more frequent need for adjuvant therapies, tracheal and pulmonary disease, and tracheostomy.

Skin warts are considered to be the main manifestation of the cutaneous HPV types, with HPV 1, 2, 3, 4, 27, and 57 being detected most frequently [14,15]. The presence of mucosal HPVs has also been reported. However, the origin and the role of these mucosal types on the skin remain unclear. Skin warts exist in different forms including common warts (*Verruca vulgaris*), plantar warts (*Verruca plantaris*), and flat warts (*Verruca plana*). Skin warts are estimated to occur in up to 10% of children and young adults, with the greatest incidence between 12 and 16 years of age. Warts occur more frequently in girls than boys. Common warts represent 70% of skin warts and occur primarily in children, whereas plantar and flat warts occur in a slightly older population. In most cases, among healthy individuals, cutaneous HPV types progress to persistent subclinical infections without causing warts or other skin lesions. The natural progression of skin warts in childhood indicates that warts spontaneously clear after 2 years without treatment in 40% of children. Depending on their location warts can be painful (e.g., on soles of the feet or near the nails), while in other cases warts are viewed as socially unacceptable when they are conspicuous (e.g., on the hands or face).

Anogenital warts in adults constitute a common sexually transmitted disease, while in children the reported incidence has been increasing dramatically since 1990 [16,17]. The clinical appearance of anogenital warts varies from subtle, skin-colored, flat warts to moist, pink-to-brown lesions found particularly in the skin creases and around the vaginal and anal openings. HPV 11 and 6 are the most frequently detected HPVs in anogenital warts in both adults and children. Cutaneous HPV types such as HPV 2 or 3 are also detected; however, their incidence is low. Among nonsexually abused children with anogenital warts, cutaneous types are more common in older children aged over 4 years, in those with a relative who had skin warts and in children with skin warts on other anatomical sites. In contrast, mucosal types are more common in girls, in children under 3 years, in children with relatives with genital warts, and in those with no warts elsewhere. The modes of HPV genital transmission in children remain controversial. Human papilloma virus (HPV) can reach a child's anogenital area by vertical transmission or by close contact, which can be either sexual or nonsexual. Nevertheless, the presence of anogenital warts in children have serious social and legal implications as it raises concerns of possible sexual abuse. Every case needs to be evaluated in detail to determine whether enough concern exists to pursue additional investigations. The commonly used upper

age limits for perinatal transmission are 12–24 months, while anogenital warts discovered among children more than 24 months of age are often assumed to have been acquired through sexual abuse. It is recommended that all children who present with anogenital warts be evaluated by a consultant with expertise in child sexual abuse and that children who are over the age of 4 years be referred routinely to Child Protection Services.

Cervical cancer remains the second most common cancer among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in 2002. Cervical cancer clusters in developing countries where 80% of the cases occur and accounts for at least 15% of female cancers. In populations of developing countries, the cumulative lifetime risk of developing cervical cancer is estimated to be in the range of 1.5–3%, whereas in developed countries it accounts for only 3.6% of all new types of cancer in women with a cumulative risk of 0.8% up to the age of 65 years. In general, the lowest rates are found in Europe, North America, and Japan. The incidence is particularly high in Latin America, the Caribbean, and south central and southern Asia. Cervical cancer clusters in the lower socioeconomic strata signal the lack of appropriate screening as one of the significant determinants of the occurrence of the invasive stages of the disease. Predictions based on the passive growth of the population and the increase in life expectancy indicate that the expected number of cervical cancers in 2020 will increase by 40% worldwide, corresponding to 56% in developing countries, and 11% in the developed parts of the world. Global mortality rates are substantially lower than the incidence, with a 55% ratio of mortality to incidence. Cervical cancer is a multistep disease. Persistent HPV infection leads to low- and high-grade SILs, which may progress to cervical cancer. Among the cervical abnormalities that develop most early lesions regress spontaneously, but the rate of regression decreases with increasing severity of SILs. Low- and high-grade SILs are common especially in women of reproductive age. Cervical cancer is a late and rare complication of a persistent HPV infection and is the end result of a chain of events that can take in excess of 10 years to unfold.

78.1.3 Pathogenesis

It is generally accepted that HPV E6 and E7 proteins from high-risk types act together to immortalize the host cell and are responsible for the oncogenic potential of HPV. It has been shown that they function as the dominant oncoproteins of high-risk HPVs by altering the function of critical cellular proteins. The expression of the E6 and E7 proteins as a consequence of viral integration is, therefore, paramount to the establishment and maintenance of the tumorigenic state (Figure 78.1). In addition, the expression of E6 and E7 increases genomic instability of the host cell, thus accelerating malignant progression [18]. E6 and E7 target important cellular growth regulatory circuits including p53 and the retinoblastoma tumor suppressor protein Rb, respectively. HPV E6 has been shown to interact with and enhance the degradation of p53, which plays an important role in cell cycle control and apoptosis in response to DNA damage, while HPV E7 disables the function of the retinoblastoma tumor suppressor protein Rb. During the last decade, it has been well demonstrated that both HPV E6 and E7 interact with the host cell to target a plethora of key host cellular

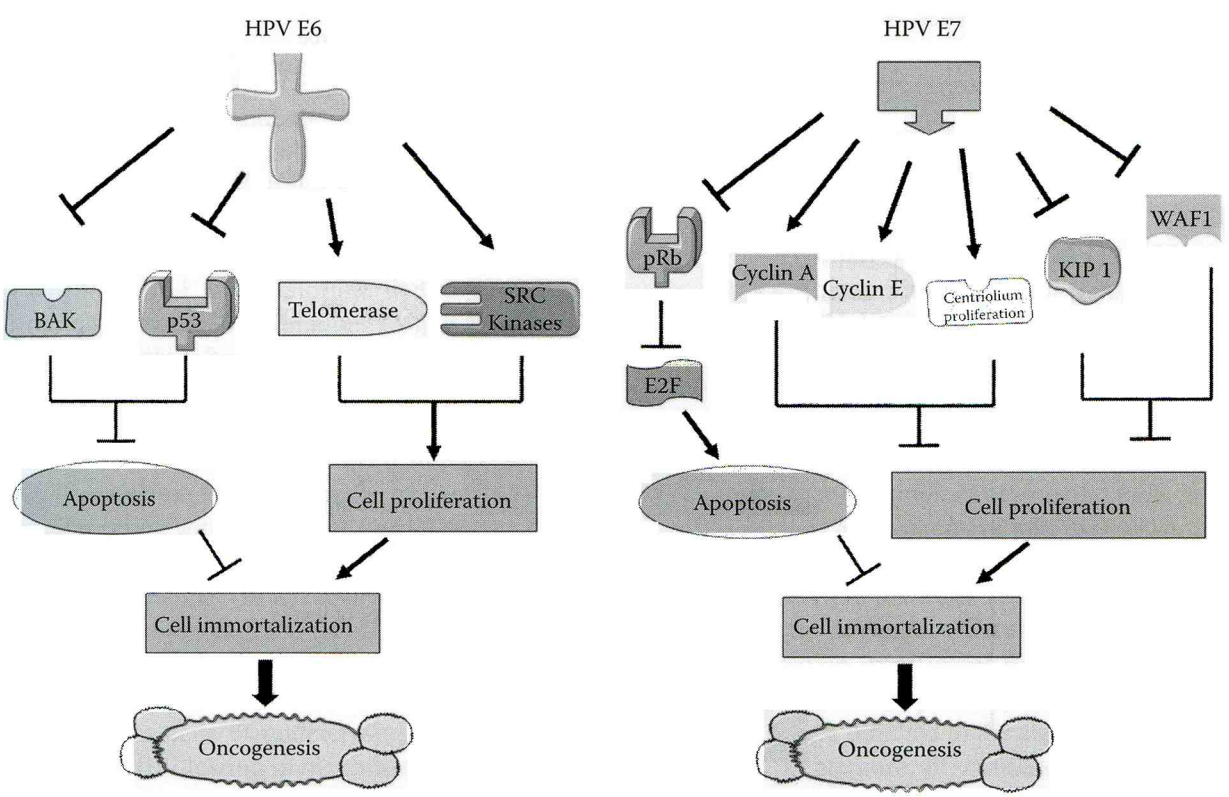

FIGURE 78.1 Interaction of HPV-E6 and -E7 oncoproteins with host cell proteins, resulting in tumorigenesis.

proteins that are involved in apoptosis and malignant cellular transformation [19].

Several of the prominent functions of the E6 protein originate from its interaction with p53 and the proapoptotic protein BAK, which results in resistance to apoptosis and an increase in chromosomal instability [1]. In addition, the activation of telomerase and the postulated inhibition of degradation of SRC-family kinases by the E6 oncoprotein appear to fulfil important functions in growth stimulation. The stabilization of the activated forms of specific members of the SRC-family kinases can contribute to the HPV-transformed phenotype. The cyclin-dependent kinase inhibitor INK4A appears to counteract these functions. E7 interacts with and degrades Rb, which releases the transcription factor E2F from Rb inhibition and upregulates INK4A. The resulting high E2F activity leads to apoptosis in E7-expressing cells. Moreover, E7 stimulates the S-phase gene cyclins A and E, and blocks the function of the cyclin-dependent kinase inhibitors WAF1 and KIP1. By inducing centriole amplification, E7 also induces aneuploidy of the E7-expressing cells, which contributes to oncogenesis. E6 and E7 can independently immortalize human cells, but at a reduced efficiency. Their synergistic function results in a marked increase in transforming activity. E6 is impaired by INK4A, whereas E7 bypasses this inhibition by directly activating cyclins A and E. In turn, E6 prevents E7-induced apoptosis by degrading the apoptosis-inducing proteins p53 and BAK.

High-risk HPV infections result in the progression to cervical cancer in only a small percentage of infected women, following a long latency period. A high percentage of infected women clear the infection by immunological mechanisms. Lasting immunosuppression represents a risk factor for viral DNA persistence and progression to cervical cancer. Viral risk factors that influence the progression of infected cells include infection with high-risk type, with specific virus variants and high viral load. Nonviral risk factors include several sexual partners, smoking, infections with herpes simplex, bacterial and protozoal infections, and genetic predisposition (Figure 78.2).

HPV DNA integration in the host cellular genome represents the critical event of malignant cellular transformation. In the normal viral life cycle, the genome replicates as episomal molecules. Even though the HPV genome is consistently retained in the episomal state in early dysplastic

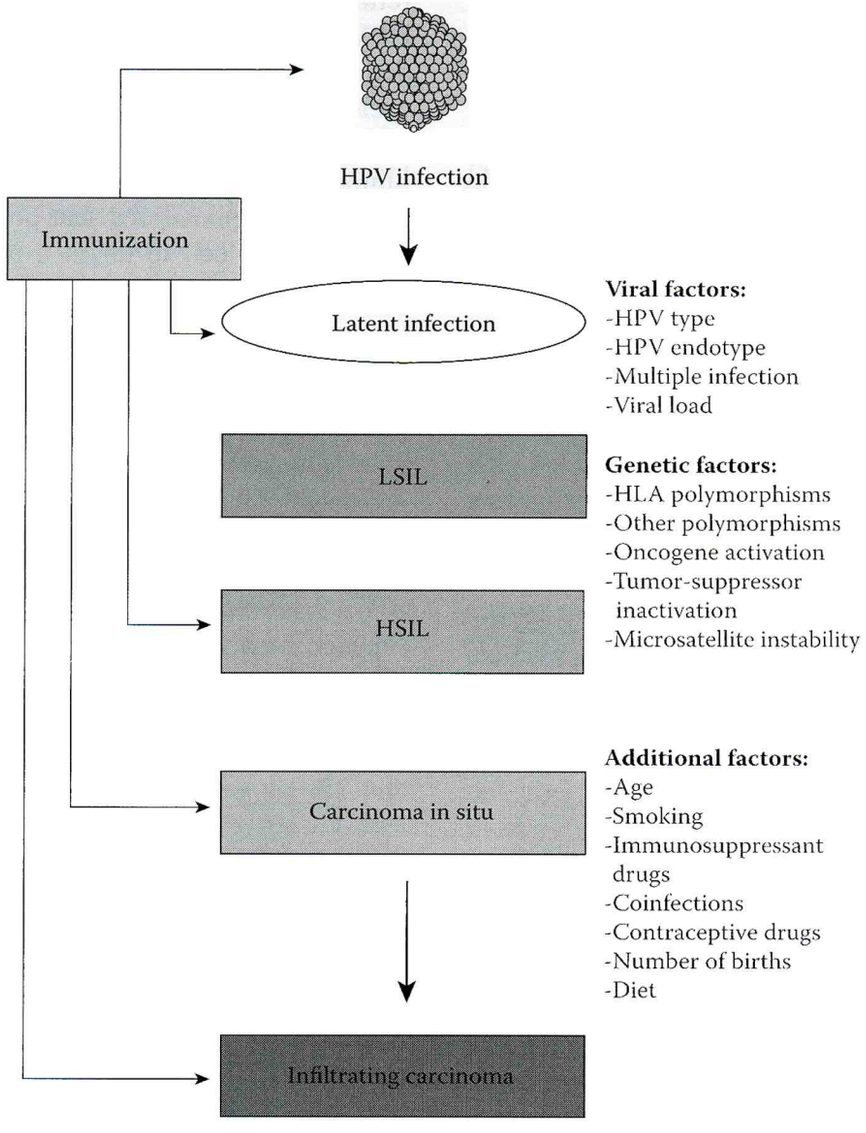

FIGURE 78.2 Viral, genetic and other predisposition factors influencing the progression of HPV-infected cells toward malignancy. LSILs, low-grade epithelial lesions; HSILs, high-grade epithelial lesions.

and low-grade lesions, it is integrated in the host chromosome in many cases of high-grade lesions and the majority of HPV-associated cervical carcinomas [20,21]. Integration is a direct consequence of chromosomal instability and an important molecular event in the progression from high-grade lesions to invasive cervical cancer. A possible explanation for the progression of the disease toward malignancy involves the structural changes that take place after HPV genome integration, leading to the deregulated expression of viral oncogenes. HPV E6 and E7, the major transforming genes, confer a much stronger transforming capacity in primary cells when they derive from integrated rather than episomal transcripts.

In addition, critical cellular genes are affected by viral integration. Coding regions are rarely affected by HPV, but gene expression and the mRNA structure can be altered by insertion of the strong HPV promoter. Some of the genes disrupted by HPV integration are known to be involved in other types of cancer, such as myc, APM1, TP63, TNFAIP2, and hTERT. Currently, about 200 HPV DNA integration sites have been mapped in primary tumor samples and cell lines. From the data analyzed it has been concluded that HPV integration sites are randomly distributed over the whole genome with a clear preference for genomic fragile sites. Viral integration is a consequence of an overall destabilization process of the chromosomal integrity in replicating epithelial cells that express the viral E6 and E7 genes. Therefore, the consequences of the structural alterations of the viral genome and the impact of cellular sequences on its transcriptional regulation appear to be more important than any functional cellular alteration caused by HPV integration.

78.1.4 Diagnosis

Cervical screening, which includes Pap smear examination and colposcopy, in organized population-based programs has been successful in reducing the incidence of cervical cancer. A high population coverage is essential for effective cervical screening, as the high incidence of cervical cancer in developing countries has been attributed to low cervical screening coverage. Women referred with abnormal cytology results should undergo colposcopic examination. Colposcopy permits assessment of the transformation zone for the presence of SILs and accurate biopsy targeting. Colposcopy generates both small, diagnostic biopsies, and excision specimens. The diagnosis of cervical cancer is based on the histopathology.

Human papilloma virus (HPV) testing was recently introduced in clinical practice with the aim of identifying women at risk of cervical cancer. This testing scheme involves: (i) primary cervical screening; (ii) triage of low-grade smear; (iii) posttreatment follow-up of CIN; (iv) cervical screening prior to vaccination; and (v) postvaccination cervical screening. HPV testing is recommended in the triage of women with atypical squamous cells of undetermined significance (ASCUS). The use of HPV testing in the follow-up of women after CIN local treatment is also strongly supported by clinical evidence [22]. Screening prior to vaccination may identify women who have already been exposed to HPV, thereby reducing the benefit derived from the vaccination. However, financial restrictions impede the prescreening of all women by HPV testing. HPV testing has also been proposed as a useful tool for primary cervical screening and the management of women with low-grade epithelial lesions (LSILs). However, recent evidence is insufficient to support HPV testing instead of the conventional cytology [23–25]. It is likely that following the introduction of the vaccination against HPV, the role of HPV testing for triage for LSILs and primary cervical screening will be reevaluated.

Since capsids of the virus are produced only in terminally differentiated squamous cells, its replication is tightly linked to squamous epithelial cell differentiation. Therefore, it is unable to establish an HPV culture in vitro. Moreover, the clinical, colposcopic and microscopic examination of exfoliated samples (pap smears) or tissue biopsies for koilocytes are both insensitive and nonspecific methods. Serology is also not suitable for distinguishing present and past infections since antibodies of the major capsid protein of the virus remain detectable for many years [26].

Several molecular assays are available for the detection of HPV infection in tissue and exfoliated cell samples, and they present different sensitivities and specificities. They are divided into: (i) target amplification assays/PCR; (ii) direct hybridization assays; and (iii) signal amplified hybridization assays.

Target amplification assays. PCR is the most widely used method for the amplification of nucleic acids. PCR-based detection of HPV is extremely sensitive and specific. The mechanism of this approach relies on a thermostable DNA polymerase that recognizes and extends a pair of oligonucleotide primers that flank the region of interest. Finally, the viral DNA is sufficiently amplified in vitro to generate adequate amounts of the target that is then directly visualized on gels. In theory, PCR is able to detect one copy of a target sequence in a given sample. In practice, the sensitivity of the PCR-based method is about 10–100 HPV viral genomes in a background of 100 ng cellular DNA. Since PCR can be performed on very small amounts of DNA (10–100 ng) it is ideal for use on specimens with a low DNA content.

Generally, HPV detection by PCR can be performed either by type-specific primers, designed to exclusively amplify a single HPV genotype, or by consensus/general PCR primer pairs, designed to amplify a broad spectrum of HPV genotypes. Unfortunately, the detection of the presence of HPV-DNA in a single sample using multiple type-specific PCR reactions separately is often a labor-intensive and expensive task [27–29]. Furthermore, the type-specificity of each PCR primer pair needs validation. On the other hand, the use of general primers is much more convenient. Usually, general primers identify a conserved region in different HPV genotypes, such as the L1 [30] or E1 regions [31]. However, most laboratories utilize consensus primers directed to the conserved L1 region.

There is a plethora of consensus on the PCR primers that can be used. The GP5+ /GP6+ pair is aimed at the L1-conserved region, but fully complement only one or a

few HPV genotypes. To compensate for the mismatches with other HPV genotypes, PCR is performed at a low annealing temperature [32–34]. The MY09/11 set contains one or more degeneracies in order to compensate for the intertypic sequence variation at the priming sites. These primers do not have to be used at a lower annealing temperature [30,35,36] because they are a combination of many different oligonucleotides. The disadvantage of this design is that synthesis of oligonucleotides containing degeneracies is not highly reproducible and often results in high batch-to-batch variation. Therefore, each novel batch of MY09/11 primers should be carefully evaluated to check the efficacy of amplification for each HPV genotype [37]. Moreover, a combination of various nondegenerate forward and reverse primers aimed at the same position of the viral genome, can be applied. Usually, combined primers may contain inosine that matches with any nucleotide. This kind of primer has the advantage that the oligonucleotides can be synthesized with high reproducibility, and PCR is performed at optimal annealing temperatures. Examples of such primer sets are PGMY [37], SPF10 [38], LCR/E7 [39], as well as a combination of the MY11 and GP6 + primers [40]. Besides the choice of primers, the size of the PCR product is also important. In general, the efficiency of a PCR reaction decreases with increasing amplicon size. Subjecting clinical samples to treatments, such as formalin fixation and paraffin embedding, degrades DNA. Consequently, the efficiency of PCR primers generating a small product is considerably higher than primer sets yielding larger amplicons [38,41].

After amplification, the sequence composition of a PCR product can be investigated in various ways, one of which is the use of restriction enzymes. Digestion of PCR products with restriction endonucleases generates a number of fragments, which can be resolved by gel electrophoresis, yielding a particular banding pattern. The restriction enzymes used for most analyses are typically *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, and *Pst*I. HPV restriction-fragment-length-polymorphism (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 (e.g., Southern blotting), identification of spurious bands can lead to uncertainty in assigning genotypes [42–47]. Consequently, the detection of multiple HPV genotypes, present in different quantities in a clinical sample, by PCR–RFLP is usually complex and the sensitivity to detect minority genotypes is limited [48].

PCR products can also be detected with a mixture of type-specific probes, such as that in an enzyme immunoassay (EIA) [34]. One good example is the HPV oligonucleotide microarray (HPVDNAChip, Biomedlab Co.) that contains 22 type-specific probes; 15 of the high-risk group (16/18/31/33/35/39/45/51/52/56/58/59/66/68/69) and seven of the low-risk group (6/11/34/40/42/43/44). Briefly, the PCR product is hybridized onto the chip, and after a washing step, hybridized signals are visualized with a DNA Chip Scanner. The sensitivity of this assay has been reported to reach 94.9%, rendering this application a diagnostic tool with significant advantages since it can discriminate the HPV genotype and identify multiple infections [49,50]. Ideally, a larger number of HPV type-specific oligonucleotides may be spotted on the Chip, although this method requires the presence of expensive equipment and may not be suitable for many laboratories. A similar assay has been released by Gen-Probe Incorporated, called the APTIMA(R) HPV Assay. This assay detects 14 high-risk HPV types in an amplified HPV nucleic acid. Specifically, it detects the mRNAs E6 and E7, which are produced in higher amounts when HPV infections progress toward cervical cancer [51].

Rapid sequencing methods of PCR products are also now available for high throughput, thus permitting application in routine clinical analysis [52]. However, sequence determination is not suitable when a clinical sample contains multiple HPV genotypes. Sequences, which represent minority species in the total PCR product, may remain undetected. In turn, this may underestimate the prevalence of infections with multiple HPV genotypes, with important consequences for vaccination or follow-up studies [53]. This was confirmed in a recent study that compared sequence analysis of SPF10 PCR products with reverse hybridization in 166 HPV-positive cervical scrapes. Compatible HPV genotypes were found in all samples. Direct sequence analysis detected multiple types in only 2% of the samples, while reverse hybridization found multiple types in 25%. The presence of multiple HPV genotypes is a common phenomenon in many patient groups. Up to 35% of HPV-positive samples from patients with advanced cytological disorders and more than 50% of samples from HIV-infected patients [54] contain multiple HPV genotypes, whereas multiple genotypes are less prevalent in cancer patients [53]. The genotype from an HPV sequence can be deduced through alignment with a set of known HPV sequences, using the BLAST software [55] of a genome database (http://www.ncbi.nlm.nih.gov). Currently, the complete genomes of various papilloma viruses have been fully sequenced. In order to designate a new type, the L1, E6, and E7 ORFs must differ by more than 10% from the closest type known. Differences of 2–10% lead to the definition of a new subtype, whereas differences of less than 2% define intratype variants [56].

Real-time PCR techniques have been developed to quantify HPV-DNA in clinical samples. Techniques that use real-time PCR technology allow for the continuous monitoring of PCR products, since dual-labeled fluorigenic probes emit fluorescence as the PCR reaction proceeds [57–59]. Reactions are usually performed in 96-well plates without the need to analyze PCR products on agarose gels, making it a useful tool for the 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. Real-time quantitative PCR is capable of quantifying over a 7-log dynamic range. Additionally, reactions can be run in multiplex with the use of different fluorochromes, in order that the starting concentrations of several target DNAs can be analyzed concomitantly [57,58]. Using this

technology, it is possible to mathematically extrapolate viral load/concentration data from reaction curves generated by monitoring PCR in real-time [57,58]. Novel real-time PCR methods have been released and can be used as high-throughput screening tools. One such example is the GenoID real-time PCR assay, the amplification of which is based on the L1 region of HPV. This assay also detects the nonintegrated copies of HPV. The assay's calibrators are designed to detect ~10,000 copies/reaction (~100 infected cells). Amplification is balanced over the genotypes, which is important in achieving optimal clinical sensitivity. The detection of high-risk HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), low-risk HPV (6, 11, 42, 43, 44/55), and the internal controls are carried out in the same reaction tube using three different color-compensated dye channels [60]. However, the exclusive optimization of this assay for the LightCycler 2.0 instrument (Roche) can be regarded as a weakness. Unlike other HPV tests, the newly released CE-marked Abbott Real-Time High-Risk HPV assay, can detect the 14 HPV genotypes with the highest risk and, in the same procedure, can identify women infected with the HPV-16 and -18 genotypes, which account for more than 70% of cervical cancer cases. The assay can rapidly identify HPV-infected patients at risk for cervical cancer by combining two diagnostic tools in one test: HPV high-risk screening and viral genotyping.

Apart from TaqMan oligo-probe technologies, SYBR-Green based real-time PCR assays utilizing the GP5+/6+ primers have also been used for HPV quantification, with a high specificity and sensitivity. Results showed excellent concordance with the EIA-reverse line blot and sequencing assays [61,62].

Although real-time PCR technology is able to provide quantitative analysis, an important point that should be considered is that high viral loads may be produced in severe disease, rather than being the cause of severe disease [62]. This suggestion is based on the fact that viral load 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.

It is also possible to look for specific viral RNA by incorporating a reverse transcriptase (RT) step before PCR amplification. Although the vast majority of HPV detection strategies used for epidemiological studies and clinical management have, thus far, been DNA-based, detection of the expression of HPV oncogenes may have significant clinical value. For example, Lamarcq et al. developed a real-time RT-PCR for HPV-16 and -18 E7 transcripts and suggested that it is more specific for the detection of symptomatic infections [63]. Wang-Johanning et al. also described an HPV-16 E6/E7 quantitative real-time RT-PCR and found that the expression increased coordinately with the severity of the lesion [64]. In another study by Cattani et al., E6/E7-RNA transcripts were detected in 18.2% of HPV DNA-positive patients with normal cytology [65]. The rate of detection increased gradually with the grade of the observed lesions, suggesting that testing for HPV-E6/E7 transcripts is a useful tool for screening and patient management, providing more accurate predictions of risk than DNA testing.

There is currently one commercially available RNA-based HPV assay, the PreTect HPV Proofer (Norchip AS Klokkarstua, Norway). This assay incorporates NASBA amplification of E6/E7 mRNA transcripts prior to type-specific detection via molecular beacons for HPVs 16, 18, 31, 33, and 45. Initial data, on the prognostic value and specificity for underlying disease, are promising [66], but the clinical value of this method compared with DNA-based assays remains to be determined in large-scale prospective studies. The physical state of the HPV genome has also been explored as a potential diagnostic marker. Integrated virus is associated with a neoplastic phenotype/high-grade disease, where loss of the regulatory E2 protein on integration results in the up-regulation of oncogenes E6 and E7.

Finally, a protocol for the amplification of papillomavirus oncogene transcripts (APOT) from cervical specimens has been proposed that allows for the distinction of episomal from integrated HPV mRNAs [20,67]. In most cervical carcinomas, HPV genomes are integrated into host cell chromosomes, whereby transcribed mRNAs encompass viral and cellular sequences. In contrast, in early preneoplastic lesions, HPV genomes persist as episomes, and derived transcripts contain exclusively viral sequences. Thus, detection of integrated-derived HPV transcripts in cervical swabs or biopsy specimens by the APOT assay points to advanced dysplasia or invasive cervical cancer. However, since the assay is based on RT-PCR protocols, sequencing steps, and is type-specific, it is not readily used in routine diagnostic testing.

Direct hybridization assays. Southern blot hybridization (SBH) and in situ hybridization (ISH) have been used, but have serious defects. The disadvantages of direct hybridization assays include low sensitivity, time-consuming techniques, and the need for possibly large amounts of highly purified DNA [68]. In fixed tissue, formalin-catalyzed DNA cross-linking resulting in DNA degradation makes SBH or RFLP impossible to be utilized. 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 [69]. Recently, new ISH assays have emerged, showing better results. First, the INFORM HPV three (Ventana Medical Systems) test can detect 13 types of oncogenic HPV (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, and 70). Results from use of this assay have shown favorable agreement with results from PCR-based assays. However, the INFORM HPV 3 still detected significantly fewer HPV-positive cases in carcinoma than did PCR [70]. Second, the HPV-CARD assay was shown to possess a high analytical sensitivity, reduce low background, have a high signal-to-noise ratio, allow for the quantification of HPV-infected epithelial cells and permit the distinction of HPV physical states [71].

To increase the throughput of a diagnostic assay, hybridizations to oligonucleotide probes can be performed in microtiter plates [34,38,75]. Biotin labeling of one of the primers generates labeled PCR products that are

then captured onto streptavidin-coated microtiter wells. Double-stranded DNA is denatured under alkaline conditions and the unattached strand is removed by washing. A labeled oligonucleotide probe is added, which hybridizes to the captured strand. Hybrids can be detected following the binding of conjugate and substrate reaction. The Roche Molecular Systems Amplicor HPV MWP assay was recently described. This method is based on the detection of 13 high-risk genotypes by a broadspectrum PCR in the L1 region, amplifying a fragment of approximately 170 bp. The heterogeneous interprimer region is detected with a cocktail of probes for high-risk genotypes. Preliminary data suggest this assay is more sensitive than HC2 for detection of the same HR-HPV types (21st International Papillomavirus Conference, Mexico, February 2004), although further work is required in prospective cohorts to assess whether this increased sensitivity is a benefit. An advantage of this method is the high throughput of the microtiter format. Therefore, the Amplicor method is suitable for distinguishing HPV DNA-positive and -negative samples as a first step in HPV diagnosis.

However, since the HC2 and Amplicor tests only differentiate between an infection with one out of 13 high-risk HPV genotypes and no high-risk HPV infection, neither allows for the individual identification of specific genotypes, nor do they identify multiple genotypes possibly involved in infection. This is regrettable as studies showed that there is a difference in the oncogenic potential between the different high-risk HPVs [76], arguing for the importance of HPV genotyping in screening and triage [77–80].

Furthermore, the CLART HPV 2 system is based on a low-density microarray that detects infections and coinfections of up to 35 of the most relevant HPV genotypes; 20 high-risk (16, 18, 26, 31, 33, 35, 39, 43, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 85, and 89) and 13 low-risk (6, 11, 40, 42, 44, 54, 61, 62, 71, 81, 83, and 84). The diagnostic sensitivity and specificity of this system can reach 98.2% and 100%, respectively.

Reverse hybridization provides an attractive tool for the simultaneous hybridization of a PCR product to multiple oligonucleotide probes. This method comprises the immobilization of multiple oligonucleotide probes in the solid phase, as well as the addition of the PCR product in the liquid phase. Hybridization is followed by a detection stage. The most frequently used reverse hybridization technology comprises a membrane strip containing multiple probes immobilized as parallel lines called the line probe assay (LiPA), line blot assay (LBA; Roche Molecular Systems) [81–84], or linear array (LA; Roche Molecular Systems). The three reverse hybridization assays require only a small amount of PCR product. A PCR product is generated, usually using biotinylated primers. The double-stranded PCR product is denatured under alkaline conditions and added to the strip in a hybridization buffer. After hybridization and stringent washing, the hybrids can be detected by the addition of a streptavidin-conjugate and a substrate, generating color at the probe line, which can be visually interpreted. These methods are judged to be advantageous in the ability to rapidly genotype HPVs present in samples with a high sensitivity and specificity [35,37,53,85–87]. By comparing LA versus LBA assays, Castle et al. found that the first was a more analytically sensitive method compared to the second, resulting in greater detection of individual genotypes, as well as an increased detection of multigenotype infection [88]. The LA assay thus translated into a more clinically sensitive, but less specific, test for CIN3 or worse, in a population of women referred to Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS) because of an Atypical Squamous Cell of Undetermined Significance (ASCUS) Pap test.

Alternative reverse hybridization methods for HPV and genotyping are the line blot assay using PGMY primers [35,89–92] and reverse line blot for GP5 + /6 + [93]. HPV DNA microarrays are based on the same principle [41,94]. Reverse hybridization methods are particularly useful for the detection of type-specific infections and multiple genotypes.

78.2 METHODS

78.2.1 Sample Preparation

Cervical exfoliated cells for HPV testing are usually collected in PBS, which is inexpensive, but requires constant refrigeration. Alternatively, these cells can be collected in other liquid-based preservation media (ThinPrep Solution, Cytyc) that can be stored at room temperature, but are expensive and flammable.

Using the phenol-chloroform extraction method, DNA is extracted from the PBS solution in which the cervical swab specimen is immersed. The PBS solution is first centrifuged at 1500 rpm for 15 min at room temperature. After discarding the supernatant, 500 µl of cell lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA) is added and the solution is incubated at room temperature for 5 min. Then, 500 µl of phenol–chloroform is added to the water phase and the mixture is centrifuged to remove the water phase. Finally, the DNA is collected by ethanol precipitation and the pellet is washed, dried and dissolved in double-distilled water.

Alternatively, there are various commercial kits for DNA extraction. One such is the Wizard kit (Promega), which is based on the precipitation of proteins. The cervical swab is again immersed in 10 ml of PBS and swirled to release the cells. Then, 250 µl of this suspension is subjected to the following protocol. The solution is spun at maximum speed in a microcentrifuge for 5 min. The supernatant is discarded, and 300 µl of Nuclei Lysis Solution (Wizard kit) is added. The solution is mixed by pipetting and incubated at 37°C for 1 h. The sample is cooled to RT, and 100 µl of protein precipitation solution (Wizard kit) is added. The solution is then vortexed and centrifuged at 13,000–16,000 × g for 3 min. The supernatant is transferred to a

new Eppendorf tube with 300 µl of isopropanol (at RT) and the solutions are mixed and centrifuged at 13,000–16,000 × g for 1 min. The supernatant is removed, and the pellet is washed with 70% ethanol and centrifuged again at 13,000–16,000 × g for 1 min. Finally, the ethanol is removed and the pellet is air dried. The pellet is dissolved in 100 µl of rehydration solution (10 mM Tris-HCl, 1 mM EDTA; pH 7.4).

The extraction procedure can also be based on the proteinase K digestion of the samples. Briefly, 250 µl of the cell suspension is spun at maximum speed in an Eppendorf centrifuge for 5 min. The supernatant is removed, and a proteinase K solution is added. The sample is incubated at 56–60°C for 2 h, and the proteinase K is inactivated at 95°C for 5 min. The sample is finally centrifuged for 5 min, and 2 µl of the top phase can be used for downstream PCR reaction.

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

Currently, novel automated DNA extraction methods have emerged. The MagNA Pure LC (Roche), DNeasy (Qiagen), and the Maxwell-16 (Promega) are unique in that they do not require a technician to extract the DNA from the samples. Collected samples can be placed directly into the robot for DNA extraction.

Measuring the intensity of absorbance of the DNA solution at wavelengths of 260 and 280 nm is used to determine DNA purity. DNA absorbs UV light at 260 and 280 nm, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration.

Using the Southern blot technique this quantified DNA can be isolated and examined further using PCR and RFLP analysis. These procedures allow differentiation of the repeated sequences within the genome.

78.2.2 Detection Procedures

78.2.2.1 Standard PCR Detection

Principle. De Roda Husman et al. [33] described a PCR assay for detection of a large spectrum of HPVs. A 150 bp fragment from the L1 region is amplified with the primer set GP5 + : 5'-TTTGTTACTGTGGTAGATACTAC-3' and GP6 + : 5'-GAAAAATAAACTGTAAATCATATTC-3'.

Procedure

1. Prepare the PCR mix (50 µl) containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 200 µM of each dNTP, 3.5 mM $MgCl_2$, 1 U thermostable DNA polymerase (*AmpliTaq;* Perkin Elmer Cetus), 50 pmol of each primer of the GP5 + /6 + primer combination, and 2 µl purified DNA.
2. For PCR amplification use the following cycling conditions: 1 cycle of 94°C for 4 min; 40 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 1–5 min. The last cycle was extended by a 4 min elongation at 72°C.
3. Visualize amplification products of predicted size using agarose gel electrophoresis.

Note: The expected product size of the PCR is 150 bp.

78.2.2.2 Real-Time PCR Detection

Principle. Saunier et al. [95] developed a TaqMan based real-time PCR assay targeting the E2 and E6 genes for specific detection of HPV16. The inclusion of primers and probe for human albumin gene provides a useful assay control.

Primer/probe	Sequence (5′–3′)
E6 HPV16 forward primer	GAGAACTGCAATGTTTCAGGACC
E6 HPV16 reverse primer	TGTATAGTTGTTTGCAGCTCTGTGC
E6 HPV16 probe	FAM-TTGACACCATTGAAAGACCCAGCGAGGAC-BHQ
E2 HPV16 forward primer	AACGAAGTATCCTCTCCTGAAATTATTAG
E2 HPV16 reverse primer	CCAAGGCGACGGCTTTG
E2 HPV16 probe	FAM-ATACCCAGCGCCGCCCCAC-BHQ
Albumin forward primer	GCTGTCATCTCTTGTGGGCTGT
Albumin reverse primer	ACTCATGGGAGCTGCTGGTTC
Albumin probe	FAM-GGACAGTACGGGTGTGTTTAGAGAGG-BHQ

Procedure

1. Prepare serial dilutions (1:10) of the pBR322-HPV16 plasmid containing 107, 106, 105, 104, 103, and 102 HPV16 DNA copies containing 50 ng/µl salmon sperm DNA, in order to make standard curves used to quantify E6 and E2 HPV16 copy number.
   Prepare the E6-PCR mix (20 µl) containing 5 mM $MgCl_2$, 1 × fast start hybridization probe buffer (Roche), 50 nM TaqMan probe, 500 mM each primer, 0.5 U uracil-DNA glycosylase (Roche), and 2 µl nucleic acid extract.
   Prepare the E2-PCR mix (20 µl) containing 4 mM $MgCl_2$, 1 × fast start hybridization probe buffer, 170 nM TaqMan probe, 500 mM each primer, 0.5 U uracil-DNA glycosylase (Roche), and 2 µl nucleic acid extract.
   For PCR amplification use the following cycling conditions: 95°C for 15 min followed by 50 cycles of 95°C for 15 sec, and 60°C for 1 min, with fluorescence acquired at the end of each 60°C step.

Note: The E2 real-time PCR was specific for HPV16, as no cross-reaction was observed for samples previously genotyped and harboring the phylogenetically related HPV31, HPV33, HPV52, HPV18, or HPV56. The reproducibility of the E2 real-time PCR was assessed using standard curves obtained from 17 independent experiments. The coefficient of variation of crossing point (Cp) obtained at each concentration was less than 4%, showing that the technique was very reproducible. The efficiency of real-time PCRs was also calculated from plasmid dilutions and from dilutions of clinical samples. Efficiency was equal to 1.9 ± 0.1 and very close to that obtained with the real-time PCR targeting E6 (2 ± 0.1).

78.2.2.3 Genotyping

Principle. Gravitt et al. [35] established a line probe assay (LiPA) for the identification of 16 different genotypes of the human papillomavirus. The assay focuses on specific sequences in the L1 region of the HPV genome. The INNO-LiPA HPV Genotyping CE is based on the principle of reverse hybridization. Part of the L1 region of the HPV genome is amplified, and the resulting biotinylated amplicons are then denatured and hybridized with specific oligonucleotide probes. These probes are immobilized as parallel lines on membrane strips. After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added, which binds to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen yields a purple precipitate and the results can be visually interpreted.

Procedure

1. Amplify the HPV DNA target, using a consensus primer set (i.e., MY09–MY11, GP5+ /GP6+).
2. Hybridize the amplified PCR product on the strip, followed by stringent wash.
3. Add the conjugate and substrate, resulting in color development.
4. Interpret visually the signal pattern.

Note: A sample is considered HPV positive if at least one of the type-specific lines or one of the HPV control lines is positive. A 100% intra- and interrun concordance was achieved for the predominant genotype present in the sample.

78.3 CONCLUSION AND FUTURE PERSPECTIVES

Currently, molecular detection of HPV DNA is the gold standard for the identification of HPV. The clinical material available in combination with the scope of studies will define the use of the HPV detection method. For the majority of clinical specimens, the accurate molecular diagnosis of HPV infection and extensive typing relies on the detection of viral nucleic acid, using consensus PCR followed by reverse hybridization. In the future, it is expected that, with the advance of technology, viral DNA extraction and amplification systems will become more rapid, more sensitive, and even more automated.

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