# Human Papillomaviruses Genotyping by Pyrosequencing Method

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Human papillomaviruses (HPV) are a group of DNA tumor viruses. There are more than 200 HPV types known to date that cause benign and malignant lesions in the skin and mucosa. Approximately 30-40 of them infect the anogenital tract. Based on their oncogenic potential in cervical cancer, they are divided into risk groups; high-, probable- or low-risk HPVs. The oncoproteins E6 and E7 of high-risk HPV genotypes have high affinities for binding and degrading tumor suppressor proteins p53 and pRb, which disrupt the cell cycle checkpoints and subsequently induce carcinogenesis. With increasing knowledge of disease outcome and vaccination program, HPV genotyping for HPV infected patients will be important for disease management. HPVs are mainly identified by molecular methods. The most applied approaches for HPV genotyping are 1) direct nucleic acid hybridization, 2) signal amplification systems, 3) target amplification systems and 4) DNA sequencing. Among these approaches, DNA sequencing is the gold-standard method as it provides sequence information. However, a challenge for DNA sequencing is the presence of multiple co-infections in a sample. This issue has been addressed by Pyrosequencing technology and multiple sequencing primers approach, which allow accurate detection and identification of multiple HPV co-infections in a sample.

**Keyword**s: Human papillomavirus; genotypes; Pyrosequencing

## 1. Human papillomavirus (HPV)

HPV causes the most prevalent sexually transmitted viral infection among men and women. It has been reported that about 80% of sexually active adults have been infected with at least one HPV type [1].

HPVs are a group of DNA tumor viruses, belonging to family *Papillomaviridea*. They are small circular double stranded DNA viruses, non-enveloped, about 55nm in diameter, with an approximately 8000 base pair (bp) genome encased in a naked icosahedral capsid. Most HPVs have eight open reading frames (ORFs) and all of them are located on one strand of the genome. The genome is divided into three regions according to their functions. The first region is the non-coding upstream regulatory region, it is 400-1000 bp long and is also known as the long control region (LCR) or the upper regulatory region (URR). Functionally, it regulates viral replication and gene transcription. The second is an early region, encoding 6 early proteins (E1, E2, E4, E5, E6, and E7), which are involved in viral replication and viral oncogenesis. The third region is a late region, which encodes the major (L1) and minor (L2) structural proteins for the viral capsid [2]. However, gene expressions of E1-E4 late transcripts are expressed simultaneously with the late proteins. They play a role in binding to cellular cytoskeleton and are involved in cell lysis in productive infections [3-5].

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To date, more than 200 HPV types have been identified based on their sequence information. A new HPV type should not share more than 90% homologous to the L1 genes sequences of known HPV types. HPV types can be further divided into subtypes, when they have a 90 to 98% sequence similarity to the corresponding type and variants are when they show no more than 98% sequence homology to the prototype.

## 2. HPV associated diseases

HPVs infect the basal epithelia of skin and mucosa, thus they have been classified as cutaneous types and mucosal types. They are epitheliotropic and do not seem to infect across different species, example, humans do not get infected by papillomaviruses from cows or rabbits. Cutaneous HPVs cause skin warts on hands and feet, for example HPV types 1, and type 2 cause plantar warts. HPV types 1, 2, and 7 are responsible for common warts, while HPV types 3 and 7 caused flat warts. HPV types 5 and 8, are known for causing epidermodysplasia verruciformis, which is a rare genetic disease but can turn cancerous by sunlight exposure. However mucosal types induce anogenital diseases; about 90% of genital warts (condyloma accuminata) are caused by HPV types 6 and 11 [6, 7], and only about 70% of cervical cancers are caused by HPV types 16 and 18 [8] (see Table 1).

**Table 1** HPV and their associated diseases

| **HPV and their associated diseases** | |
|---|---|
| **diseases** | **HPV** |
| Plantar warts | 1, 2, 4, 63 |
| Common warts | 2, 1, 7, 4 |
| Flat warts | 3, 7,10, 26, |
| Epidermodysplasia verruciformis | 5, 8, 20, 21, |
| Recurrent respiratory papillomatosis | 6, 11 |
| Oral focal epithelial hyperplasia (Heck diseases) | 13, 32 |
| Conjunctival papillomas/carcinomas | 6, 11, 16 |
| Condyloma acuminata (genital warts) | 6, 11, 42, 43, |
| Cervical low grade intraepithelial neoplasia | 6, 11, 16, 18, 31, |
| Cervical high grade intraepithelial neoplasia | 16, 18, 31,33,45,51, 52,58 |
| Cervical carcinoma | 16, 18, 31,33,45,51, 52,58 |

## 3. Risk factors of HPV

High risk HPV, HPV variants, viral load, multiple co-infections and viral integration may influence the viral-host biological interaction and play a role in the development of HPV associated diseases.

### 3.1 High-risk HPV types

Among the mucosal types, HPVs can be grouped according to their association with cervical cancer and their precancerous lesions: oncogenic high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), probable oncogenic high-risk types (HPV 26, 53 and 66), and non-oncogenic low-risk types (HPV 6, 11, 42, 43, 44, 54, 61, 70, 72, 81 and 89). Genital warts are often caused by low-risk HPVs, while cervical cancer and precancerous lesions are caused by high-risk HPVs [8]. Furthermore, it has been shown that high-risk HPV can transform human cells in tissue culture [9, 10]. The oncoproteins of high-risk HPV E6 and E7 drive cell proliferation through their association with PDZ domain proteins



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and retinoblastoma gene (pRb), and contribute to neoplastic progression, whereas E6-mediated p53 degradation prevents the repair of damaged DNA in the cellular genome. Cancers usually arise in individuals that have persistent infection and retain oncogene expression for years or decades [11].

### 3.2 HPV variants

HPV variants have different biological and biochemical properties important to cancer risk [12]. These variants often appear to have disparate geographical and ethnic origins. Most studies of HPV variants focus on high-risk HPV 16 which has 5 known variants: European (E), Asian (As), Asian-American (AA), African-1 (Af1) and African–2 (Af2). Asian-American variants have apparently higher oncogenic potential than European variants [13].

### 3.3 Viral load

Viral load refers to the HPV virus copy number in the infected cells. It has been suggested that viral load correlates to cervical disease severity [14, 15]. However, some studies showed that a high viral load alone is insufficient to induce progression from HPV infection to cervical intraepithelial neoplasia (CIN) 2, 3 and cancer [16, 17]. Furthermore, low levels of all types of high-risk HPVs are able to induce tumorigenesis [18].

### 3.4 Viral integration

Viral integration has been reported to be associated with carcinogenesis. Integrated HPV is more frequently found in high grade squamous intraepithelial lesion (HSIL) and cervical cancer than in low grade squamous intraepithelial lesion (LSIL) [19]. During carcinogenesis part of HPV E2 is deleted, and the loss of full length E2 expression leads to increased expression of E6 and E7 oncoproteins [20]. High-risk HPV E6 and E7 impair p53 and pRb functions respectively, causing the cell to escape cell cycle check point surveillance, subsequently leading to genome instability and cell immortalization [21]. Persisting HPV infection may be a consequence of viral integration.

### 3.5 Multiple HPV co-infections

Multiple HPV co-infections have been reported in the literature [22-24]. Most of these are double co-infections, but triple, quadruple and even quintuple HPV co-infections have also been detected [22, 24, 25]. Multiple HPV genotypes, usually with at least one high-risk type, were found in 11.8% of patients with normal cytology and atypical squamous cell of unknown significance (ASCUS), in 34.5% of patients at CIN1 or 2, and in 4.5% of cervical cancer tissue samples [23].

## 4. HPV detection and typing methods

HPV infection is mainly diagnosed by molecular biology methods, because culturing and *in vitro* propagation of virus are impractical and serological methods are not sufficiently sensitive [26]. Accurate molecular diagnostic techniques for HPV detection and identification are of great importance for diagnosing patients at risk, and correct diagnosis is important for patient management. Such tools are also necessary for population based epidemiological studies, vaccination trials and studies on the natural history of disease progression and outcome.

The main methods applied for HPV detection are:

1) Direct nucleic acid hybridization, such as Southern blot or dot blot hybridization and *in situ* hybridization (ISH), which localize the HPV infection on cytological or biopsy samples and enable co-



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localization with other diagnostic markers [27]. Southern blot or dot blot hybridization can detect and type HPVs directly, but they are time-consuming and are limited by their low sensitivity and the requirement for large amounts of highly purified DNA.

2) Signal amplification systems such as Digene Hybrid Capture II (HC2) system (Digene Corp., USA) is a non-radioactive immunoassay based on the hybridization of target HPV DNA to labeled RNA probes. RNA-DNA hybrids are then detected using a specific antibody and a chemiluminescence detection system [28, 29]. A cocktail of two different probes is used, one targeting against 5 low-risk HPV genotypes (HPV 6, 11, 42, 43 and 44) and the other against 13 high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). This method is widely used to identify patients susceptible to high- or low-risk HPV infection in clinics and it has been approved by the Food and Drug Administration (FDA) in the US. HC2 has been shown to be robust and reproducible and has been widely used in clinical trials worldwide as a screening assay [30]. However, this technique is somewhat limited because it cannot determine the specific HPV type(s) present in the sample. The detection limit is about 5000 copies of viral genome; therefore it is less sensitive than Polymerase Chain Reaction (PCR) technique [31, 32] and cross-reactivity of the two probe cocktails [33, 34] can further reduce their positive predictive value [35].

3) Target amplification systems or PCR is the most widely used method for target amplification method and it is the most commonly used method to detect HPV DNA because of its simplicity and high degree of sensitivity. Two approaches are used: a) Type-specific PCR using type-specific primers which only allow amplification of a single HPV genotype. This method is comparatively labour-intensive and expensive and is used primarily in research. b) Broad-spectrum PCR employing general PCR primers and consensus PCR primers to amplify a broad spectrum of HPV genotypes. The most common primers used are MY09/11 [36], PGMY [37], GP5+/6+ [22], and $SPF_{10}$ [11], which target the conserved and variable regions in the HPV L1 ORF. In order to identify patients at-risk for persistent HPV infections (the same HPV type is present >1 year), we need to know the HPV genotype. After PCR amplification, we can use different methods to acquire HPV genotypes, such as reverse line blotting (RLB) [38], restriction fragment length polymorphism (RFLP) analysis [39], and DNA sequencing [40]. RLB detects HPV genotypes through the hybridization of PCR products to different HPV probes immobilized onto nitrocellulose membrane. This method is adequately sensitive to detect specific HPV types and multiple genotypes in a single sample, but there is the possibility of false hybridization and non-specific discrimination of closely related types. RFLP is based on HPV genotype-specific restriction patterns. It is a time-consuming and labour-intensive procedure, and sometimes HPV RFLP data is difficult to interpret especially in cases of mutations. DNA sequencing is the gold-standard method for HPV genotyping. It provides sequence information for uncharacterized HPV genotypes as well as mutation information. But conventional DNA sequencing technique is not effective for the detection of multiple HPV co-infections in a single sample, because the sequence signals generated are mixed and the results are difficult to interpret. In contrast to traditional Sanger sequencing, Pyrosequencing is a real-time DNA sequencing method. Integrating the multiple sequencing primers method with Pyrosequencing allows for accurate detection of multiple HPV co-infections in one sample at a low cost [41, 42].

Another method that may be useful in HPV genotyping is mRNA detection by reverse transcription (RT) PCR and real-time PCR to identify high-risk HPV E6 and E7 expression [43, 44]. This assay can actually determine whether these transforming genes are present and active, which could be more clinically valuable to identify at-risk patients as compared to available methods [45].

## 5. Pyrosequencing technology

Pyrosequencing is a DNA sequencing technique to determine nucleic acid sequences [46]. This method is a sequencing-by-synthesis technique that employs a series of enzymatic reactions to accurately detect short nucleic acid sequences during DNA synthesis [47, 48]. In principle, when a complementary nucleotide is incorporated by DNA polymerase into an already primed DNA template (sequencing primer hybridized to target DNA), an inorganic pyrophosphate (PPi) molecule is released. The released PPi is converted to ATP by ATP sulfurylase using adenosine phosphosulfate (APS) as substrate and this



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reaction provides energy for luciferase to oxidase luciferin, and consequently light is produced a peak in the pyrogram (compare to electropherogram in Sanger sequencing). Each signal peak is proportional to the number of nucleotides incorporated (e.g. a triple dTTP nucleotide incorporation generates a triple higher peak). Apyrase is a nucleotide-degrading enzyme, which continuously degrades ATP and non-incorporated dNTPs in the reaction mixture. There is a certain time interval between each nucleotide dispensation to allow complete degradation. For this reason, nucleotide addition is performed one at a time. During the sequencing-by-synthesis process, the primed-DNA strand is extended by complementary nucleotides, and the DNA sequence signals are displayed by the signal peaks in a pyrogram on a computer monitor. Base-callings are performed with integrated software with features for related SNP and sequencing analysis. Figure 1 shows the basic principle of Pyrosequencing method.

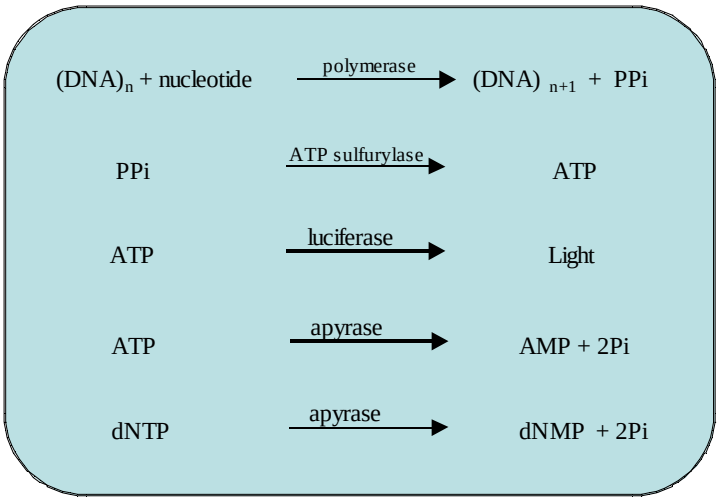


**Fig. 1** The principle of Pyrosequencing technology. For more details refer to text.

Pyrosequencing method is broadly used in many applications such as SNP genotyping [49], and for identification of bacteria [41], fungal [50] and viral typing [51]. This technique has also been applied in determination of difficult secondary structures [52], mutation detection [53], DNA methylation analysis [54], multiplex sequencing [25, 42, 55], tag sequencing of cDNA library [56] and clone checking [57].

Moreover, Pyrosequencing technology has been further developed into a massively-parallel microfluidic sequencing platform [58] by 454 Life Sciences Corporation (now acquired by Roche). The first generation of 454 sequencing platform (Genome sequencer 20 or GS20) is capable of sequencing 100 bases on average and generates up to 20 to 50 megabases (depending on the protocol) of raw DNA sequence in less than 5 hours. The company has recently released a GS FLX platform, which enables significant longer reads of 200-300 bases producing over 100 megabases per sequencing run. Microfludics Pyrosequencing method by 454 is being applied for whole genome sequencing, ultra broad sequencing [59] and ultra deep sequencing [60].

## 6. Multiple sequencing primers method by Pyrosequencing

Pyrosequencing has shown to be able to genotype HPV types by sequencing short stretches of highly heterogeneous HPV L1 [41]. Up to 25 bases are sufficient to classify different HPV genotypes found in cervical samples. However, multiple co-infections of HPV are a common phenomenon and studies show that multiple HPV infections can range from a few percent to almost 39% in different case study groups based on region and age. Conventional Sanger dideoxy sequencing and Pyrosequencing are not capable of distinguishing between different genotypes in a multiple HPV infected. When there are more than two HPV genotypes present in one sample or there is non-specific amplification in the PCR product, the



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sequence signals generated from all the genotypes result in mixed sequence signal peaks and consequently in non-interpretable sequence data both in Sanger dideoxy sequencing and Pyrosequencing. In such cases, PCR cloning is performed and different colonies are amplified and sequenced, which is time-consuming and expensive.

By using Pyrosequencing and multiple sequencing primers (MSP) methods, we can easily detect multiple infections in a sample. The MSP method employs target specific primers with each primer being specific for a specific HPV genotype. The primers are added to sequencing reaction mixture as sequencing primers and if several target species/genotypes are present in a sample, they are hybridized to the target HPV genotype(s). By using sequence pattern recognition, the sequence signals reveal each HPV genotype. The MSP method can also be used for samples with non-specific amplification products and amplicons with low yield [42]. Basically, only the primers having a specific complementary nucleotide sequence in the sample will hybridize, and thus, only the genotype related to the specific primer will result in sequence signals. MSP method eliminates the need for labour- and cost-intensive PCR cloning, and moreover, it is accurate, reliable, rapid, flexible, and cost-effective and has a general approach for microbial and viral typing.

2) Pattern recognition

Pattern recognition refers to the comparison of two sequence alignments of at least two sequence-pattern results. Pyrosequencing provides sequence signals in real-time and it is possible to differentiate these sequences aligned. A pre-programmed sequence pattern alignment can reveal each type in a multiple sequence alignment. Pattern recognition can also show the dominance or subdominance of multiple infections in the sample. Integrated software can easily identify different genotypes in a clinical sample (see figure 2).



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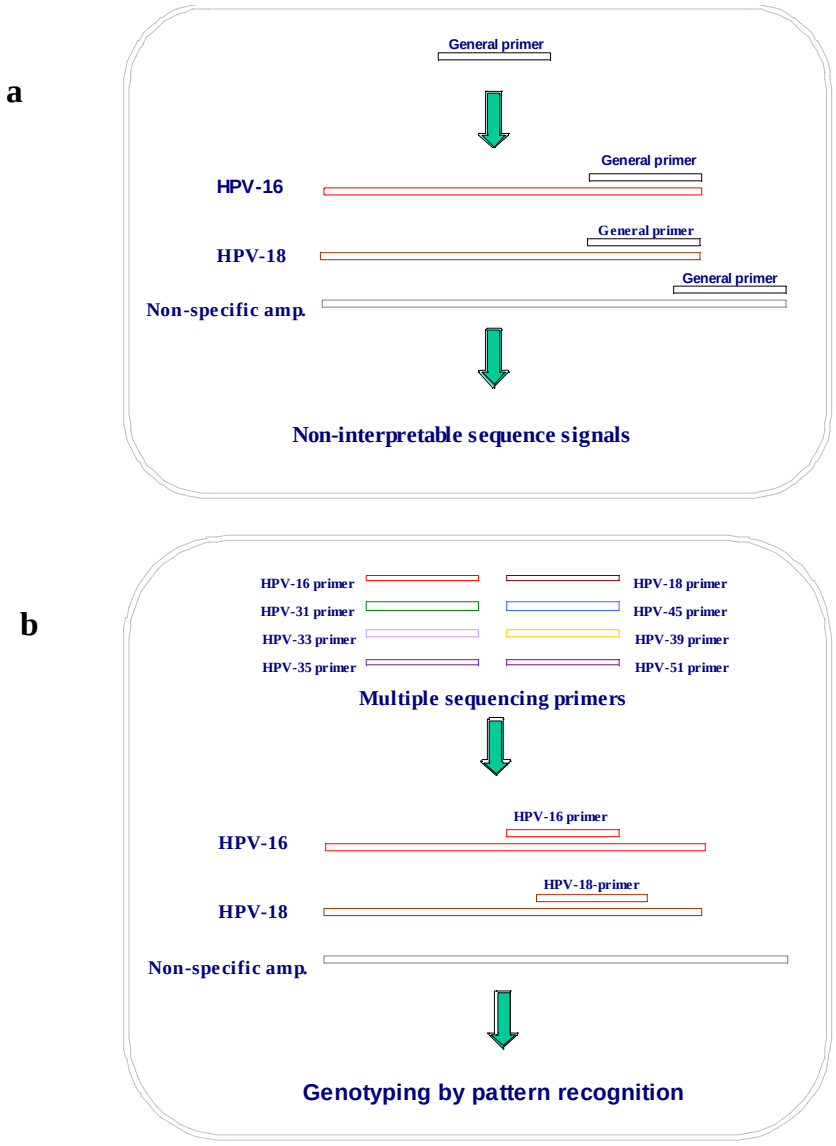


**Fig. 2** The principle of multiple sequencing primers method. In case of multiple infections and non-specific amplification products (2a), the general sequencing primer anneals to all and generates sequence results that are not interpretable. By using multiple sequencing primers method (2b), only specific primers will hybridize to target amplicons (HPV-16 and HPV-18) and the sequence results will analyzed and genotyped by sequence pattern recognition.

3) Sentinel base sequencing

MSP method has been further developed to genotype with significantly shorter reads also known as "sentinel" single base analysis, enabling multiple HPV typing in one sample. This approach focuses on identifying just one characteristic base, referred to as a "sentinel", that indicates the presence of a single or multiple HPV infections. In our study [25], the type-specific multiple sequencing primers were designed for the most prevalent high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). HPV-positive PCR products were genotyped by Pyrosequencing by four sequencing reactions using the GP5+ as general sequencing primer and three pools consisting of four HPV specific primers each.



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Each primer in each pool results in specific sequence pattern that can identify a specific HPV type (see Figure 3). This assay can easily genotype all of the twelve high-risk HPVs in a clinical sample in less than 10 minutes at a cost a very low cost by using DNA sequencing, which is the gold standard method.

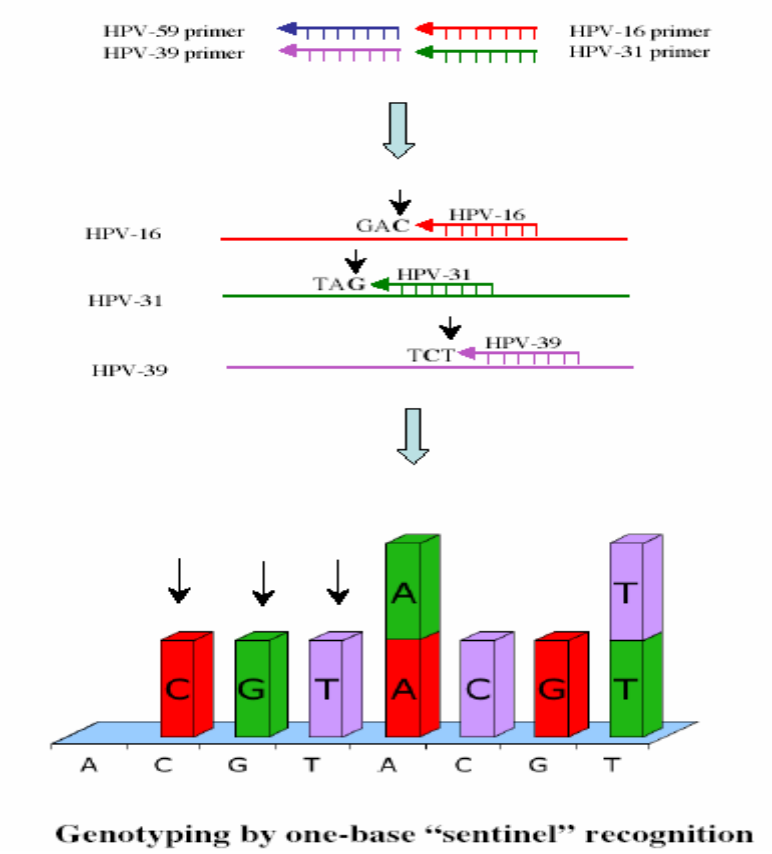


**Fig. 3** One-base "sentinel" sequencing. The assay shows genotyping of a triple HPV infection by using multiple sequencing primers method. The assay is designed in a way that each base acts as a sentinel or marker for each type.

## Concluding remarks

Pyrosequencing technology is a very versatile DNA sequencing tool that can be used for numerous applications such as SNP genotyping, short sequencing reads, microbial and viral typing. It is an excellent and cost-effective platform for HPV genotyping although multiple co-infections are an issue by conventional DNA sequencing technologies. By using multiple sequencing primers method, multiple HPV infections can be genotyped rapidly and cost- and time-effectively. The method is based on DNA sequencing, which is the gold-standard approach and the MSP method has general approach for other microbial and viral pathogens. Moreover, the new method eliminates the time-consuming and costly cloning of PCR products of multiple co-infections. Given the flexibility of the method, more type-specific primers could easily be added to expand the HPV detection spectrum and tailor it according to specific regional and demographic prevalence of not only HPV types, but also other pathogenic microorganisms and viruses.



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