Published in final edited form as:

Mol Cell Probes. 2006 ; 20(3-4): 230–238. doi:10.1016/j.mcp.2006.01.002.

Sentinel-base DNA genotyping using multiple sequencing primers for high-risk human papillomaviruses

Baback Gharizadeh^{a,1}, Biying Zheng^{b,1}, Michael Akhras^{a,c}, Mehran Ghaderi^d, Olufisayo Jejelowo^e, Björn Strander^f, Pål Nyrén^c, Keng-Ling Wallin^b, and Nader Pourmand^{a,*}

^aStanford Genome Technology Center, Stanford University, 855 California Avenue, Palo Alto, CA 94304, USA

^bDepartment of Molecular Medicine, Karolinska Institute, Center for Molecular Medicine, SE-171 76 Karolinska University Hospital, Stockholm, Sweden

^cDepartment of Biotechnology, Royal Institute of Technology, SE-106 91 Stockholm, Sweden

^dClinical Pathology/Cytology, Karolinska University Hospital, SE-171 76 Stockholm, Sweden

^eTexas Southern University, 3100 Cleburne Street, Houston, TX 77004, USA

^fOncology Center, Sahlgren's Academy, University of Göteborg, Göteborg, Sweden

Abstract

Despite the various technologies in place for genotyping human papillomaviruses (HPV), clinical use and clinical research demand a method that is fast, more reliable and cost-effective. The technology described here represents a breakthrough development in that direction. By combining the method of multiple sequencing primers with DNA sequencing, we have developed a rapid assay for genotyping HPV that relies on the identification of a single, type-specific 'sentinel' base. As described here, the prototype assay has been developed to recognize the 12 most high-risk HPV types (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and is capable of recognizing and simultaneously genotyping multiple HPV co-infections. By providing sequence information on multiple HPV infections, this method eliminates the need for labor- and cost-intensive PCR cloning. These proof-of-concept studies establish the assay to be accurate, reliable, rapid, flexible, and cost-effective, providing evidence of the feasibility this technique for use in clinical settings.

Keywords

Human papillomaviruses (HPV); DNA sequencing; Multiple infections; Multiple sequencing primers; Sentinel-base DNA sequencing; Pyrosequencing technology

1. Introduction

1.1. HPV and cervical cancer: the need for new detection systems

Human papillomaviruses (HPV) are known to play an important role worldwide in the development of cervical cancer [1,2]. To date, there are more than 80 fully characterized HPV genotypes of which approximately 40 infect the genital mucosa [3]. Based on their oncogenic

potential [1,4] these genotypes have been categorized as high-risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82); probably oncogenic (HPV-26, 53, 66); and low-risk (HPV-6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89). Given the diversity of the HPV spectrum and the high incidence of multiple HPV co-infections, accurate detection and genotyping of HPVs is key to the clinical management of cervical lesions and cancer prevention [5]. An accurate and inexpensive genotyping method would allow for detection of viral persistence and more precise monitoring of antiviral treatments [6].

1.2. Current genotyping methods

It is a difficult task to adequately culture HPV in vitro, and cytological and histological examinations reveal the consequences of a viral infection rather than quantifying or characterizing the virus itself [6]. There are several DNA-based techniques available for HPV genotyping. The majority are PCR-based assays using various PCR primer combinations, such as the GP5+/6+ and MY09/11 [7,8], SFP₁₀ [9] and PGMY09/11 [10] systems.

For genotyping purposes, these assays have been combined with a variety of hybridization techniques, including dot blots [11,12], reverse hybridization line probe assays [13,14], T-ladder generation [15], and DNA microarray [16,17]. A general drawback of hybridization techniques is that they may result in cross-hybridizations of closely related genotypes as well as non-specific hybridization.

Several non-PCR techniques have also been described, such as the Hybrid Capture II system (HCII, Digene Corp., USA), the in situ hybridization [18] and RNA-based hybridization approach [19]. HCII is a user-friendly method that enables discrimination between certain low and high-risk HPV genotypes, without identifying the actual genotype to the user. Although simple to use, the HCII system has lower sensitivity than the PCR methods [20,21].

Although DNA sequencing produces the highest resolution of nucleic acid-based genotyping [22–24], it has been limited to sequencing of clean amplicons and single HPV infections. This has been addressed with introduction of multiple sequencing primers method [25] and sequence pattern recognition [26].

1.3. ‘Sentinel-base’ genotyping

To address the current needs in HPV-associated cervical cancer, we have developed a novel and efficient assay for genotyping single and multiple HPV infections, based on a multiple sequencing primers method described in 2003 [25] in combination with Pyrosequencing technology [23,27]. In this sequencing assay, a single base acts as a sentinel, calling out the presence of a particular HPV genotype in the sample material. By careful choice of primers and multiplexing, the assay can be made to characterize the sample with respect to clinically relevant HPV genotypes.

The type-specific sentinel-base sequence characterization assay involves adding a pool of several DNA primers to a sequencing reaction containing amplified DNA. Each sequencing primer is designed to hybridize specifically to a particular HPV genotype and to generate a specific sequence pattern for that genotype. Within that sequence, the position and identity of the sentinel base signals the presence of that designated high-risk HPV type.

To make the process more efficient, multiple primers are pooled. Although in principle many primers can be used in a single pool, the current proof-of-concept assay uses just four primers per pool: a general PCR primer (GP5+) and three pools of sequencing primers. The amplified DNA is sequenced in four such pooled reactions so that 12 HPV genotypes are specifically recognized. No more than six nucleotide additions are needed for complete genotyping of the 12 HPV genotypes; the whole sequencing reaction takes no longer than 10 min.

For the sentinel-base HPV assay described here, sequencing primers were designed to recognize 12 of the most high-risk oncogenic genotypes [1]. A panel of 244 clinical samples was used to validate the current methodology using nested PCR MY09/11 and GP5+/6+ PCR primer sets, which amplify the conserved L1 region of the HPV genome and allow detection of a broad range of HPV types [7,28,29]. We further improved that assay by using a considerably lower (eight times) amount of PCR product and, moreover, employed a rapid Sepharose-bead sample preparation procedure

The novel sentinel-base recognition method proved to be highly accurate and reliable, and was especially suitable for detecting multiple HPV co-infections. In accordance with our intent to develop a method that would be not only useful in clinical settings but also cost- and time-efficient, we believe this approach to be a breakthrough worthy of further study and validation.

2. Materials and methods

2.1. HPV sequences and design of type-specific multiple sequencing primers

HPV sequences of the L1 region of HPV-6, 11, 16, 18, 26, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 67, 68, 69, 70, 71 (CP8061), 72 (LVX100), 73 (MM9), 81 (CP8304), 82 (MM4), 83 (MM7), 84 (MM8), CP6108 and IS324 genotypes were acquired through the Los Alamos National Database (<http://hvp-web.lanl.gov>). The sequences were analyzed for type-specific target sequence. The 12 type-specific sequencing primers for the high-risk HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 were designed based on the following conditions: (i) the target region for annealing each sequencing primer should have sufficient heterogeneity to allow design of type-specific sequencing primers with consideration taken for 3'-ends, as the similarity of the 3'-end with other genotypes may result in false cross-hybridization; (ii) the sequence base callings downstream of the sequencing primer (approximately 15–20 bases sequence information) should allow type-specific genotyping on BLAST/GenBank; (iii) a single base sequence should be enough for signal recognition of each genotype in each primer pool of four; (iv) for practical reasons and better differentiation, the most prevalent genotypes were distributed in the three primer pools; and (v) the sequencing primers should be designed in a way that does not result in strong self-hybridization (primer-dimers), self-looping or primer–primer cross-hybridizations that cannot be removed by single-stranded DNA-binding protein (SSB).

As shown in Table 1, the sequencing primers were mixed in three pools of four primers each. The sequencing primers were synthesized and HPLC purified by Thermo Hybaid (Germany). The final concentration of each primer in each pool was 4 pmole/μl.

2.2. Loop formation, primer–primer hybridization and cross-hybridization

To avoid false sequence signals in DNA sequencing, each type-specific sequencing primer was tested separately for self-hybridization and self-loop formation in the presence of SSB [30, 31] (Biotage, Uppsala, Sweden). The multiple sequencing primers of each pool (four primers in each pool) were also tested for primer–primer and cross-hybridization in the presence of 0.44 μg SSB in the Pyrosequencing reaction mixture when sequenced by the PSQ^{TM}HS96A System (Biotage, Uppsala, Sweden).

2.3. Sample collection and study population

The samples used in this study consist of archival cervical Pap smears and paraffin-embedded biopsies collected from western Sweden (Göteborg) and from northern Sweden (Umeå). We obtained 119 archival Pap smears from Sahlgrenska Hospital in Göteborg, 69 archival smears from Umeå University Hospital and 56 paraffin-embedded cervical biopsies from Umeå University Hospital. The cervical Pap smears retrieved from Göteborg represented a cohort of

women treated in Sahlgrenska Hospital for cervical intraepithelial neoplasia (CIN). The Pap smears from Umeå were taken from women diagnosed with CIN 2 or 3, and among the embedded biopsy samples 14 had been histologically diagnosed with CIN and 42 had been diagnosed to show invasive cervical cancer.

2.4. DNA extraction

2.4.1. DNA extraction from Pap smears—In brief, cover slips of Pap smears were removed with xylene and the cytoplasmic stain was removed in 95% ethanol. A mild lysis buffer containing 25 µl of proteinase K (20 mg/ml) (Sigma–Aldrich, St Louis, MO, USA) per 1.0 ml of buffer was used to dislodge the cells off the glass slide, and the cells were pipetted into a sterile Eppendorf tube. An additional 10 µl of proteinase K was added and digestion was carried out at 55–60 °C for a minimum of 2 h. Protein was precipitated by the addition of 100 µl saturated ammonium acetate, and centrifuged at 14,000 rpm at room temperature for 5 min. DNA was precipitated using cold ethanol and dissolved in low EDTA, TE (Tris–EDTA) buffer.

2.4.2. DNA extraction from histological samples—Tissue blocks were cut into five sections (4 µm thick) with a microtome. An empty paraffin block was sectioned in between tissue blocks to avoid cross-contamination. The sixth section was stained with haematoxylin and eosin for review to ensure that the preceding sections contained tumor tissue for PCR analysis. All tissue sections were deparaffinized twice in xylene, dehydrated in graded alcohols, and the tissue pellet was incubated in digestion buffer (100 mM NaCl, 10 mM Tris–Cl, 25 mM EDTA, 5% SDS pH8, 10 µl of 20 mg/ml proteinase K). Digestion was carried out at 55–60 °C for a minimum of 2 h. Proteinase K was inactivated by incubation at 98 °C for 10 min. After cooling, 1 µl of each sample was used for PCR.

2.5. PCR amplification

HPV was detected by nested PCR using MY09/11 and GP5+/6+ primer sets, targeting the HPV L1 open reading frame. The PCR products of MY09/MY11 and GP5+/6+ were 450 and 150 bp long, respectively. The 50 µl PCR reaction volume contained 1 X AmpliTaq PCR buffer, 10 µM of each primer, 2 mM MgCl₂, 0.2 mM dNTP, 5 µl of 2% bovine serum albumin (BSA) and 1.5 U of Taq DNA polymerase (Promega, Madison WI, USA). The PCR reaction mixture was pre-incubated at 96 °C for 1 min to denature the genomic DNA. This was followed by 40 PCR cycles starting with a denaturation step for 30 s at 94 °C and annealing at 50 °C for MY09/11 or 40 °C for GP5+/6+ for 30 s, and extension at 72 °C for 45 s. After the last cycle, a final extension at 72 °C for 5 min was carried out. The two-step nested PCR was performed first with the MY09/MY11 primers and 1 µl of the 450 bp PCR product was added to the second PCR mixture containing the GP5+/6+ primers. Ten microliters of each PCR product was run on 2% agarose gel and stained with ethidium bromide. PCR targeting human S14 gene was used to evaluate the DNA quality for PCR reaction. Positive controls included DNA extracted from CaSki cervical cancer cell line. Blanks containing no DNA were included to check for contamination.

2.6. Single-strand separation

Single-stranded DNA samples were prepared semi-automatically using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage). In brief, 5–7 µl of biotinylated PCR product was immobilized onto 2.5 µl streptavidin-coated Sepharose High Performance beads (Amersham Biosciences, Piscataway, NJ, USA) by incubation at room temperature for at least 5 min with agitation at 1400 rpm using an Eppendorf Thermomixer R (Eppendorf AG, Hamburg, Germany). Single-stranded DNA immobilized on Sepharose beads was suspended in 12 µl annealing buffer (10 mM Tris–acetate pH 7.75, 5 mM Mg–acetate). Single-stranded DNA, corresponding to 5–7 µl PCR product for each sequencing reaction, was hybridized to 4 pmole

general sequencing-primer (GP5+) as well as to the three pools of 12 sequencing primers (4 pmol of each primer) at 90 °C for 2 min followed by incubation at 10 min at 60 °C and 5 min at room temperature.

2.7. DNA sequencing by pyrosequencing technology

Sequencing by Pyrosequencing technology was performed on an automated plate-based bench-top PSQ™HS96A system at a dispensing pressure of 625 mbar with 4 ms open time and 65 s cycle time. The nucleotide dispensation order was either ACGTACG or CGTACG. A cyclic nucleotide dispensation order of *n*(ACGT) could be used for longer DNA sequencing. The sequence results were obtained in pyrogram™ formats.

2.8. PCR cloning

For verification and typing purposes a test set of 37 clinical samples that were out of our detection range, were analyzed by PCR cloning and DNA sequencing. The cloning was performed with a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA) according to company instructions. On average 10 colonies from each cloned PCR amplicon were picked for further analysis. The cloned PCR fragments were amplified with the general GP5+/6+ PCR primers and were thereafter sequenced on the PSQ™HS96A System with GP5 + as the sequencing primer.

2.9. Detection range by pyrosequencing

The quantitative gel-based exACTGene 50 bp Mini DNA Ladder (Fisher Scientific, Pittsburgh, PA) was used to determine the minimum detection limit range of PCR amplicons with the automated plate-based bench-top PSQ™HS96A system. A clinical sample infected with HPV-16 was selected for this assay. The DNA concentration of the PCR product was measured by the exACTGene 50 bp Mini DNA Ladder according to manufacturer's instructions. A dilution series of the quantitative PCR amplicon was thereafter sequenced on the PSQ™HS96A system. The determination of the minimum detection limit range of the PSQ™HS96A system was based on the diluted PCR product to the point that readable sequence information could be obtained.

3. Results

3.1. Development of sentinel-base sequencing with multiple sequencing primers

The sequences of L1 regions of HPV genotypes infecting the genital mucosa were obtained from the GenBank and aligned. Type-specific sequencing primers were designed for the high-risk HPVs-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. The first 15–20 bases of sequence information downstream of the each type-specific sequencing primer are sufficient for accurate genotyping on BLAST search. To avoid false sequence signals from self-looping, primer dimers or cross-hybridizations of the primers, which could be extended by Klenow polymerase in the presence of nucleotides in the Pyrosequencing reaction, all the primers were tested in the presence of SSB for self-hybridizations, self-looping and primer–primer cross-hybridizations, separately and in each pool. None of the sequencing primers resulted in false sequence signals. The sentinel-base sequencing detection system is set up such that each clinical sample is sequenced separately in four sequencing reactions with the general primer GP5+ and the three type-specific sequencing primer pools. Each of the four type-specific primers in the three pools will result in a unique sequence pattern containing a sentinel-base, sufficient in its identity and position to identify a designated HPV type and allow detection of four HPV genotypes for each primer pool in each sequencing reaction. Fig. 1 shows the expected sequence in each pool of HPV-16, 31, 39 and 59; HPV-18, 33, 52 and 56; HPV-45, 35, 58 and 51. Fig. 2 highlights the sentinel-base unique characterization sequence for each

genotype. For demonstration of a multiple HPV co-infection, we have simulated a triple co-infection of HPV-16, 31 and 39 in Fig. 3.

3.2. Pyrosequencing and improved sample preparation

Using the PSQ^{TM}HS96A DNA sequencing system, which uses a more sensitive CCD camera and Capillary Dispensation Tips (CDTs) permitted the use of significantly lower PCR product volumes of 5–7 µl (eight times lower than the conventional Pyrosequencing used previously) thus decreasing the cost and labor for both PCR and sequence reactions. The minimum detection range for DNA sequencing was measured to be 0.14 pmole, which is very low. The PSQ^{TM}HS96A is primarily intended for analysis of single-nucleotide polymorphisms (SNP). We have added repetitive ACGT dispensation order for sequencing in the SNP Software version 1.2. We also used a new Sepharose bead separation system using the Vacuum Prep Worktable for single-strand separation of the amplified DNA. The Sepharose bead sample preparation is rapid and a 96-well microtiter plate can be processed in 10–15 min. The sequencing reaction for sentinel genotyping takes less than 10 min.

3.3. Sequencing of clinical samples

Amplification of a test panel of 244 clinical samples by nested MY09/11 and GP5+/6+ PCR yielded amplicons of approximately 150 bp. The efficiency of the PCR amplifications was evaluated by gel electrophoresis and ethidium bromide staining. The amplicons were sequenced by Pyrosequencing in four separate reactions by the multiple sequencing primer pools, each of which included the GP5+ PCR primer and three pools consisting of four primers each. For comparison, the samples were sequenced individually using the general primer to obtain their genotype. Where necessary, HPV DNA was cloned from the sample and then sequenced. Of the 244 clinical samples, 207 were positive in our assay (85%) with 147 single infections, and 41 double, 10 triple, 7 quadruple and 2 quintuple HPV co-infections. These results are summarized in Fig. 4. The current sentinel base system covers 12 most high-risk HPV types and 37 (15%) samples were not in the detection range.

Fig. 5 shows sample results for each of the 12 high-risk HPV genotypes detectable by this assay in 12 representative clinical samples that showed single HPV infections.

Fig. 6a and d show the results from the sentinel-base method for a sample with quadruple co-infection of HPV-16, HPV-31 (detected by primer pool 1) and HPV-33 and HPV-52 (detected by primer pool 2). To validate the use of the sentinel-base method for identifying multiple co-infections, all the multiple infections were sequenced with the respective sequencing primers in separate sequencing reactions, and the sequence results were analyzed by BLAST search. Thus, the same sample was sequenced with each primer separately for confirmation (Fig. 6b, c, e and f). The results from individual sequencing runs were in agreement with the multiple sequencing primers method.

Thirty-seven samples were detected to be HPV positive by amplification but were not in the detection range. Of these, six samples were genotyped as HPV-81 by GP5+. The remaining 31 samples did not result in interpretable sequence signals by GP5+ primer due to non-specific amplification products or multiple co-infections or both. Therefore, the remaining 31 samples were cloned for DNA sequencing, and 10 colonies of each sample were sequenced using the GP5+ primer. The genotyping results of these samples are shown in Table 2. The results indicate sixteen different HPV types outside the spectrum of the current assay, of which four samples had double infections of HPV-32 and 74, HPV-43 and 67, HPV-72 and 81, and HPV-62 and 67.

We also detected sequence variations in HPV-33, -45 and -56 in the samples tested by the multiple sequencing primers method (Table 3). These sequence variations were analyzed on BLAST and they were all known sequence variations. These variations did not confound the sentinel-base sequencing pattern recognition, which were designed with natural variation in mind.

4. Discussion and conclusions

DNA sequencing is the gold standard method for microbial and viral typing, but its use has been limited to sequencing clean PCR products. Our novel assay based on a sentinel-base sequence analysis for identifying and genotyping the 12 most oncogenic HPVs is capable of genotyping samples harboring multiple infections, non-specific amplification products and samples with low yield. In two earlier studies, we introduced the principle of the multiple sequencing primers method [25] and applied this method to clinical samples with a pool of just seven sequencing primers for sequencing and genotyping HPV-6, 11, 16, 18, 31, 33 and 45 based on sequence pattern recognition [26]. Comparison of results with Sanger dideoxy sequencing has shown the multiple sequencing primers method to be an accurate and reliable approach [25] since it is based on DNA sequencing. In this study, we brought that technology to a new level with the development of a sentinel-base sequencing approach. The current study shows the utility of the combined sentinel-base approach with multiple sequencing primers for genotyping high-risk HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 in just four pools. Naturally, the primers and their downstream sequences need to be carefully chosen to provide unique sentinels.

The presence of multiple co-infections of HPV is a common phenomenon that can be of clinical significance. The percentage of multiple co-infections varies depending on the group studied and there are studies showing up to 35 and 50% of co-infections [32]. Of the 244 clinical samples analyzed, we were able to identify 60 multiple co-infections of high-risk HPVs, representing ~25% of the total number of all the samples and ~29% of the sentinel detection range.

The rationale behind the use of the general PCR primer GP5+ (as a sequencing primer) in parallel with multiple sequencing primers was to detect other HPV types that were single infections and did not contain any dominant non-specific amplification products. Finally, the sequence variations that we detected and confirmed for HPV-33, 45 and 56, indicate the power of this method for detection of HPV sequence variations.

In summary, we have developed a reliable, rapid, cost-effective HPV genotyping method that is based on sentinel-base sequence information by a multiple sequencing primers method. The sentinel-base method is remarkably user-friendly, and permits significantly more efficient and rapid genotyping. The process time for sample preparation and sequencing has been decreased considerably, and the method detects a wide range of HPV types based on one base of sequence information. The new method eliminates the time-consuming and costly cloning of multiple co-infections of HPV. The primers introduced here can also be used separately for sequencing of specific genotypes. More type-specific primers are currently being added to expand the recognition spectrum. Given the flexibility of the method, it can be scaled to recognize a greater spectrum of HPV types, or tailored for regional and demographic prevalence of HPV types, or used for other specific genotyping purposes.

Acknowledgments

This study was supported by grants from the National Institute of Health (1R21 A1059499-01 and PO1-HG000205). The other funding sources were from Tore Nissson Stiftelse (BG), the Medical Research Council, the Swedish Cancer

Society, the Swedish Medical Association, the Karolinska Institutet, the Stockholm county council and the Swedish Cancer Foundation (KLW, BZ) and NASA Grant NCC9-165 and NCC-647 (OJ,BG).

References

1. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348:518–527. [PubMed: 12571259]
2. zur Hausen H, Gissmann L, Schlehofer JR. Viruses in the etiology of human genital cancer. *Prog Med Virol* 1984;30:170–186. [PubMed: 6087412]
3. Burd EM. Human papillomavirus and cervical cancer. *Clin Microbiol Rev* 2003;16:1–17. [PubMed: 12525422]
4. Terai M, Burk RD. Identification and characterization of 3 novel genital human papillomaviruses by overlapping polymerase chain reaction: candHPV89, candHPV90, and candHPV91. *J Infect Dis* 2002;185:1794–1797. [PubMed: 12085327]
5. Giovannelli L, Lama A, Capra G, Giordano V, Arico P, Ammatuna P. Detection of human papillomavirus DNA in cervical samples: analysis of the new PGMY-PCR compared to the hybrid capture II and MY-PCR assays and a two-step nested PCR assay. *J Clin Microbiol* 2004;42:3861–3864. [PubMed: 15297550]
6. van Doorn LJ, Kleter B, Quint WG. Molecular detection and genotyping of human papillomavirus. *Expert Rev Mol Diagn* 2001;1:394–402. [PubMed: 11901854]
7. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* 1995;76:1057–1062. [PubMed: 9049358]
8. Resnick RM, Cornelissen MT, Wright DK, Eichinger GH, Fox HS, ter Schegget J, et al. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J Natl Cancer Inst* 1990;82:1477–1484. [PubMed: 2167986]
9. Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, et al. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* 1998;153:1731–1739. [PubMed: 9846964]
10. Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, et al. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000;38:357–361. [PubMed: 10618116]
11. Jacobs MV, de Roda Husman AM, van den Brule AJ, Snijders PJ, Meijer CJ, Walboomers JM. Group-specific differentiation between high-and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. *J Clin Microbiol* 1995;33:901–905. [PubMed: 7790457]
12. Ylitalo N, Bergstrom T, Gyllenstein U. Detection of genital human papillomavirus by single-tube nested PCR and type-specific oligonucleotide hybridization. *J Clin Microbiol* 1995;33:1822–1828. [PubMed: 7665652]
13. Coutlee F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, et al. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol* 2002;40:902–907. [PubMed: 11880413]
14. van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* 2002;40:779–787. [PubMed: 11880393]
15. Nelson JH, Hawkins GA, Edlund K, Evander M, Kjellberg L, Wadell G, et al. A novel and rapid PCR-based method for genotyping human papillomaviruses in clinical samples. *J Clin Microbiol* 2000;38:688–695. [PubMed: 10655368]
16. Choi YD, Jung WW, Nam JH, Choi HS, Park CS. Detection of HPV genotypes in cervical lesions by the HPV DNA Chip and sequencing. *Gynecol Oncol* 2005;98:369–375. [PubMed: 16024065]
17. Klaassen CH, Prinsen CF, de Valk HA, Horrevorts AM, Jeunink MA, Thunnissen FB. DNA microarray format for detection and subtyping of human papillomavirus. *J Clin Microbiol* 2004;42:2152–2160. [PubMed: 15131183]

18. Unger ER. In situ diagnosis of human papillomaviruses. Clin Lab Med 2000;20:289–301. [vi]. [PubMed: 10863642]
19. Lie AK, Risberg B, Borge B, Sandstad B, Delabie J, Rimala R, et al. DNA- versus RNA-based methods for human papillomavirus detection in cervical neoplasia. Gynecol Oncol 2005;97:908–915. [PubMed: 15943992]
20. Cope JU, Hildesheim A, Schiffman MH, Manos MM, Lorincz AT, Burk RD, et al. Comparison of the hybrid capture tube test and PCR for detection of human papillomavirus DNA in cervical specimens. J Clin Microbiol 1997;35:2262–2265. [PubMed: 9276398]
21. Smits HL, Bollen LJ, Tjong AHSP, Vonk J, Van Der Velden J, Ten Kate FJ, et al. Intermethod variation in detection of human papillomavirus DNA in cervical smears. J Clin Microbiol 1995;33:2631–2636. [PubMed: 8567896]
22. Feoli-Fonseca JC, Oligny LL, Brochu P, Simard P, Falconi S, Yotov WV. Human papillomavirus (HPV) study of 691 pathological specimens from Quebec by PCR-direct sequencing approach. J Med Virol 2001;63:284–292. [PubMed: 11241459]
23. Gharizadeh B, Kalantari M, Garcia CA, Johansson B, Nyren P. Typing of human papillomavirus by pyrosequencing. Lab Invest 2001;81:673–679. [PubMed: 11351039]
24. Vernon SD, Unger ER, Williams D. Comparison of human papilloma-virus detection and typing by cycle sequencing, line blotting, and hybrid capture. J Clin Microbiol 2000;38:651–655. [PubMed: 10655362]
25. Gharizadeh B, Ghaderi M, Donnelly D, Amini B, Wallin KL, Nyren P. Multiple-primer DNA sequencing method. Electrophoresis 2003;24:1145–1151. [PubMed: 12707905]
26. Gharizadeh B, Oggionni M, Zheng B, Akom E, Pourmand N, Ahmadian A, et al. Type-specific multiple sequencing primers: a novel strategy for reliable and rapid genotyping of human papillomaviruses by pyrosequencing technology. J Mol Diagn 2005;7:198–205. [PubMed: 15858143]
27. Ronaghi M, Uhlen M, Nyren P. A sequencing method based on real-time pyrophosphate. Science 1998;281:363. [see also page 365]. [PubMed: 9705713]
28. Evander M, Edlund K, Boden E, Gustafsson A, Jonsson M, Karlsson R, et al. Comparison of a one-step and a two-step polymerase chain reaction with degenerate general primers in a population-based study of human papillomavirus infection in young Swedish women. J Clin Microbiol 1992;30:987–992. [PubMed: 1315341]
29. Strauss S, Jordens JZ, Desselberger U, Gray JJ. Single-tube real-time nested polymerase chain reaction for detecting human papillomavirus DNA. Diagn Mol Pathol 2000;9:151–157. [PubMed: 10976722]
30. Ehn M, Ahmadian A, Nilsson P, Lundeberg J, Hober S. *Escherichia coli* single-stranded DNA-binding protein, a molecular tool for improved sequence quality in pyrosequencing. Electrophoresis 2002;23:3289–3299. [PubMed: 12373756]
31. Ronaghi M. Improved performance of pyrosequencing using single-stranded DNA-binding protein. Anal Biochem 2000;286:282–288. [PubMed: 11067751]
32. Goncalves MA, Massad E, Burattini MN, Villa LL. Relationship between human papillomavirus (HPV) genotyping and genital neoplasia in HIV-positive patients of Santos City, Sao Paulo, Brazil. Int J STD AIDS 1999;10:803–807. [PubMed: 10639061]

Fig. 1. Simulation of expected HPV sequence by each respective primer in each primer pool. Each graph shows the expected sequence for each genotype by using the multiple sequencing primer pool. Each pool consists of four sequencing primers (one general HPV primer and three type-specific primers).

Fig. 2. Simulation of results showing the designated sentinel base characterizing each HPV genotype. The assay is designed such that one specific base of sequence information is sufficient for genotyping. Each graph shows the sentinel-base sequence information for each HPV genotype.

Triple co-infection of HPV-16,-31 and 39

Fig. 3.

A sequence simulation of multiple co-infection of HPV-16, -31 and -39 sequenced by primer pool 1, which has the ability to detect HPV-16, 31, 39 and 59 (Fig. 1).

Fig. 4. Genotyping results by the sentinel detection method demonstrating single infections as well as multiple co-infections of HPVs.

HPV quadruple co-infection

Fig. 6. Pyrograms of a quadruple HPV co-infection sequenced and genotyped by sentinel sequencing method; a and d demonstrate HPV-16, -31 (detected by primer pool 1) and HPV-33, -52 (detected by primer pool 2). For confirmation, the same sample was sequenced with each primer separately; these results are shown in b, c, e and f.

Table 1

Designed multiple sequencing primers for 12 high-risk HPVs

Primer name	Group	Sequencing primers sequence (5'–3')	No. of bases
HPV-16	1	ATTATGTGCTGCCATATCTACTT	23
HPV-31	1	GTGCTGCAATTGCAAACAGT	20
HPV-59	1	CTACTACTTCTTCTATTCTAA	22
HPV-39	1	CTATAGAGTCTTCCATACCT	20
HPV-18	2	ACAGTCTCCTGTACCTGGG	19
HPV-33	2	AATTTTAAAGAATATATAAGACA	27
HPV-52	2	TATGTGCTGAGGTTAAAAAG	20
HPV-56	2	TACTAACATGACTATTAGTACT	22
HPV-45	3	CTCTACACAAAATCCTGTGC	20
HPV-35	3	GTTCTGCTGTGTCTTCTAGT	20
HPV-58	3	ACTGAAGTAACTAAGGAAGG	20
HPV-51	3	ACTATTAGCACTGCCACTGC	20

Multiple sequencing primers designed for sequencing and genotyping of high-risk HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59. The group of each primer is also indicated.

Table 2

Genotyping results by PCR cloning and pyrosequencing for samples out of the detection range of the 12 multiple sequencing primers

HPV genotype	No. of samples
HPV-6	3
HPV-11	1
HPV-32	2
HPV-40	1
HPV-43	1
HPV-53	2
HPV-62	5
HPV-66	1
HPV-67	2
HPV-70	2
HPV-72	3
HPV-74	1
HPV-81	9
HPV-82	1
HPV-90	6
HPV-91	1

Genotyping results of samples that were out of the detection range of the 12 multiple sequencing primers. Only six of these samples could be genotyped by GP5+ primer and the remaining 31 samples were genotyped by PCR cloning and sequencing.

Table 3

Sequence variations of HPV-33, -45 and -56 detected by multiple sequencing primers and their prevalence

HPV-genotype	HPV-33	HPV-45	HPV-56
Seq. variation 1	TGT TGA AGA ATA	CAA GTA CAT AT	GCT ACA GAA C
Prevalence	23/53	4/11	8/14
Seq. variation 2	TGg TGA GGA ATA	CAA aTA CAT AT	GCc ACt Gct C
Prevalence	18/53	3/11	6/14
Seq. variation 3	TGg TGA gGA ATt	CAg GTA CAT AT	
Prevalence	4/53	4/11	
Seq. variation 14	TGc TGA AGA ATA		
Prevalence	3/53		
Seq. variation 5	TGT gGA gGA ATt		
Prevalence	2/53		
Seq. variation 6	TGc aGA AGA ATA		
Prevalence	3/53		

Sequence variations of HPV-33, -45 and -56 that have been sequenced by multiple sequencing primers as well as the respective prevalence in the samples.