# Detection and Genotyping of Human Papillomavirus DNA Using Polymerase Chain Reaction Short PCR Fragment 10–Line Probe Assay in Abnormal Papanicolaou-Stained Cervicovaginal Smears

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**Objective**

*To evaluate the effectiveness of the short PCR fragment 10–line probe assay (SPF10-LiPA) system in detection of human papillomavirus (HPV) in abnormal Papanicolaou-stained cervicovaginal smears.*

**Study Design**

*We included 20 abnormal Papanicolaou-stained cervicovaginal smears. Samples were tested for HPV DNA detection and genotyping using the SPF10-LiPA system.*

**Results**

*All samples amplified the INF150 gene control. In 2 of 20 cases, amplification of the viral sequences was negative. Of processed samples, 18 of 20 presented HPV infection; of them, 56% showed only 1 type of HPV infection; the remaining presented ≥2 types of HPV (multiple infections). HPV16 was the most frequent specific viral in 60%, in single and multiple infections, followed by HPV18 (20%), HPV6 (10%) and HPV58 (10%). We also found HR-HPV45, 52, 58 and 68 and LR-HPV6, 11 and 70 viral DNA types. These multiple infections were detected with greater frequencies in atypical squamous cells of undetermined significance and in the low-grade squamous intraepithelial lesions.*

***Our results confirm that it is possible to identify the HPV DNA sequences from abnormal Papanicolaou-stained cervical smears using the SPF10–LiPA system. . . .***

**Conclusion**

*The SPF10-LiPA system is efficient, sensitive, fast and simple for detecting and genotyping HPV DNA in abnormal Papanicolaou-stained cervicovaginal smears, which could be enormously useful in routine investigation for better clinical management of the patient.* (Acta Cytol 2009; 53:540–547)

**Keywords:** cytology, DNA HPV, genotype, polymerase chain reaction, SPF10-LiPA.

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Persistent infection for certain types of human papillomavirus (HPV) is considered the most important risk factor in the development of cervical cancer.[1-4] At the present time, diagnosis of this infection is carried out using polymerase chain reaction (PCR)[5] which is a highly sensitive and specific methodology, that permits the detection of viral genome sequences, in cell as well as tissue samples.[6-14]

The efficiency of PCR in the detection of HPV DNA depends on the quality of the sample under study, the sensitivity of the amplifying method and genotyping applied. Various protocols for the extraction of DNA have been tried to efficiently detect HPV sequences on cervical scrapes, archival samples, Papanicolaou-stained cervical smears and cervical biopsies. Some are more effective than others under similar temperature and time conditions. Many of these procedures are cumbersome and time-consuming, with excessive manipulation providing the opportunity for contamination between samples. Simple protocols in which the proteinase K buffer is used for cellular digestion seem to be the most adequate, given that loss or destruction of genomic material is minimal.[15-18] However, DNA extracted in the traditional way with phenol-chloroform, although it is not the most commonly recommended method,[16] also permits procuring optimum DNA samples.[7,19,20]

Successful amplification of genome segments of HPV using PCR depends, to a great extent, on the sensitivity of the primers used determined by the fragment size.[8,11,21] The viral load can also influence exact detection of HPV DNA, being greater if it is found in an elevated number of copies.[16,18,22] Cervical scrapes provide a limited quantity of epithelial cells. Moreover, if some of these cells contain low viral copies, they can produce errors, depending on the samples, that have repercussions on the sensitivity of the test, above all in the case of infection by ≥ 2 HPV genotypes present in different viral loads.[13]

Consensus primers such as MY09/11,[23] GP5+/6+[24] and short PCR fragment (SPF)[8] permit the detection of broad-spectrum viral genotypes carrying out only 1 PCR, on recognition of the L1 region of the different HPV. SPF10 primer sets of the INNO–line probe assay (INNO-LiPA) (Innogenetics Inc., Gent, Belgium) are considered highly effective for the detection of 25 different types of HPV, given that it amplifies with elevated sensitivity a small segment of 65 bp, very effective in cervical samples that have been treated before PCR, including the Papanicolaou-stained cervicovaginal smears for detection of cervical cancer, in which the DNA could be damaged, degraded or degenerated.[8,9]

Genotyping of HPV DNA has been carried out using reverse hybridization.[9,18,25-27] The LiPA method, which consists of a series of oligonucleotides of viral sequences fixed over a strip of nitrocellulose, has been evaluated and validated in several research studies to determine the specific presence of HPV in various types of clinical samples, present as the only viral type or as multiple infections.[9,21,22,28-32] This method has been considered rapid, simple and highly sensitive, capable of detecting more multiple infections than direct sequencing or the restriction fragment length polymorphism method. It is also reproducible, can be automated and is easy to interpret.[9,33,34] However, its specificity has been questioned because of the possibility of cross-reactivity in multiple infections, above all, between philogenetically related viral types, which led to false positive results,[30] even though in other research it has been demonstrated that LiPA identifies simultaneously with high sensitivity multiple viral types in the same cervical sample despite the low viral load found, allowing use, in principle, in routine research.[9,22,30,31,35]

Considering the value of Papanicolaou-stained cervicovaginal smears used in routine research for retrospective analysis, the objective of this study was to evaluate the effectiveness of the SPF10-LiPA system for the detection of HPV DNA in abnormal Papanicolaou-stained cervicovaginal smears.

## *Materials and Methods*

### *Materials*

Twenty Papanicolaou-stained cervicovaginal smears used in cervical cancer screening between 1998 and 2000 in the Pathological Anatomy Service of the University Hospital Clinic of Valencia, with the diagnosis of epithelial cell abnormalities, were selected randomly: atypical squamous cells of undetermined significance (ASCUS) (8), low-grade squamous intraepithelial lesion (LSIL) (3), high-grade squamous intraepithelial lesion (HSIL) (4), squamous cell carcinoma (4) and cervical adenocarcinoma (1). These samples were reevaluated considering the cytologic criteria established by the 2001 Bethesda System.[36]

### *Methods*

*Pretreatment.* To avoid contamination, cytologic samples were individually immersed in xylene for approximately 48 hours[17] to remove the coverslips. At the end of this time, samples that still did not detach from the cover were immersed in acetone and quick detachment was observed. Later, the samples were immersed in 90°, 80° and 70° ethanol for destaining and 100 µL of phosphate-buffered saline (PBS) was added over the cell matter to facilitate the scrape with a sterile scalpel for each case. The material was transferred to a sterile Eppendorf tube that contained 200 µL PBS and stored at –20°C, until PCR was performed.

*Isolation of DNA Cells.* Cellular material was centrifuged at 13,400 rpm for 1 minute to retire the supernatant, then a lysis solution of SDS 0.5%; 10 mM Tris-HCl, pH 8; 0.15 M NaCl; 5 mM EDTA and 0.5 mg/mL proteinase K was added and incubated for 2 hours at 56°C. To deactivate the proteinase K, it was incubated for 10 minutes at 95°C. Later, the genomic material was extracted with phenol equilibrated with Tris-HCl, pH 8.0, for 20 minutes, with occasional agitation and centrifugation to 13,400 rpm during 15 minutes. Phenol/CHISAM (chloroform-isoamyl alcohol, 24:1) was added and left to act for 1 hour and centrifuged again at 13,400 rpm for 15 minutes. Finally, CHISAM was added for 30 minutes and centrifuged at 13,400 rpm for 15 minutes. The supernatant was collected in a sterile Eppendorf tube, and the previously extracted DNA was precipitated with 0.1 volume 3 M sodium acetate, pH 5.2, and 1 volume cold absolute ethanol overnight at –20°C. The pellet was washed with 70% cold absolute ethanol and centrifuged at 13,400 rpm for 15 to 30 minutes. The ethanol was completely removed and the pellet dried in an oven at 37°C. The pellet was resuspended with 5–30 µL distilled water and later stored at –20°C until amplified by PCR. This method of extraction has been standardized and used in this laboratory for tissues samples; for this study, we reduced the time and the incubation temperature adapted for lysis and digestion of loose cells.

*Amplification of HPV DNA by PCR.*[8] PCR was performed to estimate the quality of extracted DNA amplifying the interferon gene *INF* (INF150DR:ctgggatgctcttcgacctc /INT150DF:tcttttctttcccgataggt primers). The samples with a neat band of electrophoresis for *INF* were submitted to amplification of a segment of 65 bp from the L1 region of the HPV DNA using the SPF10 primers set. The reaction was carried out to a total volume of 50 µL. The mixture of the reaction containing 2 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTPs, 10 µL of the SPF10 mixture biotinylated primers set, 1.5 IU of AmpliTaq Gold DNA polymerase (Perkin-Elmer) and 10 µL of isolated DNA. The conditions of the PCR in the thermocycler were initial denaturalization and activation of the polymerase at 94°C for 9 minutes, 40 cycles in which the denaturalization was repeated at 94°C for 30 seconds, annealing at 52°C for 45 seconds and elongation at 72°C for 45 seconds, with a final 72°C for 5 minutes extension. Positive controls for HPV6 (Innogenetics) and negative controls that contained only a reaction mix were included. The visualization of the product of the PCR-SPF10 was carried out using electrophoresis gel with 3% agarose dye with ethidium bromide. If the analyzed sample contained HPV DNA, the expected band had approximately 65 bp (Figure 1).

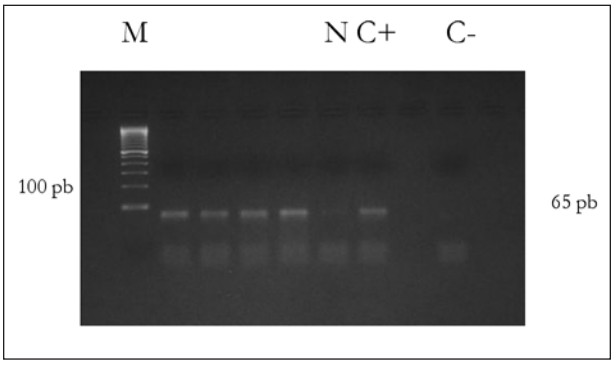


**Figure 1** Electrophoresis of the amplified HPV DNA with the SPF10 primer in some of the cytologic samples of this study. One of the samples did not show bands, so it was considered negative (N) for HPV. M = marker, C+ = positive control (HPV6, Innogenetics), C– = negative control.

*Reverse Hybridization LiPA for Genotyping of Amplified HPV DNA.*[9] The resulting HPV DNA–positive samples for the PCR-SPF10 were tested for genotyping of the associated HPV DNA using the HPV INNO-LiPA (Innogenetics). In this test, the product of PCR-SPF10 was mixed with an alkaline solution containing EDTA and placed in contact with the nitrocellulose strip that contains immobilized parallel lines of oligonucleotides for identifying the 25 specific types of HPV (types 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70 and 74) simultaneously in a single test. The strips were incubated for 1 hour at 49°C. The hybridization was proved by the presence of a purple color over the line(s) for the different viral types. The results of this hybridization were evaluated visually by comparing it with a reading card.

### *Statistical Analysis*

The data were reported in an Access database for Windows XP (Microsoft Inc., Redmond, Washington, U.S.A.). The results were represented in percentages and frequencies.

## *Results*

Twenty Papanicolaou-stained cervicovaginal smears—ASCUS (8), LSIL (3), HSIL (4), squamous cells carcinoma (4) and endocervical adenocarcinoma (1)—were studied. The average age of patients was 39.9 years, with a range of 19–72 years. All of the samples were amplified in the gene control INF150. In 10% (2 of 20) of cases the amplified viral sequences were negative.

Overall HPV prevalence was 90% (18 of 20) in the processed samples. The infection for HPV single type

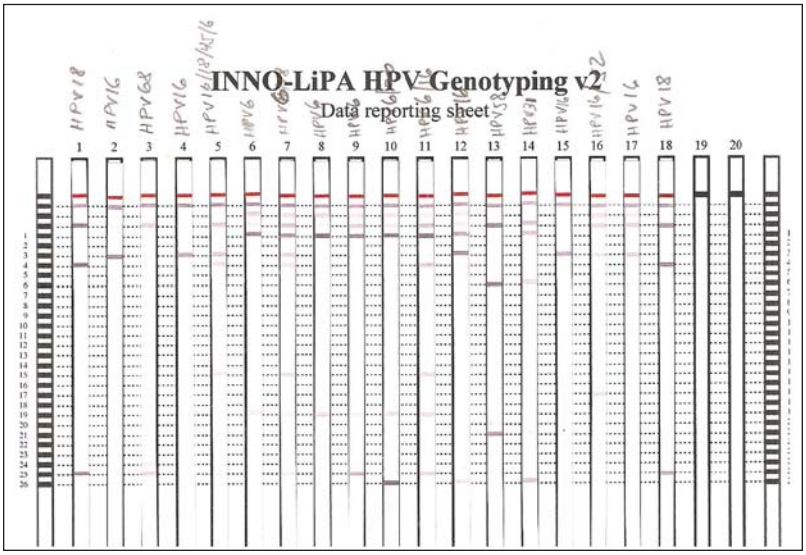


**Figure 2** Reverse hybridization in LiPA for the identification of the types of HPV present in cytologic samples in this study. Strips 19 and 20 were not used because the sample did not amplify segment 65 bp.

was 56%; the rest presented ≥ 2 types of HPV (multiple infection). In the latter group, at least 1 case of HPV was the oncogenic type. Figure 2 shows the results of HPV genotyping in the LiPA strips; the dark bands coincide, according to the reading card, with 1 or several viral types.

HPV16 was the most frequently isolated specific viral type in 60% (6 of 18) of samples, for both single and multiple infections. This was followed by HPV18 (20%), found in 2 cases: 1 HSIL and endocervical adenocarcinoma, and HPV6 and HPV58 in equal proportions (10%) (Figure 3).

The cell samples with multiple infections showed the presence of HPV DNA of LR-HPV, as well as HR-HPV. In 6 of these samples, at least 1 of the most frequent oncogenic types was detected, HPV16/18. Similarly, we found viral DNA of HPV types 45, 52, 58 and 68, considering HR-HPV and the HPV types 6, 11 and 70 nononcogenic and associated with benign lesions. These multiple infections were detected with greater frequency in the ASCUS and LSIL samples (Table I).

### *Discussion*

Although the quantity of cell specimens included in this study is not statistically significant, our results confirm that it is possible to identify the HPV DNA sequences from abnormal Papanicolaou-stained cervical smears using the SPF10–LiPA system for HPV detection and genotyping.

According to our results, Papanicolaou-stained cervical smears are excellent material for research, given that they permit examining the presence of HPV genomic sequences possibly associated with cellular alterations detected during morphologic analysis[9,12]; in this way obtaining additional samples for the molecular study can be avoided. The use of methods with high sensitivity for the detection of oncogenic viral DNA could serve as an indirect indicator of the presence of premalignant and cervical lesions.[37,38]

High cellularity and the method for recovering DNA from the smears are important determinants of successful PCR amplification of archival Papanicolaou-stained cervical smears.[16] In this study, pretreatment with xylene or acetone does not seem to affect the integrity of cellular material, possibly because the samples selected showed abundant material, for which we are in agreement that the amount of material available for amplifying PCR should be sufficient to ensure the finding of viral sequences.

Similarly, the DNA extraction procedure is of undoubted value, given that it ensures minimal degradation of DNA where the incubation time with a lysis solution is of basic importance. In this study, the extraction protocol of routine use in this laboratory was modified to perform isolation of the cell samples. Samples were left in incubation in the lysis solution overnight at 56°C, with the time reduced to 2 hours. We were able to obtain optimum material, demon-

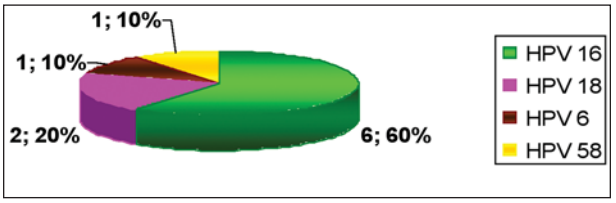


**Figure 3** Percentage distribution of types of specific HPV isolated from the cervical cytology samples studied.

**Table I** *Distribution of the HPV Types According to the Cellular Abnormalities Present in the Samples*

| Type of HPV | ASCUS (n = 6) | LSIL (n = 3) | HSIL (n = 4) | Ca (n = 4) | Adenoca (n = 1) | Total (n = 18) |
|---|---|---|---|---|---|---|
| 16 | 2 | — | 2 | 2 | — | 6 |
| 18 | — | — | 1 | | 1 | 2 |
| 6 | 1 | — | — | | — | 1 |
| 58 | — | 1 | — | | — | 1 |
| 6/58 | 1 | — | — | | — | 1 |
| 6/68 | 1 | — | — | | — | 1 |
| 16/52 | — | — | — | 1 | — | 1 |
| 16/70 | — | — | — | 1 | — | 1 |
| 6/16/18 | — | 1 | — | | — | 1 |
| 6/16/18/45 | 1 | 1 | — | | — | 2 |
| 11/16/68 | — | — | 1 | | — | 1 |

Adenoca = endocervical adenocarcinoma, Ca = squamous cells carcinoma.

strated in the amplification of the internal INF control in all cell samples, using phenol-choroform-CHISAM and ethanol for the precipitation of the genomic material from the cells previously dyed, as in other research.[15,19,39] This DNA extraction and precipitation method included the minimum number steps necessary, making the procedure simple and reliable, with the necessary precautions for reducing any source of contamination.[17]

As in this study, Poljak and Barlic[7] used phenol-chloroform-CHISAM for extraction and absolute ethanol for the DNA precipitation, finding minimal differences in the efficiency of amplification by PCR of the human β-globin and β-actin genes and later amplification of HPV L1 (MY09/11 consensus primers) from the filed and Papanicolaou-stained and nonstained smears. They considered this a quick and simple method to be used in retrospective studies and clinical correlation, as well as routine diagnosis when fresh or cryopreserved samples are not available. In contrast, Puranen et al[16] recommended not using this type of extraction, given that it could cause loss of DNA during the many stages of the process. These authors tried a protocol very similar to that used in this study but incubated the sample with a solution of lysis overnight, which could have caused excessive cellular digestion and consequently nonamplification by PCR. For this reason, we consider that the cellular digestion time, independent of the concentration of proteinase K used, should not exceed 2 hours of incubation, at temperatures between 55° and 60°C.

The reaction of PCR can be inhibited by several factors,[15,17,40] such as hematoxylin or aluminum sulfate included in the Papanicolaou stain.[41] In this research, the Papanicolaou stain and previous step of pretreatment did not appear to have interfered in the sensitivity of the HPV genomic amplification method. HPV DNA was detected in 90% of abnormal Papanicolaou-stained cervical smears using the SPF10-LiPA system, which indicates that it is possible to detect viral sequences in archival Papanicolaou-stained cell samples. Moreover, we detected HPV DNA sequences of different types, of LR-HPV as well as HR-HPV, considering that the type of samples included were treated previously for destaining, where the amount of intact HPV DNA could be scarce and degrade further in any of the steps of the process.[17]

In this study, HPV16 was the most frequent type of specific virus, followed by HPV18, coinciding with the large amount of literature. Moreover, we obtained 44% of the multiple infections with LiPA. The prevalence of multiple HPV infections differs among studies, being frequent in patients with cell abnormalities,[20,33,42] especially with ASCUS and SIL,[8,34,43] having reached 35% only in patients with HSIL.[9] However, it is possible that these multiple infections are a consequence of cross-reactivity.

The possibility of contamination from one smear to another could occur during the destaining process before amplification. In this respect, Chua and Hjerpe[15] point out that it is improbable that cross-reactivity occurs because by norm they are manipulated with gloves and strict anticontamination measures are observed during the destaining process as well as DNA extraction and PCR preparation. Other possibilities of cross-reactivity occur in the case of the presence of phylogenetically related HPV genotypes in the same sample.[44,45] The phylogenetic group of HPVs is possibly an explanation of the cross-reactivity seen in LiPA, as, for example, could occur in the case of a sample with a high HPV18 load showing a reaction in the corresponding bands of this type. Moreover, it is possible that the excess of hybrid viral sequences, also resulting from competition with the HPV45 probe, led to interpretation of this result as a false infection for HPV18 and 45. This same situation could occur also with HPV16 and 31. In this study, we performed visual interpretation of the completely dry bands, con-

sidering those bands with perfectly visible purple precipitation, without importing the coloration intensity, given that the low-intensity signal could be related to a low viral load.[25] Similarly, in the case of doubt, we agreed that it would be convenient to use additional methods such as type-specific PCR or the combination of consensus and specific primers, to confirm the presence of specific viral types associated with different viral loads, in the same sample in particular, and establish the true prevalence of multiple infections.[11,31,32,35,42] Cross-reactivity is also a common finding where cervical samples have been examined using Hybrid Capture II.[27,45,46] For this, we thought that when dealing with so sensitive a method in which the L1 region is the target of HPV, the possibility of false positive findings will always exist.

It has been confirmed that HPV types 42, 53, 56, 66 and 73 are widely identified in cross-reactivity.[27] In this study, none of these types of virus were found to be associated in the cases of multiple infections; these are possibly true multiple infections. Other studies with a greater number of samples should be performed to decide whether the infections detected by LiPA are true multiple infections or false positive results.

An interesting observation in our results was that in 2 cases with ASCUS, amplification of the viral sequences was negative. This represented true negative status, given that this methodology is capable of detecting the viral DNA even though a low number of copies are found to be fragmented or degraded.[30] This allows deducing that possibly some atypical findings are really benign changes and that research on the viral presence could lead to more precise interpretation of the cytologic findings.[47]

The method used could permit the development of retrospective studies that supply data for a better understanding of the natural history of infection by HPV and its intervention in the carcinogenesis process of the cervix in our environment. In addition, it could contribute relevant information to the clinic for better management of patients with abnormal Pap smears. Other studies are needed that include a greater number of samples to confirm the effectiveness and establish the clinical utility of the SPF10-LiPA system.

In conclusion, the SPF10-LiPA system detects and identifies HPV DNA sequences from Papanicolaou-stained cervicovaginal smears, initially used in routine cancer detection. The use of conventional protocols in DNA extraction with optimum temperatures and incubation times permits obtaining high-quality DNA samples. The SPF10-LiPA system is an efficient, fast and simple detection and typing method for the viral genome. It has elevated sensitivity that could be used in routine investigation for the initial detection of HPV for better clinical management of the patient.

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