

Chapter 2

A Real-Time PCR Approach Based on SPF10 Primers and the INNO-LiPA HPV Genotyping Extra Assay for the Detection and Typing of Human Papillomavirus

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Abstract

A highly sensitive SPF10 real-time PCR was developed to achieve simultaneous amplification and detection of the human papillomavirus (HPV) target. That way, LiPA analysis of the HPV-negative samples can be avoided, reducing workload and cost. Here, we describe in detail a SYBR Green I-based real-time PCR assay based on SPF10 primers using the LightCycler® 480 system to generate and detect HPV amplicons, which are compatible with the LiPA assay.

Key words Human papillomavirus, SPF10, Real-time PCR, LiPA, Genotype

1 Introduction

Cervical carcinoma is the third most prevalent cancer in women worldwide [1]. The causal relationship between a persistent infection with high-risk HPV and cervical cancer has resulted in the development of molecular technologies for viral detection [2, 3].

The SPF10 primers target a 65-base pair region of the HPV L1 open reading frame and enable the amplification of at least 54 genital HPV types [4, 5]. After DNA amplification, the INNO-LiPA HPV Genotyping Extra (LiPA) assay, i.e., a reverse line probe assay, can be used for the identification of 28 different HPV genotypes. This assay shows a good clinical performance compared to other commercial tests for HPV genotyping in cervical cell specimens and formalin-fixed material [6, 7]. Furthermore, the LiPA assay is commonly used in epidemiological studies and vaccination trials [8, 9].

Our group has developed a SYBR Green I-based real-time protocol for the SPF10 primers on the LightCycler® 480 to achieve simultaneous amplification and detection of the HPV target [10]. The SPF10 real-time PCR shows a detection limit of 29.7 copies for HPV6, 16, 18 and 31 and generates amplicons, which are

compatible with the LiPA. Moreover, this approach immediately indicates the HPV-negativity, making the LiPA step redundant for the HPV-negative samples. Although the HPV prevalence in cervical samples depends on various geographical, demographical and/or methodological factors, the percentage of HPV-positivity can be estimated between 7 and 18 % [11–14]. This means that the SPF10 real-time PCR would reduce the cost and workload of the LiPA assay in 93 % of the cervical samples.

2 Materials

2.1 Controls

Each run PCR should comprise a positive and a negative control:

1. Positive control contains HPV6 DNA and HLA-DPB1 DNA (INNO-LiPA HPV Genotyping Extra Amp, Innogenetics, Ghent, Belgium).
2. Use PCR Grade water (Roche Applied Science, Penzberg, Germany) as a no template control (NTC).

Store the controls at -20°C .

2.2 SPF10 Real-Time PCR

Prepare the real-time PCR master mix using the following reagents:

1. SYBR Green I (Sigma, Saint Louis, MO, USA): 1/2,000 dilution in Tris/EDTA buffer (pH 7.5) (*see Note 1*).
2. PCR buffer II, 10 \times (Applied Biosystems, Foster City, CA, USA).
3. MgCl_2 , 10 mM (Applied Biosystems).
4. dNTP mixture, 10 mM each (Roche Applied Science).
5. Biotinylated SPF10 primers (SPF10 primer set RUO, 10 μM of each primer (Innogenetics, Ghent, Belgium)).
6. AmpliTaq Gold[®] DNA polymerase, 5 U/ μl (Applied Biosystems).
7. PCR Grade H_2O (Roche Applied Science).

Store the SYBR Green dye I dilution at $2-8^{\circ}\text{C}$ protected from light for maximum 18 days (*see Note 2*) [15]. Keep all the other components of the mix at -20°C . In this protocol, the multiwell-plate-based LightCycler[®] 480 system (Roche Applied Science) is used to perform the PCR reaction.

2.3 HPV Genotyping Assay

The HPV amplicons are subjected to the INNO-LiPA HPV Genotyping Extra assay (Innogenetics) for HPV typing. Store the reagents and strips of the kit at $2-8^{\circ}\text{C}$.

The following reagents are supplied with this kit:

1. Denaturation solution.
2. Hybridization solution.
3. Stringent Wash solution.

4. Conjugate.
5. Conjugate Diluent.
6. Substrate.
7. Substrate Buffer.
8. Rinse Solution.

Fully automated processing of the assay is possible using the Auto-LiPA 48 (Innogenetics). The hybridization patterns on the strips are objectively interpreted using the LiRAS® software for LiPA HPV (Innogenetics).

3 Methods

3.1 HPV Amplification and Detection by SPF10 Real-Time PCR

A SYBR Green I-based real-time PCR is used to amplify and detect HPV DNA simultaneously. SYBR Green I is a fluorescence dye which binds only to double-stranded DNA (dsDNA) [16]. The dye has virtually no fluorescence in free solution. Upon binding to dsDNA, the specific fluorescence of SYBR Green I is greatly enhanced. The more copies of target DNA are amplified in the sample during PCR-cycling, the earlier the signal reaches a detectable threshold where it is significantly higher than background levels. The cycle at which this occurs is called the Ct (cycle threshold) or Cp (crossing point) value, which is always in the exponential phase of the PCR [17, 18]. In contrast to sequence-specific fluorescence labeled probes (e.g. TaqMan®), SYBR Green I binds to any dsDNA such as primer dimers. Therefore, a melting curve analysis has to be performed after the amplification to assess the specificity of the real-time PCR product [19].

The SPF10 real-time PCR is carried out as follows:

1. Thaw sample extracts, positive control, PCR buffer II (10×), MgCl₂ (10 mM), dNTP mixture (10 mM each), the biotinylated SPF10 primers (10 µM each), and PCR Grade water. Mix the thawed reagents and the SYBR Green I dilution (1/2,000) by vortexing and spin down. Keep all substances in the pre-cooled (2–8 °C) benchtop cooler.
2. Prepare a master mix for all samples, including the positive control and NTC plus one. Composition of the mastermix for one sample:

SYBR Green I (1/2,000)	5 µl	(final dilution 1/20,000)
PCR buffer II (10×)	5 µl	(final concentration 1×)
MgCl ₂ (10 mM)	8 µl	(final concentration 4 mM)
dNTP (10 mM each)	1 µl	(final concentration 200 µM each)

(continued)

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SPF10 primers (10 μ M each)	1.5 μ l	(final concentration 300 nM each)
AmpliTaq Gold® (5 U/ μ l)	0.3 μ l	(final concentration 1.5 U)
PCR Grade H ₂ O	19.2 μ l	
	40 μ l	

Do not vortex, but spin down the AmpliTaq Gold® DNA polymerase in a benchtop centrifuge before use. This reagent is viscous and therefore requires extra care in pipetting in order to deliver the accurate volume of reagent.

3. Vortex briefly the master mix and spin down. Distribute the master mix (40 μ l) in every well of a white LightCycler® 480 Multiwell Plate 96 with the same tip.
4. Pipette 10 μ l of the sample extract into the master mix. Add 10 μ l of the positive control to the positive control well and add 10 μ l PCR Grade water to the negative control well.
5. Seal the Multiwell Plate with LightCycler® 480 Sealing Foil.
6. Place the Multiwell Plate in a swinging-bucket centrifuge and balance it with a suitable counterweight. Centrifuge for 2 min at 1,500 $\times g$.
7. Load the Multiwell Plate into the LightCycler® 480 system.
8. Run PCR with the corresponding program on the LightCycler® 480 system (*see Note 3*).

		Ramp rate (°C/s)	Fluorescence acquisition
AmpliTaq Gold® activation	95 °C for 10 min	4.4	None
Amplification	95 °C for 30 s	4.4	None
40 cycles	52 °C for 45 s	2.2	None
	72 °C for 45 s	4.4	Single
Melting curve	95 °C for 5 s	4.4	None
	52 °C for 1 min	2.2	None
	97 °C	/	Continuous
Cooling	40 °C for 10 s	1.5	None

9. Use the Fit Points method implemented in the LightCycler® 480 software version 1.5 to obtain a C_p value for each sample.
10. Perform a melting temperature (T_m) calling analysis for the SYBR Green I format using the LightCycler® 480 software

version 1.5. The T_m corresponds with the maximum of the negative first derivative of the melting curve. Samples with a T_m in the range of 79–83 °C are considered HPV-positive (*see* Fig. 1). Samples without a sharp peak on the melting peak chart or with weak peaks (width: 0, height: 0), or with a T_m outside the predefined range are considered as HPV-negative.

11. Store the Multiwell Plate at –20 °C (*see* **Note 4**) or subject the HPV amplicons immediately to the LiPA assay.

3.2 HPV Genotyping by the LiPA Assay

The LiPA assay is based on the reverse hybridization principle. Biotinylated HPV amplicons are denatured and hybridized to specific oligonucleotide probes immobilized on the strip. After stringent washing, streptavidin-conjugated alkaline phosphatase is added and binds to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen gives a purple/brown precipitate (*see* Fig. 2). The results can be interpreted visually or by the LiRAS® HPV software.

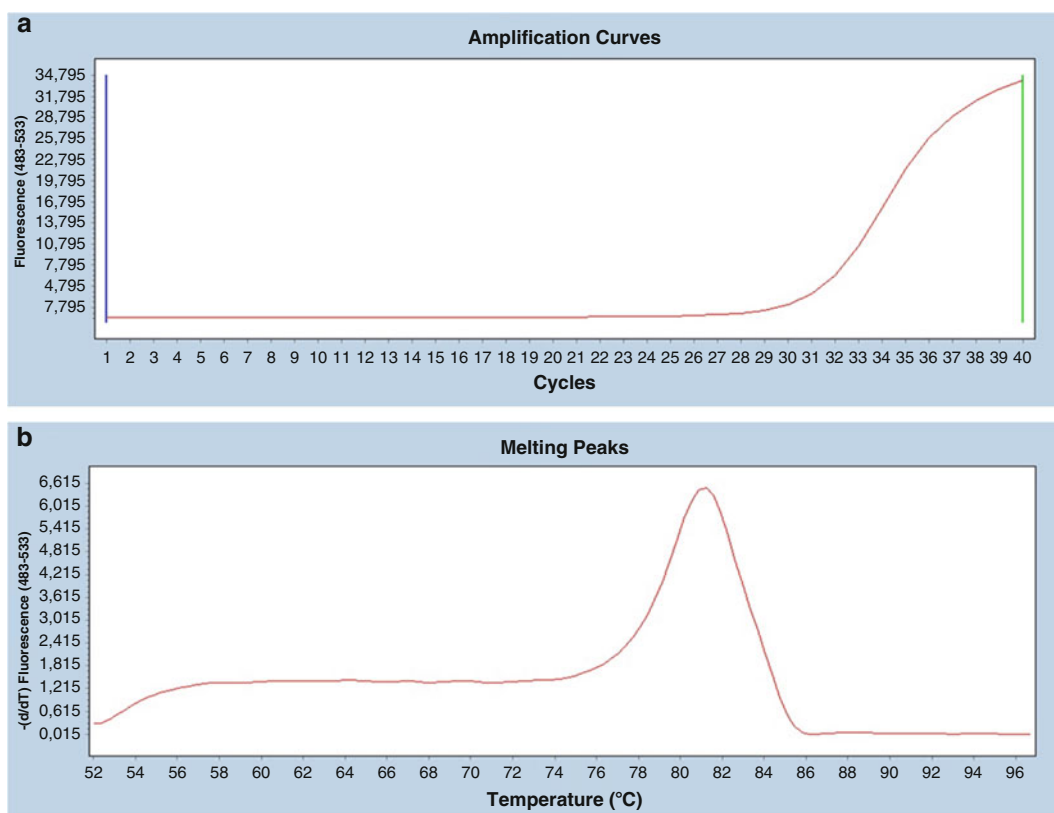


Fig. 1 Example of an amplification plot and melting peak for HPV detection by SPF10 real-time PCR. (a) Amplification plot of SPF10 real-time PCR. (b) Melting peak showing a HPV16-positive sample, T_m 81 °C

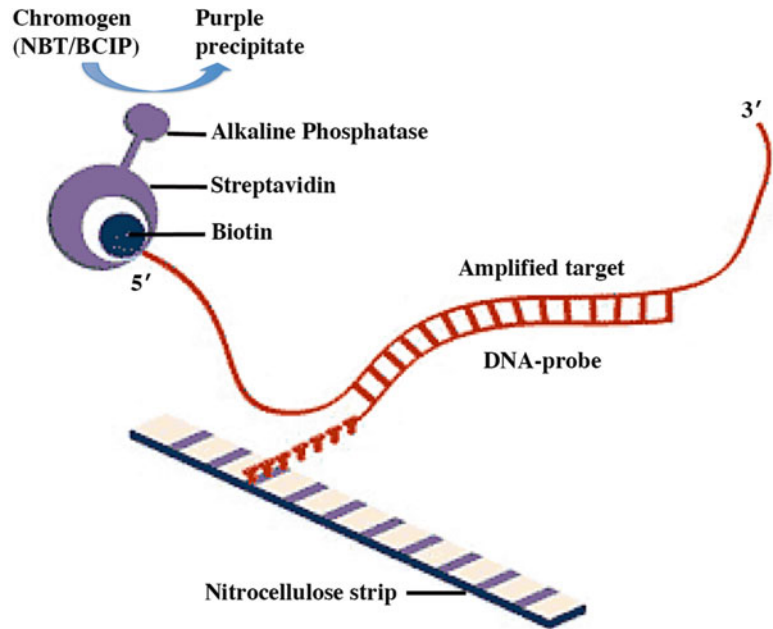


Fig. 2 Principle of the LiPA assay

3.2.1 The LiPA Protocol

The LiPA protocol is carried out using the Auto-LiPA 48 and consisted of the following steps:

1. Bring all reagents and the tube containing the strips to room temperature (RT) (20–25 °C) approximately 1 h before use. All these components should be returned to the refrigerator (2–8 °C) immediately after use.
2. Heat the shaking water bath to 49.5 or 50 °C (*see Note 5*). Prewarm the Hybridization solution and Stringent Wash solution to at least 37 °C but do not exceed 50 °C. Mix before use.
3. Prepare the LiPA reagents according to the number of samples. Each LiPA run contains HPV-positive samples, a positive amplification control and a NTC.
4. Rinse Solution should be diluted 1/5 in distilled H₂O to make Rinse working solution. Prepare 8 ml Rinse working solution for each LiPA well plus 20 ml (i.e., total volume needed = 8 × number of wells + 20). This solution is stable for 2 weeks at 2–8 °C.
5. Conjugate should be diluted 1/100 in Conjugate Diluent to make Conjugate working solution. Prepare 2 ml Conjugate working solution for each LiPA well plus 10 ml (i.e., total volume needed = 2 × number of wells + 10). This solution is stable for 8 h at RT if stored in the dark.
6. Substrate should be diluted 1/100 in Substrate Buffer to make Substrate working solution. Prepare 2 ml Substrate working

solution for each LiPA well plus 10 ml (i.e., total volume needed = $2 \times \text{number of wells} + 10$). This solution is stable for 8 h at RT if stored in the dark.

7. Using a clean forceps, remove the required number of strips from the tube (one strip per sample). Place the strips in the wells of the LiPA tray and put an identification number above the marker line using a pencil.
8. Pipette 10 μl Denaturation solution into the upper corner of each well from the LiPA tray.
9. Add 1 μl of amplified biotinylated sample (*see Note 5*) to the Denaturation solution. Allow denaturation to proceed for 5 min at RT.
10. Place the LiPA tray in the shaking water bath at 49.5 or 50 °C. Add 2 ml prewarmed Hybridization Solution to each well and incubate for 1 h.
11. Wash each strip twice with 2 ml prewarmed Stringent Wash solution for 10–20 s.
12. Incubate each strip in 2 ml prewarmed Stringent Wash solution in the shaking water bath at 49.5 or 50 °C for 30 min.

The following steps are carried out on a shaker at RT:

13. Wash each strip twice using 2 ml Rinse working solution for 1 min each.
14. Add 2 ml Conjugate working solution to each well and incubate 30 min while shaking.
15. Wash each strip twice using 2 ml Rinse working solution for 1 min and wash once more using 2 ml Substrate Buffer.
16. Add 2 ml Substrate working solution to each well and incubate for 30 min while shaking.
17. Stop the color development by washing the strips twice with 2 ml distilled water while shaking for at least 3 min.
18. Using forceps, remove the strips from the wells and place them on absorbent paper. Let the strips dry completely and fix them to the data reporting sheet. The uppermost line is the marker line. The conjugate control line under the marker line aids correct alignment of the strips on the data reporting sheet.

3.2.2 Interpretation of the LiPA results

The hybridization patterns can be interpreted visually using the INNO-LiPA HPV Genotyping Extra Reading Card supplied with the kit. Our group employed the LiRAS® HPV software to obtain an objective interpretation of the results.

1. Each clear purple/brown line should be scored as positive.
2. The first line is the Conjugate Control line. This line controls for the addition of reactive Conjugate and Substrate working

solution during the detection procedure. It should always be positive and have the same intensity on each strip in the same test run.

3. The second line is a human DNA control. Because of the absence of HLA-DPB1 primers in the real-time PCR master mix, this line should always be negative.
4. A sample is considered HPV-positive if at least one of the type-specific lines or one of the HPV control lines is positive.
5. If a positive band is obtained on the strip for the NTC, the entire run should be considered as invalid and the complete procedure should be repeated.

4 Notes

1. SYBR Green I should be diluted in Tris/EDTA buffer of pH 7.5 but not in a buffer of pH 8.0. Tris-buffered solutions of pH 8.0 at RT will change to about pH 8.5 when stored at 4 °C. This increase is sufficient to render the dye unstable and to generate SYBR Green I degradation products, which are potent PCR inhibitors. Therefore, SYBR Green I has to be diluted in Tris/EDTA buffer of pH 7.5 [15].
2. After 21 days, SYBR Green I exhibits an inhibitory effect on PCR reactions [15].
3. The melting program is 5 s at 95 °C followed by an increase of 2.2 °C/s from 52 °C up to 97 °C. The complete run takes 2 h and 7 min.
4. Thaw the Multiwell Plate with the real-time PCR amplicons. Centrifuge the plate for 2 min at 1,500 × *g* and carefully remove the seal from the plate. After that, the samples can be subjected to the LiPA assay.
5. The high amplification efficiency of the SPF10 real-time PCR results in higher amplicon concentration than with conventional PCR, leading to cross-reactivity when the LiPA assay is performed under default conditions. Performing the LiPA assay at an increased hybridization temperature and stringent wash incubation temperature (49.5 or 50 °C) in combination with a lower amplicon volume (1 µl) eliminated this cross-reactivity [10].

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