

# Improvement of DNA Extraction for Human Papillomavirus Genotyping from Formalin-Fixed Paraffin-Embedded Tissues

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## Introduction

**M**UCOSAL HUMAN PAPILLOMAVIRUSES (HPVs) play a crucial role in the development of cervical carcinoma, the third most common malignancy in women worldwide.<sup>1,2</sup> These viruses are classified as high risk (HR) or low risk (LR), depending on their transforming ability.<sup>3,4</sup> Formalin-fixed paraffin-embedded (FFPE) tissues represent the most frequent form of tissue storage in pathology departments. These archival tissues represent a potentially useful resource for retrospective epidemiological studies. Several HPV studies have used FFPE specimens to correlate HPV genotypes with histological classification,<sup>5-7</sup> to establish the geographical distribution of HPV genotypes,<sup>8</sup> or to search for these viruses in other primary cancers.<sup>9,10</sup>

In these tissues, the detection of viruses by molecular biological tools based on molecular hybridization between DNA or RNA targets and specific probes<sup>11</sup> is problematic because fixation causes damage to nucleic acids. Formalin fixation induces protein-protein and protein-nucleic acid cross-linking.<sup>12</sup> As a result of protein-nucleic acid cross-linkages, it is difficult to separate DNA from histones and to obtain pure nucleic acids at extraction.<sup>13,14</sup> The fixation of tissues also leads to the fragmentation of nucleic acids, such that polymerase chain reaction (PCR) methods that amplify a smaller portion of the viral genome are most effective.<sup>15,16</sup>

The aim of this study was to test whether HPV genotyping techniques used routinely in our diagnostic laboratory could be applied to FFPE tissues. For recovery of HPV DNA from FFPE tissues, we had to adapt extraction protocols to obtain viral DNA useful for PCR amplification. We tested different extraction protocols for HPV DNA, mainly to simplify this step, and we tested two genotyping methods. The first genotyping method involved sequencing of the L1 open reading frame. This method links PCR amplification of a conserved HPV L1 segment using GP5+/GP6+ consensus primers with automated sequencing of amplified PCR products. The second method used the Greiner Bio-one (GBO) Papillo-

Check<sup>®</sup> DNA microarray, which enables the detection and genotyping of 24 different HPV types (18 high risk and 6 low risk) from DNA preparations of human cervical cytology specimens.

## Materials and Methods

### Sample collection

The study included a total of 46 FFPE tissue specimens collected from 36 cervical conizations performed between 2008 and 2011 and analyzed in the pathology laboratory at the Université de Nice. Mean age of the women at time of conization was 38 years. Histological analysis of the tissue specimens showed: 13% cervical intraepithelial neoplasia (CIN) grade 1, 69% CIN 2-3, 11% without lesion, and 7% condyloma.

From each tissue block, fifteen 5- $\mu$ m thick sections were cut and placed in a 1.5-mL microcentrifuge tube and submitted to our laboratory. To minimize the potential for PCR contamination, the blocks were handled with gloves and the microtome was cleaned by changing the microtome blade between each block that was cut.

### First protocol of DNA extraction

**Deparaffinization with xylene.** The paraffin was removed by 5-minute incubation in 1 mL of xylene (Carlo Erba reagents) and centrifugation at 15,000 g for 2 minutes. The supernatant was removed and this step was repeated twice. Then the sections were immersed twice in 1 mL absolute ethanol (Carlo Erba reagents) for 5 minutes and centrifuged at 15,000 g for 2 minutes, and the supernatant was removed. The sections were then incubated in 1 mL 70% ethanol for 5 minutes and centrifuged at 15,000 g for 2 minutes, after which the supernatant was removed.

**Proteinase K lysis.** The tissue sections were incubated overnight with 500  $\mu$ L proteinase K solution (20 mM pH 7.8

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TABLE 1. PRIMERS USED IN THE POLYMERASE CHAIN REACTION ASSAYS

PCR	Target	Oligo	Sequence	Amplicon
Actin	Human actin	Reverse primer	5'-GCCTTCATACATCTCAAGTTG-3'	650 bp
		Forward primer 1	5'-AAGTACTCCGTGTGGATC-3'	
		Forward primer 2	5'-GATGCGTTGTTACAGGAAGT-3'	300 bp
GP5+ /GP6+	L1 HPV	Forward primer GP5+	5'-TTTGTACTGTGGTAGATACTAC-3'	150 bp
		Reverse primer GP6+	5'-GAAAAATAAACTGTAAATCATATTC-3'	
PapilloCheck	E1 HPV	Forward primer	unknown	350 bp
		Reverse primer		

HPV, human papillomavirus.

Tris [RP Normapur<sup>TM</sup>], 20 mM EDTA [gen apex], SDS 0.2% [Rectapur<sup>TM</sup>], 3 mg proteinase K [Invitrogen]) at 56°C.

**NucliSENS<sup>®</sup> easyMAG<sup>®</sup> extraction.** Five hundred microliters of the lysate was added to 2 mL NucliSENS easyMAG lysis buffer, which was then homogenized and incubated for 10 min at room temperature. Then 140  $\mu$ L of NucliSENS easyMAG magnetic silica was added. The DNA was isolated using the "specific B" program on the NucliSENS easyMAG instrument. Elution was performed in 60  $\mu$ L NucliSENS easyMAG extraction buffer 3.

#### Second protocol for DNA extraction

**Proteinase K lysis.** The paraffin sections were incubated overnight in 600  $\mu$ L proteinase K solution (50 mM pH 8.5 Tris [RP Normapur], 1 mM pH 8 EDTA [gen apex], 0.5% Tween<sup>®</sup>20 [Sigma], and 3 mg proteinase K [Invitrogen]) at 56°C.

**Heat treatment.** The sections were then incubated at 100°C for 10 min and centrifuged at 4°C at 15,000 g for 30 seconds. The lysate was recovered by aspiration under the paraffin, which collected as a white ring at the surface.

**NucliSENS easyMAG extraction.** Same procedure as for first protocol.

#### Third protocol

Proteinase K lysis and heat treatment were identical to the second protocol but without extraction by the easyMAG instrument.

#### Actin PCR

PCR amplification of actin was used as a positive control in assessing amplifiable sample DNA. As shown in Table 1, we used different sets of primers that produced different lengths of amplicon (S1 = 650 bp and S2 = 300 bp). The assay was optimized for template quantity, denaturation temperature, duration of each step, number of cycles, and addition of gelatin to the mix. To avoid contamination with amplicons we used the Uracyl-N-glycosylase (UNG Roche) system. PCR was performed using the QIAGEN HotStarTaq<sup>®</sup> PCR kit: 200 ng of purified DNA, 5  $\mu$ L of buffer 10 $\times$ , 100 ng of each primer, 0.5  $\mu$ L of HotStarTaq<sup>®</sup> DNA polymerase, 1  $\mu$ L of UNG, 1.5  $\mu$ L of mix dNTP (ATP, CTP, GTP 23 mM [Invitrogen] and UTP 30 mM [Roche]), 14  $\mu$ L of MgCl<sub>2</sub> and 1  $\mu$ L of gelatin 0.05% (type B, from bovine skin: Sigma).

After 10 minutes of incubation at 25°C to activate UNG and 15 minutes at 95°C to activate HotStarTaq, PCR was performed for 55 cycles of 94°C for 1 minute, 53°C for 1 minute, 30 seconds, and 72°C for 4 minutes. The PCR product was electrophoresed in 2% agarose (Biorad) gel and was visualized by staining with ethidium bromide.

#### HPV genotyping using sequencing

The assay was based on L1 consensus PCR with GP5+ /GP6+ primers (Table 1) yielding a 150-bp amplicon using the HotStarTaq DNA polymerase QIAGEN. PCR amplification of the open reading frame of L1 was performed with the QIAGEN HotStarTaq PCR kit: 200 ng of purified DNA, 5  $\mu$ L of buffer 10 $\times$ , 100 ng of each primer, 0.5  $\mu$ L HotStarTaq DNA polymerase, 1  $\mu$ L of UNG, 1.5  $\mu$ L of mix dNTP (ATP, CTP, GTP 23 mM [Invitrogen] and UTP 30 mM [Roche]), 14  $\mu$ L of MgCl<sub>2</sub>, and 1  $\mu$ L of gelatin 0.05%.

PCR was performed for 55 cycles of 94°C for 1 minute, 50°C for 1.5 minutes, and 72°C for 4 minutes. The PCR product was electrophoresed in 2% agarose gel and was visualized by ethidium bromide staining.

HPV DNA was identified by sequencing 5  $\mu$ L of L1 PCR product with dRhodamine Terminator Cycle Sequencing Ready Reaction Kit ABI PRISM<sup>®</sup> using the same primers as for the initial amplification. Sequencing was run for 10 minutes at 96°C, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes and analyzed (after precipitation) using an ABI PRISM 3100 genetic analyzer (Perkin-Elmer). Sequence alignments of L1 were performed with Sequence Navigator software<sup>TM</sup>.

#### HPV genotyping on PapilloCheck DNA microarray

HPV genotyping was performed by PapilloCheck HPV DNA chips (GBO) from 200 ng of purified DNA. After extraction, a 350-bp fragment of the E1 open reading frame, an external PCR control, and a DNA fragment of the human ADAT1 gene were amplified with specific primers in the presence of 5  $\mu$ L of purified DNA sample. Slides were scanned and analyzed with CheckReport software<sup>TM</sup> (GBO). The oligonucleotide microarray detects 18 HR HPV (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) and six LR HPV (HPV 6, 11, 40, 42, 43, 44). PapilloCheck microarray contains 28 probes, including 24 HPV probes and four control probes (orientation, hybridization, external PCR, and ADAT1), each in five replicate spots. The manufacturer's protocol for cervical brush specimens was followed, except that the quantity of HotStarTaq DNA polymerase QIAGEN was increased (2  $\mu$ L instead of 0.2  $\mu$ L) and gelatin was added (0.001%).

TABLE 2. EXTRACTION PROTOCOL EFFICIENCY EVALUATED BY DOWNSTREAM ACTIN POLYMERASE CHAIN REACTION RESULTS

Protocol	Xylene deparaffinization	Proteinase K lysis	Extraction	Actin PCR-positive results	
				650-bp fragment	300-bp fragment
P1	Yes	SDS buffer	NucliSENS easyMAG	(30/46) 65%	100%
P2	No	Tween buffer	NucliSENS easyMAG	100%	100%

This table summarizes the results of actin PCR of DNA isolated from 46 formalin-fixed paraffin-embedded (FFPE) tissues via two different extraction protocols.

P1, first protocol; P2, second protocol; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

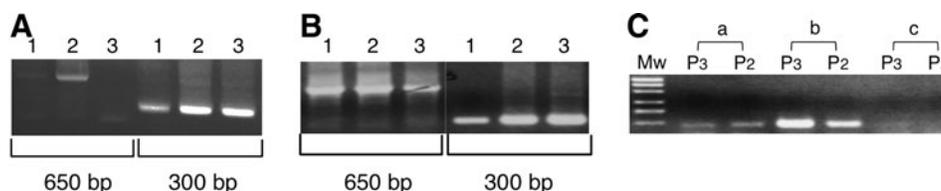


FIG. 1. Polymerase chain reaction (PCR) gel migration. (A) Actin PCR after the first (A) and second (B) protocols, from three different samples (1, 2, 3). (C) Human papillomavirus (HPV) PCR (GP5+ /GP6+ primers) after the second (P2) or third (P3) protocol from three samples (a, b, c).

## Results

### Actin PCR

To test the efficiency of DNA extraction from FFPE tissues, we used PCR amplification of the human actin gene as a positive control. We chose two different sets of primers to amplify two fragments of the actin gene of different lengths (300 bp and 650 bp). The second extraction protocol without xylene gave better results, as shown in Table 2 and Fig. 1. The 650-bp fragment of human actin was amplified successfully from all FFPE tissues subjected to the extraction protocol without deparaffinization in xylene (Fig. 1). These results showed that this second extraction protocol could amplify a long DNA fragment, and so HPV genotyping was only performed on the 46 samples after DNA extraction by the second protocol.

### HPV genotyping

HPV DNA extracted via the second extraction protocol was genotyped by sequencing in 50% of the assays (16

patients; Table 3). To improve these results, we tested a third protocol (without the easyMAG step), which involved less handling of the formalin-fixed, fragile DNA. With this third extraction protocol, HPV DNA was identified in 55% of the assays (Table 3). Thus, there was no significant difference between the genotyping results using these different extraction protocols. HPV DNA was identified by sequencing in 57% of FFPE samples, but without multiple genotypes detected.

Using the PapilloCheck kit (with 2  $\mu$ L of HotStarTaq DNA polymerase QIAGEN instead of 0.2  $\mu$ L), HPV DNA extracted via the second extraction protocol (with the easyMAG step) was identified in 74% of the assays, and HPV DNA extracted with the third protocol was genotyped in 58% of the assays (Table 3). Among the 46 FFPE tissues (82% histologically classified as CIN, 7% as condyloma, and 11% with no lesions), HPV DNA was identified by PapilloCheck in 72% of the cases. In these latter samples genotyped with PapilloCheck, 26% showed multiple genotypes (Table 4), and the most frequent HPV type in CIN2-3 samples was HPV16 (Fig. 2).

TABLE 3. HUMAN PAPILOMAVIRUS GENOTYPING RESULTS

HPV genotyping method <sup>a</sup>	Protocols of DNA extraction	Proteinase K lysis (Tween buffer)	Heat treatment	NucliSENS easyMAG extraction	Results by assays			Results by FFPE tissue samples			
					Total	Positive	Negative	Total	Positive	Negative	Multiple infection
Sequencing	2	X	X	X	32	16 (50%)	16	46	26 (57%)	20	0
	3	X	X		22	12 (55%)	10				
PapilloCheck	2	X	X	X	34	25 (74%)	9	46	33 (72%)	13	12 (26%)
	3	X	X		26	15 (58%)	11				

<sup>a</sup>Genotyping was performed by sequencing 32 HPV DNA samples extracted by the second extraction protocol, including automated extraction with the easyMAG instrument, and for 22 samples extracted by the third extraction protocol, without automated extraction by the easyMAG instrument. PapilloCheck kit was used to test 34 HPV DNA samples extracted via the second extraction protocol and for 26 samples extracted via the third extraction protocol. Among the 46 formalin-fixed paraffin-embedded (FFPE) tissue samples, some were tested with both extraction protocols. A result was considered positive if at least one HPV genotype was identified.

TABLE 4. HUMAN PAPILLOMAVIRUS GENOTYPING RESULTS FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES BY SEQUENCING AND PAPILOCHECK

	Samples	Sequencing	PapilloCheck	
No HPV	9	—	—	
Single HPV type	1	—	HPV44/55	
	1	—	HPV51	
	1	—	HPV58	
	2	—	HPV31	
	2	—	HPV16	
	1	HPV6	∅	
	1	HPV66	∅	
	1	HPV16	∅	
	1	HPV18	∅	
	7	HPV16	HPV16	
	1	HPV18	HPV18	
	2	HPV33	HPV33	
	1	HPV35	HPV35	
	1	HPV56	HPV56	
	1	HPV58	HPV58	
	1	HPV6	HPV6	
	Multiple HPV types	1	∅	HPV16 HPV58
		1	∅	HPV73 HPV53 HPV40 HPV6
1		∅	HPV6 HPV73 HPV58	
1		∅	HPV6 HPV73	
1		HPV16	HPV16 HPV52 HPV43 HPV68 HPV40	
1		HPV16	HPV16 HPV33	
1		HPV16	HPV16 HPV6 HPV66	
1		HPV16	HPV16 HPV6	
1		HPV56	HPV56 HPV11	
1		HPV66	HPV33 HPV66	
1		HPV66	HPV11 HPV82 HPV66 HPV56	
1		HPV73	HPV73 HPV53	

—, negative; ∅, noninterpretable results (multiple sequences by sequencing or invalid controls by PapilloCheck).

**Discussion**

DNA from archived FFPE tissue can be used for papillomavirus genotyping, but fixation causes damage to nucleic acids, induces protein-protein, and protein-nucleic acid cross-

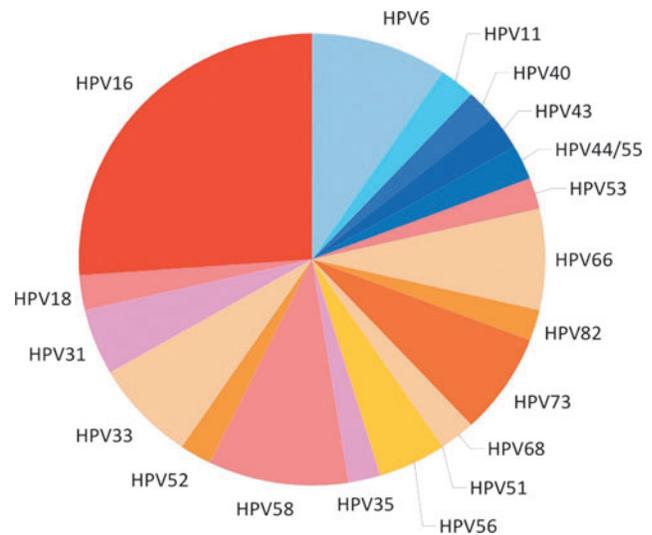


FIG. 2. HPV genotypes in high-grade lesions (CIN2-3). Between the 46 samples, 32 presented high-grade lesions (CIN2-3). In these 32 samples, we found 42 HPV types, 81% of high-risk (HR) HPV and 19% of low-risk (LR) HPV. The most frequent genotype was HPV16 (26%). Twenty-five percent of these high-grade lesions were positive for different HPV genotypes.

linking, and can impair PCR. This is problematic because an amplification step by PCR is necessary for HPV genotyping. We were particularly interested in the performance of different extraction protocols prior to genotyping by PapilloCheck kit from Greiner Bio-One. This PapilloCheck DNA chip incorporates several controls (orientation control, hybridization control, HPV PCR control, and sample control) to validate results and allows the detection of multiple HPV infections, which is of considerable potential interest when HPV genotyping is performed on fixed tissues.

In the present study we compared different DNA extraction techniques for HPV detection in FFPE cervical specimens. As demonstrated by other studies, removal of the xylene deparaffinization step saves time, avoids loss of material by accidental pipetting of tissue fragments, and most importantly increases the amplifiable DNA yield (Fig. 1).<sup>17</sup> Moreover, traces of xylene may inhibit PCR amplification and in multiple studies no step is added for removal of paraffin.<sup>18-20</sup> In fact, prolonged digestion with proteinase K appears to be sufficient.<sup>21-24</sup> Our study also suggests that the addition of an extraction step by the easyMAG instrument is suitable for purifying DNA from FFPE tissues, as described in other studies,<sup>25</sup> but this did not appear to give a significant improvement in our results.

In conclusion, our results suggest that DNA extraction is more efficient with the protocol without xylene deparaffinization and that HPV genotyping is more efficient with the PapilloCheck kit which detects multiple genotypes. Indeed, with this kit, we detected HR HPV in 78% of CIN2-3 FFPE tissues, from 200 ng of DNA extracted with or without easyMAG automation (if genotyping was negative with the second protocol, we tested the third), and the presence of appropriate controls could validate results if a smaller amount of purified DNA was used. This new method of DNA extraction enables the use of archival FFPE tissues for HPV genotyping and

could improve the monitoring of women at risk of developing malignancy in cases in which HPV genotyping could not previously be performed.

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### Author Disclosure Statements

Authors have nothing to disclose.

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