Human papillomavirus quantification in urine and cervical samples using a general real-time PCR on Mx4000 and Lightcycler systems.

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ABSTRACT

During the last decade, growing efforts have focused on HPV detection using liquid-hybridization, conventional PCR and real-time PCR based methods to increase the overall proportion of patients participating in cervical cancer screening procedures. We proposed a new general HPV DNA real-time PCR on Mx4000 (Stratagene) and Lightcycler (Roche Diagnostics) usable for both cervical scrape specimens and urine samples. A linear range was obtained from 5 DNA copies to 8-log DNA copies/mL and intra- and inter-assay were between 1.8 and 4%. Cervical carcinoma / HPV DNA screening was performed in 333 consecutive women referred for gynecological examination at the University Hospitals of Angers and Brest and enrolled in the PapU study. Among cervical specimens (n=333), 45% were positive for HPV DNA with a mean viral load at 5.00 log/mL (+/- 1.73). Among urine samples (n=177), 37% were positive with a significant 50-fold lower mean viral load (3.77 +/- 1.32 log/mL, p<0.0001). Kappa agreement for HPV DNA between cervical and urine was excellent (93%). Thus, we developed a highly sensitive and quantitative general HPV DNA real-time PCR method that allows mass screening of patients with HPV infection. The ongoing longitudinal and prospective multicentric PapU study should give us the opportunity to validate this method adapted to HPV DNA screening in urine samples in a larger population.

Key words: HPV DNA – quantification – real time PCR – urine detection
Human Papillomaviruses (HPV) are epitheliotropic viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia. More than a hundred different types of HPV have been identified to date, of which 40 have been reported in anogenital infections. In a recent multianalysis involving 1918 women in 11 case-control studies (14) fifteen HPV (HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) were classified as high risk (HR-HPV) and associated with precancerous lesions of the cervix, 3 were classified as probable HR-HPV (26, 53, 66) and 12 as low risk, i.e. not associated with the development of cervical carcinoma (LR-HPV type 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108). Because of the strong association between HPV infection and cervical cancer, detection of HPV DNA in cervical samples may be an option for identifying women at risk of developing cancer (13). However, cervical sampling is uncomfortable, time-consuming and requires a degree of skill. Self-collected cervical sampling was not found to be as efficient as sampling done by a physician (19). Therefore, about 40% of the women in France presenting a cervical carcinoma have never been screened. Moreover, it would be easier to use urine specimens as is done with molecular detection of Chlamydia trachomatis (7, 21). This would simplify mass screening and survey of HR-HPV female carriers.

Efficient HPV culturing remains elusive and the clinical performance of serological assays is still poor. Thus diagnosis of HPV infection is based almost entirely on molecular tools, including liquid hybridization (e.g., Hybrid capture, etc.), Southern and dot blot hybridization with HPV type-specific probes, type-specific PCR and general-primer PCR. Several general PCR primers have been developed to detect a broad spectrum of HPV genotypes. The majority of large studies to date have been performed with the MY 09/11, the GP5+/6+ and the SPF10 general-primer sets allowing the amplification of the L1 HPV region. Various methods have been described for detection and identification of HPV genotypes after amplification with general PCR primers, such as Amplicor HPV assay combined with linear
array (LA, Roche Diagnostics) or SPF10-Line probe assay (LiPA, Innogenetics) showing similar results (10, 15, 18, 19, 23, 24).

In this study, we propose a new, highly sensitive, real-time general PCR that will allow quantification and typing of more than 50 HPV genotypes. This method can also be used for urine samples, permitting mass screening of HPV genital infections.

**MATERIALS AND METHODS**

**Patients and specimen collection.** Cervical scrape samples were collected from women consulting a gynecologist at the following units: the Department of Obstetrics and Gynecology of the Angers University Medical School Hospital; the Department of Obstetrics and Gynecology of the Brest University Medical School Hospital; the Angers antivenerial dispensary; and the Angers Women and children protection unit. The samples were prospectively assessed for HPV screening. Patients were proposed participation (with informed consent) in the PapU study, a prospective longitudinal multicentric study for HPV DNA detection in urine and cervical samples, that started in 2004 and is currently underway (up to 2007). HPV positive patients were invited for a follow-up visit after 6 to 12 months. Both cytobrush of cervical scrapes in 2SP medium (2 mL) and, when included in our study, first-stream urine (5 to 10 mL) specimens were sampled for each patient and stored at -80°C while awaiting analysis.

**DNA isolation.** DNA was extracted from 200 µL of cervical samples using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France), as recommended by the manufacturer. Briefly, sample lysis was obtained by proteinase K digestion at 56°C for 10 min and the lysate was then loaded on the Qiamp column. After 2 washes, DNA was eluted with 200 µL of elution buffer provided in the kit. For DNA extraction from urine, the QIAamp viral RNA mini kit from Qiagen was used. As recommended by the manufacturer, 1 mL of urine was centrifuged
1 hour at 23000g and the pellet was resuspended with 200 µL of sterile phosphate buffer. After lysis, the suspension was loaded onto the Qiamp column; DNA was then washed twice and eluted with 50 µL of elution buffer. Extracted DNA was kept at -80°C before PCR analysis.

**Plasmid and cell controls.** HPV type 6 plasmid (pHP6) was previously constructed in Angers virology laboratory using the pMos Blue vector (Amersham, France). A 73-base amplified DNA in the L1 region was introduced in the unique EcoRV restriction site of pMos Blue. The insert sequence was controlled by sequencing pHP6 using the Ceq2000 capillary system (Beckman) and Blast analysis was performed on the NCBI web site. Plasmid concentration was measured by DNA spectrophotometric analysis at 260 nm (160 ng/µL or 5x10^10 DNA copies/µL).

SiHa cell line, kindly provided to the Brest virology laboratory by Dr Marianne Coste (Virology lab, Nantes, France), is considered to contain one to two copies of HPV 16 per cell (average of 1.5 according to our calculation). These cells were cultured in MEM medium and 10% fetal calf serum, trypsinized at confluence and pelleted; cells were then resuspended in sterile phosphate buffer to get 10^7 HPV DNA copies per mL. Five 10-fold serial dilutions in phosphate buffer from this suspension were done. DNA was extracted from these pure and diluted cell suspensions with the QIAamp DNA mini kit as realized for cervical samples.

We used pHP6 and SiHa cells to assess the sensitivity and the reproducibility of our method.

**Real-time PCR.** HPV DNA amplification and quantification were carried out in the Mx4000 (Stratagene Europe, Amsterdam, The Netherlands) and in the Lightcycler (Roche diagnostics, Meylan, France), used respectively in the Angers and Brest virology laboratories. A SYBR green protocol was assessed on these automates with modified SPF10 general primers (Table 1) allowing detection of about 40 HPV genotypes (9). Modification with 4
more bases at the 5' end of the original SPF primers adjusted to several HPV genotypes allowed hybridization at 60°C and a two amplification steps protocol at 60 and 95°C for real-time PCR. Addition of a biotin in the 5' end of reverse primers allowed direct typing of HPV positive samples using the Line probe assay (LiPA). Forward and reverse modified SPF primers were pooled at 2.5 pmol/µL. Five µL of extracted DNA from samples was added to SYBR Green master mix (Stratagene for Mx4000 or Roche Diagnostics for Lightcycler) and forward and reverse modified SPF primers at 100 nM in a final volume of 25 µL for Mx4000 or 20 µL for Lightcycler. Thermal cycling was one step at 95°C for 10 min, followed by 45 cycles at 95°C for 30 sec and 60°C for 1 min using Mx4000 or 95°C for 15 sec and 60°C for 30 sec using Lightcycler. A melting curve was added between 55 and 95°C. Expecting melting temperature for HPV L1 SPF amplicons was around 72°C (+/- 1°C) using Mx4000 and 77°C (+/- 1°C) using Lightcycler.

Cell GAPDH DNA from each sample was tested in parallel, as internal control and cell number evaluation, in the same thermal run with the TaqMan universal master mix (Applied Biosystem), forward and reverse GAPDH primers at 300 nM and Fam-Tamra GAPDH probe at 250 nM (Table 1). Results were expressed in threshold cycle number (Ct), the number of cycles at which the fluorescent signals enter the log-linear phase were consistently obtained. The number of cells in each sample was estimated by the following calculation obtained after several cell counts and GAPDH Ct measures: \( n(\text{cells}) = 10^{exp[(-0.2861 \times \text{CtGAPDH} + 12,602) - 1]} \); r = 0.96.

Series of 10-fold diluted SiHa cells and diluted pHP6, corresponding to respectively 5, 50, 500, 5000, 50 000 DNA copies of HPV16 and 25, 250, 2500, 25 000, 250 000 DNA copies of HPV6, were run in parallel to plot the standard curves for HPV DNA quantification in samples. Triplicate dilution series were included as standards in each run. HPV copy number in samples was evaluated using regression analysis. Corresponding Ct were reported to the
standard curve allowing sample results expressed as negative (no Ct) or positive, and in the
latter, quantitatively in $\log_{10}$ DNA copies/mL of cervical media or urine. All assays were
performed in duplicate on Mx4000 and in a single test on Lightcycler.

Optimization of our protocols on Mx4000 and Lightcycler was obtained by evaluation of
primer concentrations in a range of 100 to 300 nM, $\text{MgCl}_2$ concentration for Lightcycler
master mix from 3 to 5 mM and thermal hybridization temperature between 52 and 62°C on
Mx4000 in 10-fold diluted pH6 and SiHa as DNA standards in triplicate.

Genotyping using reverse hybridization by the INNO-LiPA HPV genotyping system.
Twenty µL of the biotinylated real-time PCR product from positive samples were added to
denaturation solution and incubated with one strip in hybridization solution from the INNO-
LiPA kit (Innogenetics, Ghant, Belgium). Each membrane strip contained 25 oligonucleotides
specific for 25 different HPV types (namely types 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43,
44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70 and 74). The strips were washed twice in
stringent conditions and then incubated with an alkaline phosphatase-labeled streptavidin
conjugate for color development by addition of 5-bromo-4-chloro-3-indolylphosphate and
nitroblue tetrazolium in substrate buffer. After a final washing and drying, the strips were
visually interpreted using a grid as previously described (10).

Statistical analysis. Qualitative parameters were analyzed by using the Fisher exact test
and quantitative parameters by using the Mann-Whitney non-parametric test and Pearson's
correlation factor. Sensitivity of HPV detection in urine compared to cervical samples was
expressed in percentages with 95% exact confidence intervals. Cohen's kappa constant was
calculated to evaluate the measure of kappa agreement ($K$ value) for HPV detection between
urine and cervix. A $p$ value higher than 0.05 and a $K$ value higher than 0.60 were considered
as significant. All statistical analysis was performed using the software SPSS 10.1 for
Windows (statistical package of services solutions, SPSS Inc. Chicago IL, USA).
RESULTS

Optimization of our general HPV protocol. The best primer concentrations were defined at 100 nM with 98.8% efficacy. Increasing reverse pooled primer concentration did not modify Ct results of diluted pH6 and SiHa extracted DNA but using forward pooled primers higher than 200 nM showed non-specific positive Ct results. Biotinylation of reverse primers did not affect PCR efficacy compared to non-biotinylated primers (respectively 98.8 vs 99.1% efficacy, p>0.05). Hybridization temperature assays between 52 and 62°C gave specific results over 58°C. Below this level, non-specific positive results were observed. A temperature of 60°C was found to be most appropriate for the thermal cycling program. The best MgCl$_2$ concentration was defined at 4 mM for the Lightcycler protocol. A 5 mM concentration gave similar Ct results but a 3 mM gave higher Ct levels with thus lower sensitivity (about 5-fold).

Development of real-time PCR to amplify and quantify HPV DNA. Mx4000 and Lightcycler protocols gave very similar results (Table 2). Threshold levels of 50 and 95% of positive samples were evaluated using 10-fold and 4-fold diluted pH6 and SiHA cells (Table 2). The lowest detection threshold was defined at about 5 HPV DNA copies. Linearity assays were obtained up to 8 log$_{10}$ DNA copies/mL. Inter and intra-assays reproducibilities upon 10 runs were below 5%. The slope was excellent (-3.22) showing high efficiency (98%). Results using pH6 or SiHa cells, containing respectively HPV6 and 16 types, were similar (Ct at 31.65 vs 31.37 for 500 HPV DNA copies and Ct at 28.63 vs 28.28 for 5000 HPV DNA copies).

Evaluation of HPV DNA quantitative real-time PCR on clinical samples. Cervical scrape samples were obtained from 333 women followed in Angers and Brest hospitals. Among them, 177 participated in the PaPU study. A panel of 10 positive samples was evaluated in both Mx4000 and Lightcycler protocols. Median values were comparable (4.83
log DNA copies/mL for Mx4000 vs 5.04 log/mL for Lightcycler, with a mean difference of 0.21 log and a Pearson's correlation of 0.79), confirming our plasmid results. All cervical samples were positive for GAPDH DNA showing the presence of cells in all samples (Ct=25.60 +/- 5.66, corresponding to about 620,000 cells with a range from $10^3$ to $7 \times 10^6$ cells). Most of the urine samples presented cells (150 out of 177 had a positive GAPDH Ct with an average Ct at 29.71, corresponding to about 16,000 cells with a range from 0 to $3 \times 10^5$ cells).

HPV positive samples were found in 150 cervical specimens out of 333 (45%) and 66 urine samples out of 177 (37%). Good agreement was obtained for HPV detection in cervical and urine samples, with a Kappa test at 93% (Table 3). However, HPV viral load was 50-fold higher in cervical samples than that in urine (respectively 5.00 +/- 1.73 vs 3.77 +/- 1.32 log/mL, p<0.0001). Levels of viral load in cervical specimens were correlated to levels in urine ($r=0.81$, $p=0.009$). Discordant results between positive cervical and negative urine samples showed more often low viral loads (below 3 log/mL). No correlation was found between viral load in both samples and level of cells (41% of cervical specimens and 62% of urine samples were not correlated to cell levels estimated by GAPDH Ct levels, $r=0.64$ and 0.54 respectively, $p>0.05$).

Higher viral load was observed in high-grade squamous intraepithelial lesions (HGSIL) than that of normal cytology (respectively 5.52 +/- 1.95 vs 4.26 +/- 1.42 log/mL, p=0.008). High risk (HR) HPV type 16 was found most frequently (34.9%) followed by type 66 (14.9%), type 31 (10.8%), type 33 (9.5%), type 56 (8.6%) and type 18 (6.8%). Mixed types accounted for 31.7% of the patients with always one or more HR-HPV types whereas low risk (LR) HPV types alone were found in 8.7% of cases. Higher viral load was observed with HR-HPV types than that of LR-HPV (5.52 +/- 1.95 vs 4.26 +/- 1.42 log/mL, p=0.01). Among HR-
HPV types, the highest viral loads were observed with types 16, 18, 31 and 33 (5.99 to 6.49 log/mL) compared to types 56 and 66 (5.17 to 5.29 log/mL, p=0.02).

**DISCUSSION**

We have reported herein a highly sensitive (about 5 copies), linear (up to about 100 millions of copies) and quantitative real-time general PCR for HPV DNA detection. This method, on which relies the diagnosis of HPV infection, was adapted to two commonly used real-time PCR systems (Lightcycler from Roche Diagnostic and Mx4000 from Stratagene) with comparable results. Previous studies have suggested that methods with higher sensitivity could make HPV detection in urine possible (2, 3, 6, 20). The urine approach should facilitate HPV detection in women who do not access to a gynecologist and should be more attractive to patients whatever their social origin. HPV positive patients (about 10% expected) would thus be referred to a gynecologist for clinical and cyto-histological diagnosis in the cervix.

Therefore, we have adapted the SPF10 PCR method previously described (9) to real-time PCR, showing higher sensitivity to detect more HPV infections from almost 57 different genotypes, compared to two other general PCR based methods using the GP5+/6+ and MY09/11 primer sets. These primers (SPF, GP and MY) allowed amplification of different overlapped fragments in the HPV L1 region, between base/nucleotide 6500 and 7000 from HPV, with respectively 65, 150 and 450 basepairs. The smallest fragment obtained with the SPF10 primers was the most appropriate for real-time PCR thermal conditions, since only small PCR fragments are required. Also, type-specific assay is possible using the SPF10 amplicons with biotinylated reverse primers on the Line probe assay (LiPA) developed by Innogenetics (10). We showed in this study that the use of biotinylated primers in real-time PCR did not affect amplification and SYBR green detection. We also optimized the SPF10...
primers to allow a 60°C hybridization temperature for real-time PCR optimal thermal condition.

A highly sensitive method may be required in order to realize the highest negative predictive value (NPV) for detecting HPV-associated cervical carcinoma (i.e. no detected HPV is related to no risk of developing a carcinoma). Amplification detection assays, such as PCR-based methods, are more sensitive than liquid hybridization tests such as Digene Hybrid capture (HC) II assay, as demonstrated previously for several viral molecular markers, such as cytomegalovirus DNA, hepatitis B DNA and more recently for HPV DNA (15). Indeed, Perrons et al (15) showed that HC II assay could detect only 56% of positive HR HPV DNA using the SPF10 PCR method tested in parallel whereas the latter could detect all HC II positive HR HPV. They also demonstrated that lower sensitivities of HC II were obtained for normal or ASCUS Pap smears (respectively 28 and 45% compared to 55 and 69% using the SPF10 PCR method), related perhaps to lower viral load in these groups of patients. HC II assay is reported to have an analytical sensitivity at 1 pg of HPV DNA corresponding to about 5000 DNA copies and to be less sensitive than PCR methods which allowed cut-off levels of about 5 to 500 copies (12). However, PCR methods may be impeded by inhibitors as shown in cervical lavages (11), urethral swab specimens (22) or urine samples (3). It is important to evaluate the efficacy of the PCR using internal controls and to use the best DNA extraction procedure. Therefore we used the GAPDH DNA cell control to assess the presence of cells in samples and elimination of inhibitors, which have been found in 7 to 19% of endocervical samples and about 50% of urethral, and urine samples. GAPDH DNA has been found to be a stable marker for cell counting estimation (8). Other cell markers, such as β-globin, were shown to be insensitive to some inhibitors in 8% of cervical lysates (11). Appropriate DNA extraction procedures have been shown to eliminate these inhibitors as demonstrated by Brinkman et al for HPV extraction in urine samples (3). These authors found that urine could
inhibit HPV DNA amplification 10^6-fold more compared to water using a type 16 HPV
diluted plasmid. They showed that by using the silica-based viral DNA/RNA extraction
method from Qiagen, as we used in this study, they were able to allow reduction of inhibitor
effect of more than 10^5-fold.

A reliable HPV DNA quantification method is required for viral load measures. High viral
load seems to be indicative of viral persistence and disease development, whereas low viral
load is more often associated with viral clearance (5, 21). Although the HC II assay has not
been validated as a quantitative test, it was shown in these studies that high HPV RLU ratio
was related to the risk of developing HGSIL. However, more precise methods have to be
evaluated for this purpose. The recent availability of real-time PCR methods allows such an
approach (17). However, clinical viral load thresholds have to be defined. Using a specific
HPV type 16 quantitative method, Pretet et al. (17) showed that a viral load higher than 10^7
copies per mL may contribute to lesion evolution towards CIN 2 to 3, corresponding to more
than 100 pg/mL using the HCII test. We also observed that higher viral load in patients (over
10^5 copies per mL) was related to HGSIL. Variation in clinical threshold emphasizes the need
of standardization of the methods with a control panel (18). HC II assay is the only US Food
and Drug Administration approved method and the lack of a reference method makes the
constitution of a control panel difficult. We also showed in this study that viral load was not
correlated to cell levels. Therefore, we do not agree with Pretet and collaborators who chose
to normalize HPV copy numbers according to sample cellularity assessed by albumin PCR
(17). These authors found good correlations between their method and the HC II assay
whether expressed in copies per mL or per 10^3 cells. However, we did not find a correlation
when HPV DNA was expressed in copies per mL or per 10^2 cells. Indeed, we observed
samples, mostly urine but also cervical specimens, with high viral load and few cells.
Normalization of HPV value might contribute to under or over estimation of the viral load.
Finally, it is important to express HPV results with specific genotype. Indeed, the recent study from Perron et al (15), showed that among followed HPV positive patients assessed by SPF10-LiPA method (60% of the included patients), 28% were positive with the same genotype at 6 months and 7% positive with a different genotype; the first would be considered as persistent infection whereas the second as "resolved-acquired" infection. Both situations were considered as persistent infection using the HCII assay. They also observed, as others did (4, 16), cross-reactivity between HR and LR HPV; 13 out of 20 LR HPV assessed by the SPF10-LiPA system were found as HR HPV using HCII assay. Cross-hybridization has been found with at least 22 different LR HPV with the current HR HPV HCII probes (16).

In conclusion, we developed a highly sensitive and quantitative general HPV DNA real-time PCR method that allows mass screening of patients for HPV infection. This method allows specific genotyping using the LiPA system. Our method also seems to be sensitive enough to allow HPV detection in urine samples with high performance, owing to improvement of the whole PCR process as suggested by the recent study of Daponte et al (6). The ongoing longitudinal and prospective multicentric PapU study should give us the opportunity to validate this approach by using this method in a larger population and at the new age of vaccination against HPV infections.

ACKNOWLEDGMENTS

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REFERENCES


TABLE 1. Sequences of HPV general primers derived from SPF10 primers (9) and of GAPDH DNA cell primers and TaqMan probe.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Forward HPV primers</th>
<th>Reverse HPV primers</th>
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<tbody>
<tr>
<td>HP 1E</td>
<td>5'-AACGTGCICAGGGICACAATAATGG-3'</td>
<td>5'-biotCGGCTAGTATCIAACAGTAACAAA-3'</td>
</tr>
<tr>
<td>HP 1F</td>
<td>5'-ATAAGGCGAGGGICATAACAAATGG-3'</td>
<td>5'-biotCGAGTGCTATCAGTAACAAA-3'</td>
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<tr>
<td>HP 1G</td>
<td>5'-AACGTGCICAGGGICATAAAATGG-3'</td>
<td>5'-biotCGGGGTAGTATCIAACTACAGTAACAAA-3'</td>
</tr>
<tr>
<td>HP 1H</td>
<td>5'-AACGTGCICAGGGICATAAAATGG-3'</td>
<td>5'-biotCGGGTAGTATCIAACTACAGTAACAAA-3'</td>
</tr>
<tr>
<td>Reverse HPV primers</td>
<td>5'-biotCGGCTAGTATCIAACAGTAACAAA-3'</td>
<td>5'-biotCGAGTGCTATCAGTAACAAA-3'</td>
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<tr>
<td>HP 2Cb  biot</td>
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<td>HP 2Db  biot</td>
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<td>HP 2Ebiot</td>
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<td>5'-biotCGGGTAGTATCIAACTACAGTAACAAA-3'</td>
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<td>GAPDH Forward primer</td>
<td>5'-GGACTGAGGGCTCCACCTTT-3'</td>
<td>5'-GGACTGAGGGCTCCACCTTT-3'</td>
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<td>GAPDH Reverse primer</td>
<td>5'-GGACTGAGGGCTCCACCTTT-3'</td>
<td>5'-GGACTGAGGGCTCCACCTTT-3'</td>
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<tr>
<td>HT probe</td>
<td>5'-Fam-CATCCAAGACTGGCTGCTCCCTGC-Tamra-3'</td>
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Biot: 5'end biotinylated primers; Fam: 6-carboxyfluorescein; Tamra: 6-carboxy- tetramethyl-rhodamine; GAPDH: glyceraldehyde phosphate dehydrogenase
<table>
<thead>
<tr>
<th>Test results</th>
<th>Mx4000</th>
<th>Lightcycler</th>
<th>HPV DNA protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thresholds 50 and 95% (HPV DNA copies/assay)</td>
<td>15 and 150</td>
<td>25 and 200</td>
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<tr>
<td>Linearity ($\log_{10}$ DNA copies/mL)</td>
<td>1.17 – 8.39</td>
<td>1.39 – 7.69</td>
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<td>Reproducibility at 500 copies (mean Ct; SD; CV %)</td>
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<tr>
<td>Intra-assay</td>
<td>31.62; 0.58; 1.8</td>
<td>31.65; 0.69; 2.2</td>
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<tr>
<td>Inter-assay</td>
<td>32.59; 0.65; 4.0</td>
<td>33.19; 0.70; 3.2</td>
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</tbody>
</table>

Ct : cycle threshold; SD: standard deviation; CV: coefficient of variation
TABLE 3. Overview of the 177 patients tested for HPV DNA in urine and cervical paired samples (CS)

<table>
<thead>
<tr>
<th>Test results</th>
<th>Positive CS</th>
<th>Negative CS</th>
<th>Total</th>
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<tr>
<td>Positive urine</td>
<td>62</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>Negative urine</td>
<td>6</td>
<td>105</td>
<td>111</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>109</td>
<td>177</td>
</tr>
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</table>

Sensitivity with urine = 91.2%, Specificity with CS = 96.3%, Agreement = 94.4%, Kappa test = 93.2%