Simultaneous Detection, Genotyping and Quantification of Human Papillomaviruses by Multicolor Real-Time PCR and Melting Curve Analysis

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ABSTRACT

Long-term infection of high-risk human papillomavirus (HPV) is the leading cause of cervical cancer while infection of the low-risk HPV is the major reason for condyloma acuminata. An accurate, rapid and convenient assay that is able to simultaneously detect, genotype and quantify HPV is of great clinical value and yet remains to be achieved. We developed a three-color real-time PCR assay that is able to analyze 30 predominant HPVs types in three reactions. The amplification curves indicated the presence of HPV, melting curve analysis identified the HPV genotype, and Cq value determined the quantity. We applied this assay to 647 cervical swab samples and the results were compared with a commercial genotyping system. The proposed assay had a limit of detection of 5~50 copies per reaction and a dynamic range of 5×10¹~5×10⁶ copies per reaction. Comparison study showed that the overall sample concordance with the comparison method was 91.6% and the type agreement was greater than 98.7%. Quantification study demonstrated that the viral loads of HPV 16 in 30 samples with cervical intraepithelial neoplasia (CIN) III lesions were significantly higher than those with CIN I lesions or CIN II lesions and the results were concordant with the comparison method. The increased information content, high throughput, and low cost would facilitate the use of this real-time PCR-based assay in a variety of clinical settings.
INTRODUCTION

HPV is one of the most important and complex targets in molecular diagnosis (30, 31). The importance lies in its direct association with cervical cancer and the complexity is caused by its large number of genotypes (4), which should be differentiated in order to clarify the disease risks and to implement vaccine program (22, 23). Moreover, quantification of HPV is required to determine disease status and monitor the progression of HPV infection (12, 17, 24, 29). Preferably, a diagnostic method for HPV infection should enable simultaneous detection, genotyping and quantification of HPV. Ideally, such a method should be undertaken in a high throughput, easily automated and cost-effective way, in particular, when considering its use in large-scale population screening programs (21, 27).

A variety of assays for detection and/or genotyping for HPV have been described. The hybrid capture 2 (HC2, Qiagen Inc, Gaithersburg, Maryland, USA), cleared by the US Food and Drug Administration (FDA), is considered the gold standard to detect 13 high-risk HPVs from cervical scrapings and has been used in routine screening for years (16). However, this assay does not allow identifying the specific HPV type and cannot discriminate multiple infections. These deficiencies have been solved partially with the introduction of PCR-based assays by using consensus or type-specific primers combined with numerous post-PCR analysis procedures, such as genotype-specific hybridization (7, 13, 15), minisequencing (9, 25), sequencing (1, 6), etc. Most of these assays allow identification of a broad range of HPV types, with the major disadvantage of being expensive and difficult to automate due to the complicated post-PCR analysis.

Real-time PCR-based methods, being free of post-PCR analysis, have gained increasing
popularity in HPV diagnosis. This trend is reflected in the recent reports of several automated commercial systems. For example, the newly FDA-approved cobas 4800 HPV test features fully automated sample preparation combined with real-time PCR technology to detect 14 high-risk HPV genotypes (8). Although designed as a qualitative test utilizing real-time PCR technology, the test also provides cycle number that is generally indicative of the amount of viral DNA present in the sample. Similar features are found in the Abbott RealTime High Risk (HR) HPV assay (2). LG Life Sciences (Seoul, Korea) has also developed a real-time PCR assay (AdvanSure HPV Screening real-time PCR), which can detect and distinguish the signals for HPV 16/18 from 39 non-16/18 types as well as an internal control in a single reaction (11). Because current real-time thermocyclers have limited number of detection channels and are thus unable to distinguish many different fluorescent dyes, the above real-time PCR-based systems preclude full genotyping of dozens of HPV types in a single reaction. Alternatively, type-specific real-time PCR approaches have been developed with high sensitivity and specificity, however, many separate reactions are required in order to cover all common HPV types (20).

To maintain the simplicity of real-time PCR while overcoming the channel limitation, we hereby described a new strategy that allows simultaneous detection, quantification, and genotyping of 30 HPV types in three reactions on a three-color real-time PCR machine. Our previous work had demonstrated that dual-labeled, self-quenched probes could be used for both real-time PCR detection and melting curve analysis under asymmetric PCR conditions (10). Because real-time PCR detection could offer quantitative information while the melting curve analysis reveals the sequence information, a combination of them would allow a simultaneous quantification and genotyping from one sample in
a single assay. By assigning a predefined melting temperature to one HPV type, up to five different
HPV types could be differentiated in one detection channel. A multi-channel real-time PCR machine
would therefore allow a large number of HPV types to be differentiated in one reaction. The
established assay was evaluated regarding the sensitivity, accuracy and dynamic ranges. Both the
typing and quantification results were compared with commercial and approved assays by analyzing
clinical samples.

MATERIALS AND METHODS

Clinical samples and synthetic plasmids. Cervical swabs were obtained from 647 females, ages
19–68 years (mean, 33 years; median, 32 years), 494 of them were referred to Molecular Diagnostics
Laboratory, Maternal and Child Health Hospital of Xiamen, China, for a routine gynecologic
examination, and 153 of them were for colposcopy after suspected diagnosis of HPV infection.
Genomic DNA was extracted from each swab using a kit supplied with the HPV GenoArray test kit
(Hybrbio Ltd., Chaozhou, China). DNA extracts were first used for GenoArray analysis. After storage
at -20°C for 3–4 months, the same DNA extracts were thawed and used for real-time PCR assay. The
Research Ethics Committee of Xiamen University approved the study protocol.

Plasmids containing GP5+/GP6+ flanked region of the respective 30 HPV types were synthesized
by Sangon (Shanghai, China) based on the type-specific sequences provided by Genome Sequencing
Projects in GenBank (http://www.ncbi.nlm.nih.gov). The concentration of each plasmid was
determined by ND-1000 spectrometer (Nanodrop Technologies, Rockland, Delaware, USA). To
determine the limit of detection (LOD) of the real-time PCR assay for each HPV type, the plasmids
were diluted to a series of concentrations of $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, and $10^0$ copies/μL in the presence of 20 ng of SW-480 cell genomic DNA (HPV negative) for each concentration. These plasmid preparations were also used to study the reproducibility of the $T_m$ measurement as well as the specificity of HPV genotyping.

**Primers and probes.** The design of consensus primers for the 30 HPV types was based on the previously reported GP5+/GP6+ primer pair, which proved to be able to efficiently amplify 23 mucosotropic HPV types (3). We introduced 2 degenerate bases in GP5+ and 2 degenerate bases in GP6+, the resulted GP5+/GP6+’ primer pair could amplify all the 30 HPV types efficiently. One additional primer pair was designed for amplification of human β-globin gene ($HBB$), which was used as an internal positive control (IPC). The sequences of the primers are given in Table S1.

Thirty dual-labeled fluorogenic probes were designed for the 30 respective HPV types. The rule of the probe design was that each probe would hybridize with its specific target at a distinct melting temperature ($T_m$) value, and that each probe would have no cross-hybridization with any other known mucosotropic HPV types detectable at above 40°C. Moreover, the $T_m$ decrease induced by the intratype variants within each type would cause no crossover with other HPV types. The $T_m$ value of each probe-target hybrid was controlled by the length and G/C content of the probe. According to our experimental design, three reactions were used to test 30 HPV types. Reaction 1 tests 10 high risk HPV types (HPV 68, 58, 82, 59, 73 in Cy5 channel, HPV 31, 33, 16, 35, 18 in ROX channel). Reaction 2 tests 5 high risk HPV types (HPV 56, 39, 45, 52, 51 in Cy5 channel) and 5 low risk HPV (HPV 66, 53, 26, 11, 6 in ROX channel). Reaction 3 tests 10 low risk HPV types (HPV 72, 70, 61, 54, 64, 67, 71, 73, 31, 33).
In all three reactions, *HBB* was detected in FAM channel. Therefore, each reaction contained 10 HPV probes and 1 *HBB* probe. Five of the HPV probes were labeled with 5'-ROX and 3'-BHQ2, another five HPV probes were labeled with 5'-Cy5 and 3'-BHQ2, and *HBB* probe was labeled with 5'-FAM and 3'-BHQ1. The sequences of probes are given in Table 1.

**Real-time PCR assay.** The real-time PCR assay was performed in 3 separate reactions. Each reaction (25 μL) contained 75 mmol/L Tris-HCl (pH 9.0), 20 mmol/L (NH₄)₂SO₄, 4 mmol/L MgCl₂, 0.01% (V/V) Tween 20, 1 unit Taq DNA polymerase, 200 μmol/L of each dNTP, 100 nmol/L GP5+' primers, 1 μmol/L GP6+' primers, 100 nmol/L IPC-F primers, 1 μmol/L IPC-R primers, and 250 nmol/L of each of the 10 HPV and *HBB* probes. In each reaction, 5 μL template DNA was added. The PCR and dissociation were performed in the Applied Biosystems 7500 (Foster City, California, USA). PCR started with denaturation at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 40°C for 32 s, and 75°C for 30 s. Melting curve analysis consisted of 95°C for 15 s, 40°C for 60 s, and 85°C for 15 s with the ramp rate between 40°C and 85°C set to 5%. Fluorescence signal was acquired at the annealing step of each cycle during amplification and throughout the final ramp between 40°C and 85°C in three detection channels: FAM (510 nm), ROX (610 nm) and Cy5 (660 nm). The existence of HPV in the sample was judged by the appearance of the amplification curve, HPV genotype was called by the predefined *Tₘ* value in melting curve.

**Quantification of HPV in a sample.** The amount of HPV was determined by the Cq value of the
amplification curve and calculated by its external calibration curve. For a sample infected with a single HPV type. Quantification can be directly achieved through the C_q value. For a sample infected with multiple types of HPV that occur in different channels or identical channel but different reactions, each type could be quantified separately like in the single infection. If the co-infected HPV types occur in identical channel of the same reaction, the C_q value obtained represents the sum of co-infected types. To calculate the amount of individual genotype, the quantity ratio of different genotypes needs to be determined. We explored this situation by using co-infection of HPV 16 and HPV 18 as a model. These two genotypes occur in ROX channel of reaction 1. Firstly, we hypothesized that that the quantity ratio of HPV 16 to HPV 18 had a linear relationship with the height ratio of their respective melting peak according to previous reports (32). This hypothesis was confirmed by the observation that in the range of 90:10 to 10:90, the quantity ratio of HPV 16 to HPV 18 was linear to the height ratio of their melting peaks and this linear relationship (y = -3.58 + 3.99X, where “y” is the concentration ratio and “X” is the peak height ratio) (R^2 = 0.9905) kept constant in their concentration range between 5 × 10^2 copies to 5 × 10^6 copies per reaction (see Fig. S1 in the supplemental material). These observations allowed us to deduce the quantity ratio of these two genotypes from the melting curves. Secondly, we reasoned that once the equations of the calibration curve of both HPV 16 and HPV 18 are known, the equation of the calibration curve of their mixture at certain ratio could be obtained. To do so, five mimic samples containing different percentage ratios of HPV 16 to HPV 18 plasmid DNA (100:0, 75:25, 50:50, 25:75, and 0:100) were prepared, their respective equation of the calibration curve was obtained by detecting the C_q values at a series of different concentrations (see Fig. S2 in the supplemental material). Five linear equations were
obtained from the five ratios in terms of $C_q = a + b \times \log(\text{viral load})$, where “$a$” is the initial value, “$b$” is the slope. Using a polynomial regression fit, “$a$” could be obtained as a function of the percentage of HPV 16 (Y) as: $a = 43.30 - 6.731Y + 2.971Y^2 \ (R^2 = 0.9980, \text{equation 1})$ and “$b$” as: $b = -3.717 + 0.712Y - 0.331Y^2 \ (R^2 = 0.9906, \text{equation 2})$. Therefore, using equation 1 and 2, an equation for the mixture of HPV 16 to HPV 18 at a certain ratio could be calculated. Consequently, a quantification guideline was formulated to quantify both HPV 16 and HPV 18 of different ratios (see Quantification rule in the supplemental material). This quantification is also applicable to other dual, triple or even higher level of multiple infections.

Comparison methods. The HPV GenoArray test is a Chinese FDA-approved assay for HPV genotyping. It is an L1 consensus primer-based PCR assay and is capable of typing 21 HPV genotypes (15). The assay utilized a flow-through hybridization technique by actively directing the targeting molecules toward the immobilized probes within the membrane fibers, with the complementary molecules being retained by the formation of duplexes. After a stringent wash, the hybrids were detected by the addition of the streptavidin-horseradish peroxidase conjugate, which binds to the biotinylated PCR products, and a substrate to generate a purple precipitate at the probe dot. The results were interpreted by direct visualization. The Diagnostic kit for HPV (Type 16 and 18) is a Chinese FDA-approved real-time PCR assay for quantification of HPV 16 and HPV 18 (Kehua Ltd., Shanghai, China). This is a 5′-nuclease assay that can quantify both HPV 16 and HPV 18. Sequencing with type-specific primers (28) was used to confirm those discrepant samples detected by the new method and the comparison methods. Statistical analysis of the comparison data was carried out by using the
RESULTS

The proposed assay is a 3-tube, 3-color, duplex real-time PCR combined with melting curve analysis. Its temperature program contains two stages: amplification and melting, which can be finished in a consecutive procedure within 2.5 h. A typical result from a sample infected with HPV 16 is shown in Fig. 1. The appearance of an amplification signal from ROX channel of reaction 1 demonstrated that the sample was HPV positive. The $T_m$ value obtained from the melting curve identified HPV 16 according to the predefined $T_m$ values (63.4°C). The quantity of HPV 16 in the sample was determined to be $2.19 \times 10^5$ copies/μL by referring the $C_q$ value (21.7) to the external calibration curve of HPV 16.

Using plasmid DNA as amplification template, the LOD of the assay was 5~50 copies per reaction. The amplification efficiency was 84.63%~103.68%. A linear relationship ($R^2>0.99$) between $C_q$ value and logarithmic DNA concentration was achieved in the range of $5\times10^1$~$5\times10^6$ copies per reaction. All HPV types could be identified by their corresponding $T_m$ values, which mostly had a window of larger than 4°C between neighboring HPV types in the same channel (Table 1).

The $T_m$ values listed in Table 1 were obtained from the plasmid template containing sequence of the reference HPV genotype. To detect whether the presence of intratype variants could cause $T_m$ overlapping between two neighboring genotypes in one channel, five HPV genotypes were studied using synthetic oligonucleotide as target mimics to hybridize with their respective probes. The results showed that the $T_m$ shifts caused by these variants were smaller than 2°C and none of them could induce crossover with neighboring types.

The new assay was then subjected to evaluation by a blind test of 647 cervical swab samples and...
the results were compared with the GenoArray method. The overall agreement between the two methods was 91.6% (593/647). Among the 54 discrepant samples, 12 samples were found to be infected with HPV types not included by GenoArray technique. These 12 samples were all concordant with sequencing results. Of the remaining 42 discrepant samples, sequencing analysis revealed that 35 (83.3%) samples were concordant with our assay and 7 (16.7%) samples agreed with the GenoArray method. We then compared the type-specific concordance between the two assays (Table 2). Except for the 9 HPV types that were not included in the GenoArray, the concordance between the two assays was higher than 98%. The degree of concordance (κ) was more than 0.75 for all the genotypes with the exception of HPV 44. There were totally 48 discrepant results (from 42 samples) in 2×2 typing results (from 647 samples), yielding an overall disagreement rate of 0.18%. Sequencing results demonstrated that, among these 48 discrepant typing results, 41 results (85.4%) agreed with our method and 7 results (14.6%) agreed with GenoArray method.

By referring to the histopathological results, we observed that the real-time PCR assay had a higher positive detection rate of high-risk HPV than the GenoArray method (74.8% vs. 69.9%) in samples with CIN lesions. With CIN II or higher (CIN II+) in the histopathologic results as a clinical reference, the sensitivity for the real-time PCR method was 93.8% [95% confidence interval (CI), 89.1%–98.5%], the specificity was 44.6% (95% CI, 34.9%–54.3%), and the positive predictive value was 30.9%, whereas the sensitivity of the comparison method was 90.6% (95% CI, 84.9%–96.3%), the specificity was 47.9% (95% CI, 38.1%–57.7%), and the positive predictive value was 31.5%. When the number was summarized for each genotype, HPV 16 and HPV 52 were found to be the two most common high-risk genotypes.
Among all HPV types, only the viral load of HPV 16 was reportedly relevant with the CIN status of cervical cancer (24, 26, 29). Among the 647 samples, 30 HPV16-positive samples had CIN lesion information and their viral loads were thus detected. The results showed that the viral loads in samples with CIN I lesions ranged from 2.73 to 5.41 log copies/μL (medium, 4.6 log copies/μL). The viral loads were more concentrated in higher order in samples with CIN II lesions (from 3.68 to 5.32 log copies/μL, median, 4.7 log copies/μL). The highest viral loads were detected in samples with CIN III lesions, and more condensed distribution of high viral loads was observed (from 5.26 to 5.84 log copies/μL, median, 5.6 log copies/μL). To examine the difference of HPV 16 viral loads among the three CIN statuses, an independent samples t test was performed. The results demonstrated that HPV 16 viral loads had no significant difference between CIN I and CIN II states (P = 0.236) while HPV 16 viral loads in CIN III states was significantly higher than both CIN I and CIN II states (P < 0.001).

To validate the above quantification results, the same samples were analyzed using a commercial real-time PCR-based assay. Comparison between the results of the two methods was carried out by both regression analysis (5) and Bland-Altman analysis (19). Regression analysis showed that the slope approached to 1 (1.039, 95% confidence interval, 0.987 to 1.091) and the Y intercept approached to 0 (-0.238, 95% confidence interval, -0.493 to 0.017) and the correlation coefficient (R) between two methods was 0.9908 (P < 0.001) (Fig. 2A). Bland-Altman analysis gave a mean difference of -0.12 log10 (95% confidence interval, -0.37 log10 to 0.12 log10) of viral loads between the two methods while all of the 30 samples differed by less than 0.5 log10 (Fig. 2B). The above results together demonstrated that our method and the comparison method had no significant difference in quantification of HPV 16 virus load.
DISCUSSION

We developed a real-time PCR-based assay that could simultaneously detect, differentiate and quantify 30 HPV types in three reactions, enabling multilayer information to be retrieved from a sample but with no extra manipulations. Such an advantage is attributed to the detection mode of “one channel, multiple genotypes” realized by combined use of multicolor real-time PCR and melting curve analysis. By comparison, existing real-time PCR assays use either “one channel, one genotype” or “one channel, mixed genotypes” mode. The former requires extra reactions for additional HPV types while the latter, though it can detect more HPV genotypes in one channel, is unable to differentiate them.

The key feature of the described assay is the use of multiple type-specific probes with predefined \( T_m \) values in one reaction to identify the respective HPV genotypes. This was achieved by the use of the dual-labeled, self-quenched probes (10), which allowed us to design probes with fine adjusted and well separated \( T_m \)s in one channel. The \( T_m \) values obtained through melting curve analysis proved to be highly reproducible under constant reaction conditions (18). Our results from 168 positive samples showed that all the \( T_m \) values obtained were all concordant with their predefined values regardless of the HPV concentrations. This concordance in \( T_m \) value was also seen when we tested the same samples on other real-time PCR machines like Stratagene 3005p (La Jolla, California, USA) and Bio-Rad CFX-96 (Hercules, California, USA). A systematic \( T_m \) shift of 0.5–1.5°C was observed in certain HPV types due to varied melting program as previously reported (14). By simply adjusting the predefined \( T_m \) values in these machines, genotyping could be performed as reproducibly as the ABI
There is a potential problem in that HPV type may be falsely identified if cross-reactivity occurs between probes or if intratype variants exist in HPV (1). To avoid any possible cross-reactivity, all probes were designed to have at least 4-5 bases different from each other so that no cross-hybridization could be measured at above 40°C. To overcome the destabilization effect caused by intratype variants, the probe binding region was chosen to make the variant sites be close to the terminal. Otherwise, inosine was used to base pair with the variant site. The experiment with mimic targets demonstrated that no crossover took place with those intratype variant-containing targets in spite of somewhat Tₘ reduction. The clinical validation study showed that 3 samples were found to have intratype variants and none of them was misidentified.

Although the value of viral load is still controversial, inclusion of its measurement could improve the effectiveness of HPV-based screening and triage strategies (17). Moreover, comprehensive quantification method for common pathogenic HPV is helpful to clarify type-specific association between viral load and CIN lesions (24, 26, 29). As a real-time PCR assay, our method enabled quantification of HPV regardless of whether the sample was of single infection or multiple infections. At current stage, the quantification procedure for multiple infections seemed complex, but it could be simplified or even automated through a computer program with preinstalled calibration curves.

The clinical validation study was based on a large clinical sample consecutively enrolled from a population-based screening program. The results confirmed that the new method was comparable with the GenoArray method in HPV genotyping despite that our method had a higher positive detection rate. One reason for the higher positive rate of our method should be that our assay tested more HPV types.
than the GenoArray method (30 vs. 21 types). For example, among the 54 discrepant samples, 12
samples were found to be infected with HPV types not included by GenoArray technique. Another
reason might be that our assay has higher analytical sensitivity than GenoArray. In the 48 discrepant
tying results, 41 results agreed with our method. By looking back at their quantification data, we
noticed that 23 of 41 discrepant results had copy number in between 50-3000 copies per reaction, all
of which were nevertheless detected as negative by the GenoArray method. The limit of detection of
our method was 5~50 copies per reaction for all the 30 HPV types, while the GenoArray was reported
to be 10~50 copies per reaction for HPV 16 and HPV 18 only (15). In fact, GenoArray is based on the
color intensity judged by naked eyes, while our method is through fluorescence detection by real-time
PCR. The subjective judgment of GenoArray might vary with the observer and therefore the exact
limit of detection can differ from the reported one. Indeed, the user of GenoArray was often confused
with the weak dot blots found in some samples. Nevertheless, there were 7 discrepant samples that
turned out to be inconsistent with our method but agreed with sequencing. Among them, 4 of them
samples had at least 1 concordant HPV type and therefore would not have been misclassified as HPV
negative in a screening situation. The other 3 samples were all detected as positive by both GenoArray
and sequencing but negative by our method. This false negative results reported by our method could
be due to DNA degradation during long term storage (3~4 months) or some potential interference.
When compared with reported PCR-based methods, our method had a combined advantage of
real-time PCR and solid-phase hybridization-based method while eliminating their respective
shortcomings. As a real-time PCR based-method, it has advantages such as ease-of-use, high
throughput and the ability of quantitative detection. On the other hand, the melting curve analysis step

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offers full genotyping that so far has only been achieved by solid-phase hybridization. Currently, real-time PCR-based commercial systems all detect HPV 16/18 and non-16/18 types as well as an internal control in a single reaction. Thus all those non-16/18 types are classified as one type and therefore losing the genotype information (2, 8, 11). While type-specific real-time PCR can target independently one or a few types in one reaction, it will need multiple reactions when detecting dozens of HPV types. Consequently, the throughput would be substantially decreased while the labor and the overall cost would be increased when testing a large number of samples (20).

One important advantage of the new assay is its adjustability of throughput in different clinical settings. For example, 30 samples could be tested per run in a standard 96-well real-time thermocycler, and 120 samples could be tested per run if changed to a 384-well model. As most of the time is spent in the amplification stage, the combined use of extra standard PCR machines can further increase the throughput if quantification is omitted. In our experience, as many as 360 samples could be analyzed within 8 h by combined use of 4 standard PCR machines and 1 real-time PCR machine. Such flexibility in throughput could satisfy clinical requirements of different levels. The throughput could be further increased if we use four instead of three detection channels. In this assay, our aim was to develop an assay that can be used on mainstream real-time PCR machines and we therefore chose the three most commonly used channels, i.e., FAM, ROX and Cy5. It is easy to add one channel to increase the number of HPV that can be detected in one reaction. For example, if 14 high risks HPV are detected only, a single reaction can be designed to cover all 14 HPV types by using the principle of our current method on a 4-color real-time PCR thermocycler. The only constraint of this assay is that the T_m gap between two neighboring types should be large enough to avoid the influence of
temperature fluctuation caused by the machine and the reaction system. Such a gap restricts the overall number of HPV types differentiated within one channel. Lastly, the consumable cost of this assay is approximately 3 USD per sample, which is much cheaper than most existing commercial assays.

In summary, the described assay enabled simultaneous detection, typing and quantification of 30 HPV types in 3 reactions. The probe-based detection nature ensured the reliability of this method while the closed-tube operation mode significantly reduced post-PCR manipulations, time requirements and carryover contamination. Furthermore, the high throughput and low cost would also facilitate its acceptance in clinical settings.

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Table 1. The probe sequences, amplification efficiencies and the T_m values of 30 HPV types in the real-time PCR assay.

<table>
<thead>
<tr>
<th>Type</th>
<th>Probe sequence (5'-3')</th>
<th>Efficiency (%)</th>
<th>T_m value (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 18</td>
<td>ROX-TCTACACGATCTCTCTGTACCTGGGCAATATGATGCTACCA-BHQ2</td>
<td>85.59</td>
<td>74.6</td>
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<tr>
<td>HPV 35</td>
<td>ROX-TGCTGTGCTTCTATGTGACAGTACATATAAATGAC-BHQ2</td>
<td>103.17</td>
<td>67.0</td>
</tr>
<tr>
<td>HPV 16</td>
<td>ROX-TTATGTTGCTGCCATATCTACTTTTGCAAAAT-GA-BHQ2</td>
<td>99.52</td>
<td>63.4</td>
</tr>
<tr>
<td>HPV 33</td>
<td>ROX-ACAAGTAACATGACATACATCA-BHQ2</td>
<td>85.10</td>
<td>55.8</td>
</tr>
<tr>
<td>HPV 31</td>
<td>ROX-CAAACGATGATCATTAT-BHQ2</td>
<td>93.41</td>
<td>50.8</td>
</tr>
<tr>
<td>HPV 73</td>
<td>CY5-TGATTGCTACACAGGTGACTAAGCTTTGCTACGACTA-ACGTACC-BHQ2</td>
<td>103.56</td>
<td>73.7</td>
</tr>
<tr>
<td>HPV 59</td>
<td>CY5-CTCTCTCTATCTCTTCTTCTTCTATCTCTAAATTCAA-BHQ2</td>
<td>95.94</td>
<td>66.9</td>
</tr>
<tr>
<td>HPV 82</td>
<td>CY5-CTAATCCATCTGCTGACATG ACTAAATCAATCCA-AAGGAGGT-CAC-BHQ2</td>
<td>94.88</td>
<td>63.3</td>
</tr>
<tr>
<td>HPV 58</td>
<td>CY5-TGAAAGTAACATAGGACAGTGAC-BHQ2</td>
<td>88.69</td>
<td>58.3</td>
</tr>
<tr>
<td>HPV 68</td>
<td>CY5-TTGTCTACTACTAAGAATGCAATTTGA-BHQ2</td>
<td>99.16</td>
<td>52.0</td>
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<tr>
<td>HPV 6</td>
<td>ROX-TGCTACCGTAAATCACATCTTCTACACATACACCAATCTCGAT-BHQ2</td>
<td>99.87</td>
<td>71.1</td>
</tr>
<tr>
<td>HPV 11</td>
<td>ROX-CTACCTGCTCAATCCTACACATACCAATTTCAAT-TGAT-C-BHQ2</td>
<td>97.72</td>
<td>66.0</td>
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<tr>
<td>HPV 26</td>
<td>ROX-ATTAGGTAATTATCTACACGACATGCTC-ACT-BHQ2</td>
<td>84.63</td>
<td>62.0</td>
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<tr>
<td>HPV 53</td>
<td>ROX-CCACACATGCTTACTACACTACA-TTCA-BHQ2</td>
<td>100.27</td>
<td>57.1</td>
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<td>HPV 66</td>
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Table 2. Kappa values and P values by McNemar’s test for HPV types detected by two assays.

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<th>GA</th>
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<th>Agreement (%)</th>
<th>κ value (95% CI)</th>
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a The results for 168 concordant and 42 discordant samples after initial analysis are shown.

b rtPCR, real-time PCR assay.

c GA, GenoArray assay.

d CI, confidence interval.

e Strength of agreement considered perfect.

f Strength of agreement considered very good.

g Strength of agreement considered good.
Figure legends

Fig. 1. Readout of the real-time PCR assay. A typical result from a sample infected with HPV 16. One positive amplification curve appears only in ROX channel of reaction 1. The melting peak of HPV 16 is shown in red line, the melting peak of other 29 HPV types are shown in grey lines and the melting peaks of IPC are shown in green lines. All melting curves were first normalized between 0 and 1, and the data between 0.4 and 1.0 are shown. The Cq value is marked in the amplification curve. R1, R2 and R3 represent reaction 1, 2 and 3, respectively.

Fig. 2. Comparison of the HPV 16 quantification results between the new method and commercial Kehua kit. (A) Regression analysis of the two methods. (B) Bland-Altman analysis for the two methods. The solid line indicates the mean log10 difference, and the dotted line indicates the borders of the 95% confidence.
HPV 16 quantified by our method (log_{10} copies/μL) 
HPV 16 quantified by Kehua kit (log_{10} copies/μL) 

N = 30 
Mean log_{10} difference: -0.12 
95% CI: -0.37 ~ 0.12