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The evaluation of loop-mediated isothermal amplification-quartz crystal microbalance (LAMP-QCM) biosensor as a real-time measurement of HPV16 DNA

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A B S T R A C T

We have previously developed quartz crystal microbalance biosensor integrated with loop-mediated isothermal amplification (LAMP-QCM) for human papillomavirus (HPV) type58 DNA detection. Infection with HPV, particularly HPV16, remains a serious health problem due to its major risk factor contributing to cervical cancer. In the present study, LAMP-QCM biosensor was evaluated in terms of a quantitative assay for copy number of HPV16 DNA in cervical samples compared to quantitative PCR using TaqMan assay (TaqMan-qPCR). The detection limit of LAMP-QCM was found to be 10 fold more sensitive than TaqMan-qPCR with 100% specificity and 7.6% imprecision. Different plot of HPV16 DNA copy number using Bland–Altman analysis revealed 94% correlation between LAMP-QCM and qPCR. We therefore concluded that the developed LAMP-QCM biosensor provides a possible rapid and sensitive assay for HPV16 DNA quantification in a routine laboratory.

1. Introduction

The presence of high-risk human papillomavirus (Hr-HPV) in cervical lesion is a prognostic marker among women diagnosed with pre-malignant cervical hyperplasia (Burd, 2003; Dehn et al., 2007; Hong et al., 2010; Tabora et al., 2008). An increase of HPV16 viral load, a common Hr-HPV, has been reported as a surrogate marker for cancer progression (Wanram et al., 2009). Sensitive and rapid methods for HPV DNA are still needed. The principle of the QCM sensor is based on the detection of deposited mass change resulting from binding between the immobilized probe and specific target (Kleo et al., 2011; Natphopsuk et al., 2013). The simplicity and real-time response promotes its use for screening in mass population. Recently, we have reported QCM biosensor integrated with loop-mediated isothermal amplification (LAMP) for viral DNA detection of HPV58 (Prakrankamanant et al., 2013). However, the application of LAMP-QCM for quantitative analysis has not yet been evaluated. The aim of the present work is to evaluate the efficiency of the LAMP-QCM for estimating of HPV16 DNA copy number in clinical samples compared with the quantitative PCR (qPCR) using TaqMan® probe. To the best of our knowledge, this study is the first quantitative analysis of infectious organisms using LAMP-QCM biosensor. We found that the biosensor showed a significant correlation with qPCR and with higher test sensitivity.

2. Materials and methods

2.1. Cervical tissues

A total of 31 cervical tissues including 17 dysplasia and 14 cancers were collected from patients at the tumor clinic of Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. This study was approved by the Ethics committee of Khon Kaen University for Human Research (HE531211). The CaSki cell line was used as a positive control for estimating HPV16 DNA. Tissue DNA was extracted using Wizard® SV Genomic DNA purification system (Promega Corporation, Madison, WI). DNA samples were stored at −20 °C until assayed.

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Table 1
Primer sets for HPV16 DNA amplification by LAMP-QCM assay.

Primer	Primer sequence (5′–3′)	Genome position
F3	TCGGTTGTGCGTACAAAG	756–773
B3	AGCCTCTACATAAAACCATCC	933–913
b-FIP	*Biotin*-TGGGGCACACAATTCCTAGT-CACACACGTAGACATTCGT	838–819/**TTTT**/774–792
BIP	TCAGAAACCATAATCTACCATGGC-ATTACATCCCGTACCCTCTT	846–869/**TTTT**/912–893
LF	CCCATTAACAGGTCTTCCAAAGT	815–793
LB	CCTGCAGGTACCAATGGGG	874–892

2.2. TaqMan-quantitative PCR (TaqMan-qPCR)

Quantification of viral copy number of HPV16 DNA was determined by amplifying E6 region using TaqMan® probe (Applied Biosystems, Framingham, MA) according to the previous report (Wanram et al., 2009). The reaction was conducted in 25 μL containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.25 mM dNTP, 4 mM $MgCl_2$, 300 nM of each primer, 100 nM of E6-FAM probe, 2.5 units of *Taq* DNA polymerase and 100 ng of DNA. PCR condition was started with one cycle of 2 min at 50 °C, 10 min at 95 °C and followed by 40 cycles of two-step cycling at 95 °C for 15 s and 60 °C for 60 s. The copy number of HPV DNA was estimated from a standard curve as previously described (Wanram et al., 2009).

2.3. QCM system and quartz surface preparation

The QCM system used in this study is composed of QCM chamber and a heating box assembly generating a constant temperature for LAMP reaction with an accuracy ±1 °C as previously described (Prakrankamanant et al., 2013). An one inch diameter of 9 MHz AT-cut quartz crystals with polished gold electrode (gold area = 1.34 cm^2, thickness = 185 μm) was purchased from Maxtek (Maxtek Inc., Santa Fe Springs, CA). The thiol self-assembled monolayer on the gold surface was freshly prepared by injecting 10 mM 3-mercaptoproprionic acid (MPA) (Sigma–Aldrich, St. Louis, MO) for 1 h at room temperature (25 °C) using flow injection mode with 200 μL/min flow rate. After rinsing with ethanol and distilled water, the crystal was subsequently activated in solution containing 50 mM *N*-hydroxysulfosuccinimide (NHS) and 200 mM 1-ethyl-3-(3-dimethylaminopropil) carbodiimide (EDC) (Fluka, Switzerland), and rinsed with distilled water. The QCM was then immobilized with 0.1 mg/mL of avidin (Sigma–Aldrich, St. Louis, MO) in 0.05 M *N*-[2-hydroxyethyl] piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer for 50 min. An excess avidin was removed by rinsing with buffer. The residual reacting sites were blocked with 1 mM ethanolamine–HCl, pH 8.3 (Sigma–Aldrich, St. Louis, MO) for 30 min.

2.4. Detection of HPV16 DNA copy number by real-time LAMP-QCM assay

HPV16 DNA was amplified by LAMP reaction following the previous report (Saetiew et al., 2011). The copy number of DNA generated was monitored with the QCM analytical software. Briefly, 50 μL LAMP reaction mixture contained 0.2 μM each of outer primer (F3, B3), 1.6 μM each of inner primer (b-FIP, BIP), 0.8 μM each of loop primer (LF, LB), 6 mM $MgSO_4$, and 0.5 M betaine (Sigma–Aldrich, St. Louis, MO). All LAMP primers are shown in Table 1. DNA sample (100 ng) was added into reaction mixture and pre-warmed at 59 °C for 5 min. To obtain constant temperature and stable signal in the chamber, the HEPES buffer was injected into the chamber and left until this reached the constant temperature at 59 °C. The LAMP reaction was started by adding 8 units of *Bst* DNA polymerase (large fragment; New England Biolabs, Beverly, MA) and inoculated into the QCM system. The amplified biotinylated LAMP products were bound with the immobilized avidin in which the signal was real-time monitored and analyzed by software of the RQCM system (RQCM, Maxtek Inc., Santa Fe Springs, CA). The copy number of HPV16 DNA in clinical samples was also compared with results obtained by TaqMan-qPCR.

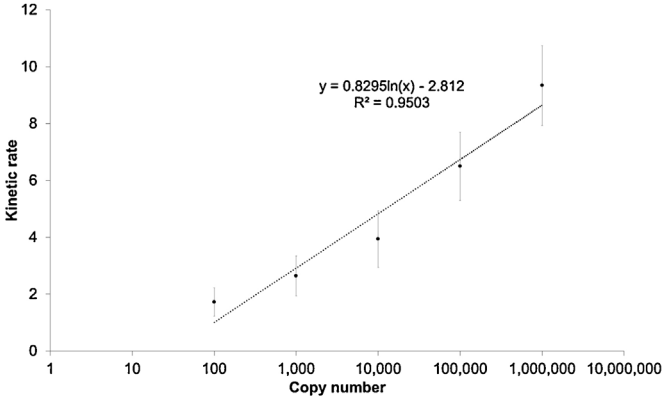

Fig. 1. Standard curve of HPV16 DNA copy number measurement showing significant logarithmic correlation with kinetic rate with the LAMP-QCM.

Table 2
Comparison of analytical characteristics between LAMP-QCM and TaqMan qPCR for HPV16 DNA copy number.

	LAMP-QCM	TaqMan qPCR[a]
Sensitivity (copies)	100	1000
Specificity (%)	100[b]	100
Precision (%CV)	7.6[c]	9.2[d]
Analysis time	30 min	3 h
Detection method	Surface-mass detection	Fluorescence

[a] Results are obtained from Wanram et al. (2009).
[b] Results are obtained from Fig. 2.
[c] Within-run precision at 100 copies.
[d] within-run precision at 1000 copies.

2.5. Statistical analysis

The method agreement between LAMP-QCM and TaqMan-qPCR was evaluated using correlation coefficient (R^2) and Bland–Altman analysis (GraphPad Prism 5.0, La Jolla, CA). *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

We recently published the successful development of LAMP assay on a QCM system (Prakrankamanant et al., 2013). The real-time monitoring data for LAMP-QCM reduced analysis time with good diagnostic performance and improved the positive rate of HPV58 DNA detection with 100% sensitivity and 90.5% specificity compared to conventional LAMP. This system has a potential for real-time quantitative assay which is applicable to other DNA targets. At present, we therefore used HPV16 DNA quantification as a model to demonstrate the applicability of real-time LAMP-QCM in clinical practice.

The real-time monitoring data for LAMP reaction at different viral DNA copy numbers on QCM are shown in Fig. S1 (supplementary data). No change was observed in the negative control. The kinetic rate calculated from the slope of frequency change was correlated with viral copy number expressing a linear regression of $y = 0.8295\ln(x) - 2.812$ with $R^2 = 0.9503$ (Fig. 1). The comparison of analytical performance between LAMP-QCM and TaqMan-qPCR is

Table 3
The analytical precision of LAMP-QCM for HPV16 DNA measurement.

Precision	HPV-16 DNA		
	$\Delta f_{mean} \pm SD$ (Hz)	Estimated DNA (copies)	%CV
Within-run ($N = 4$)			
Low level[a]	35.8 ± 2.7	132	7.6
High level[b]	120.1 ± 8.3	12,673	6.9
Between-run ($N = 12$)			
Low level[a]	35.8 ± 2.9	132	8.1
High level[b]	119.1 ± 10.6	12,005	8.9

[a] 100 copies of HPV16DNA from CaSki cell line.
[b] 10,000 copies of HPV16DNA from CaSki cell line.

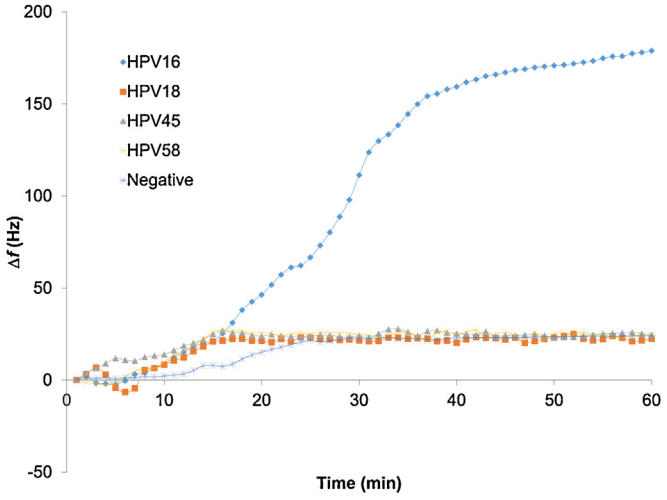

Fig. 2. The analytical specificity of LAMP-QCM for HPV16 DNA measurement. The response signals obtained from other HPV subtypes are found within the background range of non-template reaction.

summarized in Table 2. LAMP-QCM showed 10 fold higher sensitivity and more rapid analysis time than with TaqMan-qPCR.

CaSki cell line containing known HPV16 DNA at 60–600 copies per cell (Meissner, 1999; Pattillo et al., 1977) was prepared with low and high HPV16 DNA at 100 and 10,000 copies, and used as positive control for LAMP-QCM assay. The inaccuracy of the HPV16 DNA detection was also estimated from the difference between the measured copy numbers (132 ± 22 and 12,005 ± 34) and the known copy numbers of CaSki (100 and 10,000 copies) resulting the inaccuracy at 32% and 20%, respectively (Table 3).

The lower detection limit at 100 HPV16 DNA copies is equivalent to 1.0 pg/mL. The precision of LAMP-QCM expressed in coefficient of variation (%CV) for both within-run and between-run at 100 copies was found to be 7.6% and 8.1%, respectively (Table 3). No cross-reaction between LAMP primer set of HPV16 with HPV18, 45 and 58 was observed as shown in Fig. 2. Both LAMP-QCM and TaqMan-qPCR methods indicate the acceptable %CV of less than 15% (Luo et al., 2006) as shown in Table S1. All HPV subtypes used including HPV16, 18, 45 and 58 were previously reported as the representative common HPV subtypes covering 80% in the study population (Chansaenroj et al., 2014; Chinchai et al., 2012; Natphopsuk et al., 2013).

The measurement of HPV16 DNA copy number in 31 cervical samples using LAMP-QCM and TaqMan-qPCR is listed in Table 4. The median copy number measured by LAMP-QCM was 2.5×10^4 copy number (ranged from 1.6×10^3 to 8.4×10^8 copy number), while TaqMan-qPCR was 2.8×10^4(ranged from 1.1×10^3 to 6.8×10^8). A significant correlation is shown in Fig. 3 with $R^2 = 0.9877$ ($P < 0.001$). The agreement between methods was

Table 4
The copy number of HPV 16 DNA measured by TaqMan qPCR and LAMP-QCM assay in 31 cervical tissues..

No. of cervical tissues[a]	TaqMan qPCR (copy number)[b]	LAMP-QCM (copy number)[b]
DS 01	13,136	13,881
DS 02	31,781	32,279
DS 03	5171	4690
DS 04	126,949	121,572
DS 05	2316	2017
DS 06	21,287	22,483
DS 07	28,451	25,363
DS 08	50,140	46,343
DS 09	145,210	137,148
DS 10	5570	5291
DS 11	19,055	17,666
DS 12	349,365	359,780
DS 13	12,664	9668
DS 14	13,572	13,881
DS 15	11,396	10,907
DS 16	4565	4158
DS 17	4164	4690
CX 01	437,158	457,878
CX 02	25,862,658	21,644,467
CX 03	68,426	66,535
CX 04	718,431	741,608
CX 05	2,500,076	2,194,725
CX 06	59,227	52,280
CX 07	6848	6734
CX 08	2756	1788
CX 09	849,350,003	680,344,247
CX 10	94,203	95,526
CX 11	130,231	121,572
CX 12	1617	1104
CX 13	2,361,541	1,945,467
CX 14	10,972	10,907
Median	28,450	25,363

[a] DS represents for cervical tissues with dysplasia. CX represents for invasive cervical cancer tissues.
[b] All data were normalized by the equal amount of 100 ng DNA template used in the reaction.

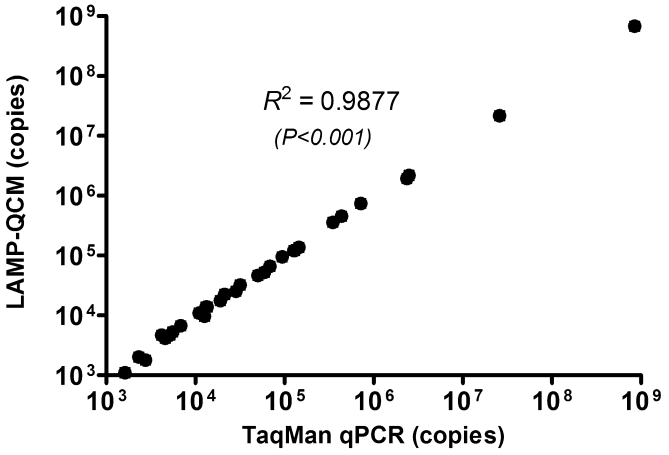

Fig. 3. The correlation of viral copy number of HPV16 DNA measured by the LAMP-QCM assay and TaqMan-qPCR. A significant correlation ($R^2 = 0.9877$, $P < 0.001$) was observed.

further analyzed using Bland–Altman analysis as shown in Fig. 4. The percentage of difference in copy number showed 29 of 31 data locating within the 95% CI. Two data, CX08 and CX1, deviated from 95% CI (Table 4). We found that both samples harbored low viral copy number. Thus, the deviation might be due to the different detection limit between LAMP-QCM and TaqMan-qPCR. Taken together, this result implies the acceptable accuracy of LAMP-QCM compared to TaqMan-qPCR.

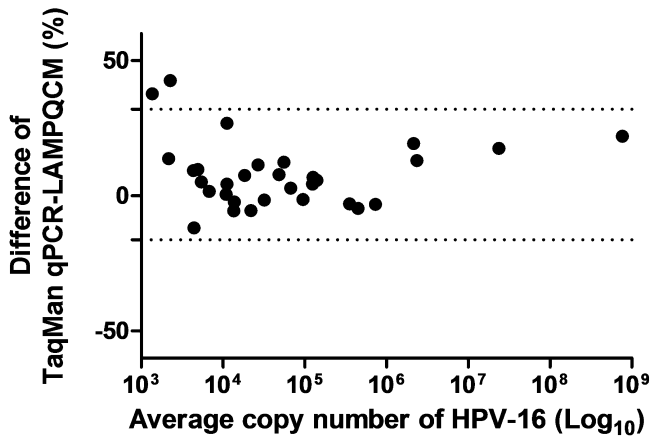

Fig. 4. Comparison of different HPV16 DNA copy numbers between TaqMan-qPCR and LAMP-QCM assay in cervical samples using Bland–Altman analysis. The 94% (29 of 31) of data was within 95% confidence interval limit.

A factor that influenced the sensitivity of LAMP-QCM for viral copy number quantification was considered to be the excess of biotinylated primers remaining from LAMP reaction. However, this limitation can be minimized by the optimization of biotinylated primers used in LAMP reaction. Moreover, kinetic measurement of slope in mass change on the biosensor can correct the background caused by the excess biotinylated primers. Up to date, the most sensitive biosensor technology integrated with LAMP for DNA target has been reported with surface plasmon resonance (SPR) (Chuang et al., 2012). The LAMP reaction could be directly detected by measuring the refractive index of SPR method with disposable cartridge. The performance of SPR is found at 2 fg/mL with rapid detection at 20 min. The limit of SPR use may depend on cost effectiveness for possible commercialization. QCM is known as a label-free biosensor system where detection can be performed by direct binding regardless of probe reagents. Immunosensor based QCM has been reported for specific and rapid detection of several bacteria, for example, *Salmonella typhimurium* with limit of detection (LOD) at 100 cfu/mL (Su and Li, 2005). Salam and coworkers have recently shown the utilization of gold nanoparticles coated with *Salmonella* antibody to amplify the sensitivity which enhance the LOD to 20 cfu/mL (Salam et al., 2013). On the other hand, the linearity of detection is significantly reduced to 1000 cfu/mL, which limiting its use for quantitative analysis. Currently, there is no report using QCM for quantitative purposes. The initial study in this report with label-free LAMP-QCM facilitates its use for the estimation of viral DNA copy numbers and may be expected to be commercially competitive.

4. Conclusion

In summary, we demonstrated the acceptable analytical performance of the HPV16 DNA quantification by LAMP-QCM. This data exhibits the potential applicability of LAMP-QCM for quantitative measurement in clinical practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.12.005.

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