

Sensitivity Evaluation of SYBR Green I, SYBR Safe and Calcein Dyes for Detection of Human Papillomavirus 16 by Loop-Mediated Isothermal Amplification

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ABSTRAK

Amplikasi isoterma berpengantara gelung (LAMP) merupakan teknik amplifikasi gen yang menghasilkan produk akhir iaitu mendapan keruh magnesium pirofosfat yang boleh dianalisis dengan hanya menggunakan mata kasar. Penggunaan pewarna interkalat yang sesuai adalah penting kerana ia boleh meningkatkan sensitiviti dan mengurangkan keputusan positif palsu dan negatif palsu untuk pengesanan. Kajian ini bertujuan untuk membandingkan prestasi tiga pewarna interkalat yang berbeza; SYBR Green I, SYBR Safe dan pewarna berasaskan calcein di dalam asai LAMP HPV-16 melalui visualisasi oleh mata kasar, elektroforesis gel dan mesin masa-nyata turbidimeter. Reaksi LAMP dilakukan menggunakan amplifikasi kit Loopamp DNA berisipadu sebanyak 25 µl. Campuran reaksi dieram pada suhu 60°C selama 60 minit dan ditamatkan menggunakan suhu 80°C selama 5 minit dalam mesin masa-nyata turbidimeter. Untuk pengesanan menggunakan mata kasar, SYBR Green I dan SYBR Safe telah dicairkan dalam 1:10 DMSO dan telah ditambah ke dalam tiub yang mengandungi campuran reaksi selepas proses amplifikasi berlaku manakala calcein ditambah sebelum proses amplifikasi. Sensitiviti asai telah disiasat menggunakan pencairan DNA HPV-16 yang berkepekatan bermula dari 10¹ salinan/µl to 10⁸ salinan/µl. Ketiga-tiga pewarna mempamerkan keputusan yang sama dari segi sensitiviti dengan had pengesanan adalah 10³ salinan/µl. Penambahan calcein di dalam asai menunjukkan masa pengesanan bertambah selama 10 minit dengan menggunakan mesin nyata-masa turbidimeter. Prestasi ketiga-tiga pewarna interkalat untuk pengesanan mata kasar adalah setanding dan boleh digunakan untuk aplikasi pemeriksaan titik akhir dalam asai HPV-16, manakala dengan mesin

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nyata-masa turbidimeter, penambahan calcein melambatkan masa pengesanan selama 10 minit.

Kata kunci: pewarna interkalat, human papillomavirus, amplikasi isoterma berpengantara gelung

ABSTRACT

Loop-mediated isothermal amplification (LAMP) is a gene amplification technique whereby the amplification products are commonly visualized as turbidity by naked eye in the presence of magnesium pyrophosphate precipitation. An appropriate intercalating dye is important as it could increase the sensitivity and reduce the false positive and false negative results for the detection. The study aimed to compare the performance of three different intercalating dyes; SYBR Green I, SYBR Safe and calcein-based dyes in HPV-16 LAMP assay by naked-eye visualization, gel electrophoresis and real-time monitoring. The LAMP assay was carried out using a Loopamp DNA amplification kit in 25 μ l volumes. The reaction mixture was incubated at 60°C for 60 mins and terminated at 80°C for 5 mins in a real-time turbidimeter. For naked eye detection, SYBR Green I and SYBR Safe were diluted at 1:10 of DMSO and was added to the solution after the reaction was completed while calcein was added before the amplification process. The sensitivity of the LAMP assay was investigated ranging from 10^1 copies/ μ l to 10^8 copies/ μ l of the HPV 16 DNA template. All three dyes exhibited similar results in term of sensitivity with the detection limit of 10^3 copies/ μ l. Addition of calcein dye showed decrease in detection time by 10 mins by real-time turbidimeter. The performance all three dyes for naked-eye detection are comparable and can be used for endpoint screening applications in HPV 16 assay, whereas in real-time evaluation, addition of calcein delay the detection time by 10 mins.

Keywords: dyes, human papillomavirus, loop-mediated isothermal amplification

INTRODUCTION

Human papillomavirus (HPV) infection is one of the risk factors for the development of oral cancer which ranks sixth in the cancer incidence seen worldwide (Goot-Heah et al. 2012). It is a small-sized DNA virus and comprises 8 kb, circular, double stranded DNA genome (Ramqvist &

Dalianis 2010). In Malaysia particularly, oral cancer associated with HPV is ranked 11th most common form of cancer seen in clinical practice (Goot-Heah et al. 2012). Up to date, there are about 200 types of HPV which were found to be associated with skin and mucous tissue (Burd 2003). The association of HPV with juvenile laryngeal papillomatosis was reported.

A study conducted by Siti Aishah et al. (2006) reported two cases of juvenile laryngeal papillomatosis containing HPV viral genomes in Malaysia. Among all, HPV genotype 16 (HPV 16) is one of well-known risk factors for the development of oral cancer, representing >95% of all HPV positive squamous cell carcinoma (OSCC) (Luo et al. 2011).

Several molecular methods, including loop-mediated isothermal amplification (LAMP), have already been introduced for the detection of HPV-16 (Hagiwara et al. 2007; Luo et al. 2011; Livingstone et al. 2016). LAMP is a gene amplification method that can amplify nucleic acids in less than 60 mins under isothermal conditions without a thermocycler which provides highly sensitive and specific results (Notomi et al. 2000). High amplification of nucleic acids was achieved using four to six oligonucleotide primers which recognizes six to eight distinct regions on the target DNA/RNA sequence (Tanner et al. 2015).

LAMP amplicons can be detected by two ways, either by direct visual assessment by adding a fluorescent intercalating dye such as ethidium bromide (Moradi et al. 2012), SYBR Green I (Parida et al. 2011), SYBR Safe (Nicolasora et al. 2014) and propidium iodide (Singh et al. 2015) after the reaction is completed or metal indicators such as calcein (Tomita et al. 2008), hydroxynaphthol blue (HNB) (Goto et al. 2009; Cardoso et al. 2010; Ahmadi et al. 2012) and magnesium pyrophosphate (Moradi et al. 2012, Almasi et al. 2013) prior to the reaction, or using real time monitoring by

measuring the increase in fluorescence of DNA binding dyes (Parida et al. 2008; Tanner et al. 2015). Various fluorescent dyes have demonstrated different sensitivities for both visual endpoint and real-time detection of in LAMP assay (Ding et al. 2015). This study was conducted to compare the sensitivity of pre- and post-loaded fluorescent dyes (SYBR Green I, SYBR Safe and calcein) by naked eye visualization, gel electrophoresis and real-time detection of LAMP for detection of HPV16.

MATERIALS AND METHODS

ATCC STRAINS

The reference strain used in this study was HPV16 plasmid, commercially obtained from the American Type Culture Collection (ATCC 45113D).

OLIGONUCLEOTIDE PRIMERS

The type-specific primers of HPV16 for LAMP amplifications were designed from E6/E7 gene encodes HPV viral oncogenes by using free software (Primer Explorer V4, Tokyo, Japan) (<http://primerexplorer.jp/e>) based on the Genbank sequence information (accession number: NC001526.2). Six primers were used in LAMP reactions (Table 1). The Basic Local Alignment Search Tool (BLAST) on National Centre for Biotechnology information (NCBI) website was used to check for cross-reactivities of the primers. These primers were synthesized and purchased from the

Table 1. Details of LAMP primers used for the detection of HPV 16

Virus	Primer	Sequences (5'-3')
Human Papilloma-virus 16	F3	ATGCACCAAAAAGAGAAGCTG
	B3	AGCATATGGATTCCCATCTC
	FIP	GCAGCTCTGTGCATAACTGT-CAATGTTTCAGGACCCACA
	BIP	AGAATGTGTGTACTGCAAGCAA-TCCCGAAAAGCAAAGTCAT
	LF	GGTAACTTTCTGGGTCGCTCC
	LB	CAGTTACTGCGACGTGAGGT

First Base Laboratories Sdn. Bhd. (First Base, Kuala Lumpur, Malaysia) in the lyophilized form. Each of the primers was dissolved in DNase & RNase-free water (Gibco, MA, USA) and adjusted to 100 mM as stock solution and stored at -20°C prior to use.

SENSITIVITY AND SPECIFICITY

The HPV 16 plasmid was diluted ranging from 10¹ to 10⁸ copies/μl for a sensitivity test in LAMP assay. Other HPV plasmids (HPV-types 16, 18, 35, 43, 56) were used for specificity analysis.

LAMP REACTION

The LAMP assay was carried out using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan). The reaction mixture (25 μl) contained 40 pmol of each FIP and BIP, 5 pmol of each F3 and B3 primers, 2 μl of HPV-16 plasmid, 1 μl of *Bst* DNA polymerase and 12.5 μl of reaction mix. For the acceleration of the LAMP reaction, 20 pmol of each LF and LB was added to the reaction mixture and incubated at 60°C for 60 mins in a Loopamp real-time turbidimeter (LAA-500) (Eiken Chemical Co.,

Tochigi, Japan), followed by 80°C for 5 mins to terminate the reaction. The amplification results were analysed by naked eye detection using SYBR Green I (BioTeke, Beijing, China), SYBR Safe (Invitrogen, California, CA) and calcein dyes (Eiken Chemical Co., Tochigi, Japan). The electrophoretic analysis was analysed for further confirmation of the naked eye visualization and the turbidity of magnesium phyrophosphate were recorded using Loopamp real-time turbidimeter (Eiken Chemical Co., Tochigi, Japan).

NAKED-EYE VISUALIZATION OF LAMP PRODUCTS

SYBR SAFE

One microlitre of 1:10 diluted SYBR Safe was added to the reaction mixture after amplification and the colour change was observed. The positive reaction turned green while negative reaction retained the original orange colour.

SYBR GREEN I

One microlitre of 1:10 diluted SYBR Green I was added to the reaction mixture after amplification and the

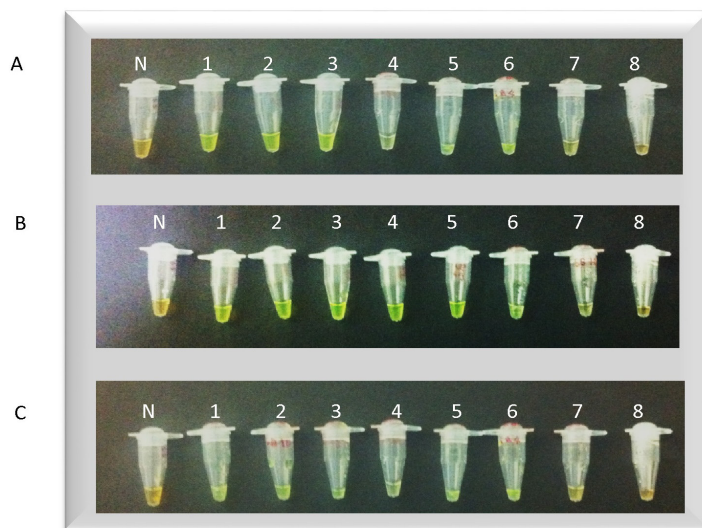


Figure 1: Naked eye visualisation of serial diluted HPV 16 using different fluorescent dyes. A) SYBR Green I, B) SYBR Safe, C) Calcein. N: Negative control; 1: 10^8 copies/ μ l; 2: 10^7 copies/ μ l; 3: 10^6 copies/ μ l; 4: 10^5 copies/ μ l; 5: 10^4 copies/ μ l; 6: 10^3 copies/ μ l; 7: 10^2 copies/ μ l; 8: 10^1 copies/ μ l.

colour change was observed. The positive reaction turned green while negative reaction retained the original orange colour.

CALCEIN DYE

One microlitre of FDR calcein dye was added to the reaction mixture before the amplification. The positive reaction turned green while negative reaction retained the original orange colour.

ELECTROPHORETIC ANALYSIS OF LAMP PRODUCTS

Five μ l of LAMP product was run on a 1.5% agarose gel and visualized under UV light and documented by the Image Analyzer (Giles Scientific, USA). The positive reaction showed a ladder-like band pattern.

REAL-TIME ANALYSIS OF LAMP PRODUCTS

Real-time turbidimeter was used to measure the turbidity of magnesium pyrophosphate, a byproduct of the LAMP reaction. The graph of reaction time corresponds to turbidity of the assay were recorded and analysed.

RESULTS

SENSITIVITY BY NAKED EYE VISUALIZATION

The colour of the positive LAMP products exhibited green by the addition of SYBR Green I, SYBR Safe and calcein dyes, while the negative reaction retained the original orange colour. As shown in Figure 1, the

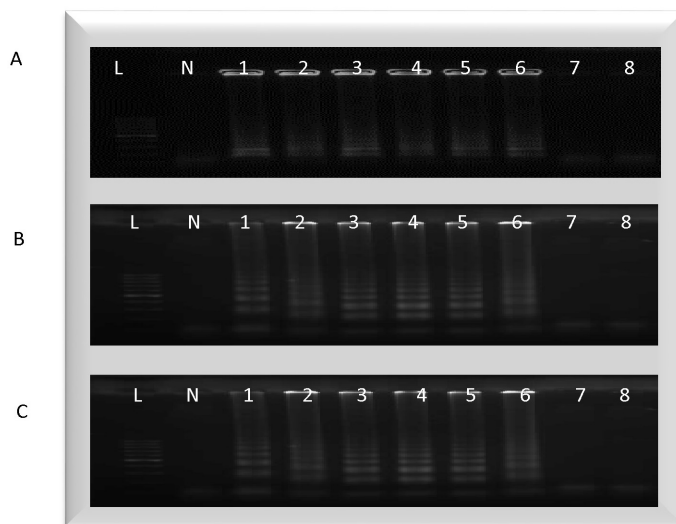


Figure 2: Gel electrophoresis analysis of serial diluted HPV 16 using different fluorescent dye on 1.5% agarose gel. L: Ladder 100 bp (ThermoFisher, MA, USA). A) SYBR Green I, B) SYBR Safe, C) Calcein. N: Negative control; 1: 10^8 copies/ μ l; 2: 10^7 copies/ μ l; 3: 10^6 copies/ μ l; 4: 10^5 copies/ μ l; 5: 10^4 copies/ μ l; 6: 10^3 copies/ μ l; 7: 10^2 copies/ μ l; 8: 10^1 copies/ μ l.

detection limit of HPV16LAMP assay was 10^3 copies/ μ l for all three different dyes used in this study.

SENSITIVITY BY AGAROSE GEL ANALYSIS

The ladder-like band pattern in agarose gel indicated positive LAMP reaction. As shown in Figure 2, the detection limit of HPV16 LAMP assay was 10^3 copies/ μ l for all three different dyes used in this study. The electrophoretic analysis was in accordance with the naked eye visualization analysis.

REAL-TIME ANALYSIS

The real-time analysis monitors the turbidity (Df) with the threshold time (Tt). As shown in Figures 3-5, the Tt of LAMP using SYBR Green I and SYBR Safe occurred at 12 mins, while the

Tt of LAMP using calcein was at 22 mins. SYBR Green I and SYBR Safe have shorter Tt compared to calcein dye. Using SYBR Green I and SYBR Safe, the amplification result could be detected as early as 12 mins (Figures 3 & 4). Compared to calcein dye, the earliest detection was at 22 mins of the amplification process (Figure 5). The sensitivity of the assay was in accordance with naked eye visualization and agarose gel results which up to 10^3 copies/ μ l was detected.

SPECIFICITY OF THE ASSAY

The specificity of the HPV 16 LAMP assay was evaluated using several HPV viruses (HPV18, HPV35, HPV43, HPV56). Results confirmed that the designed primers were specific for HPV16 as none of the tested HPV viruses were detected (data not shown).

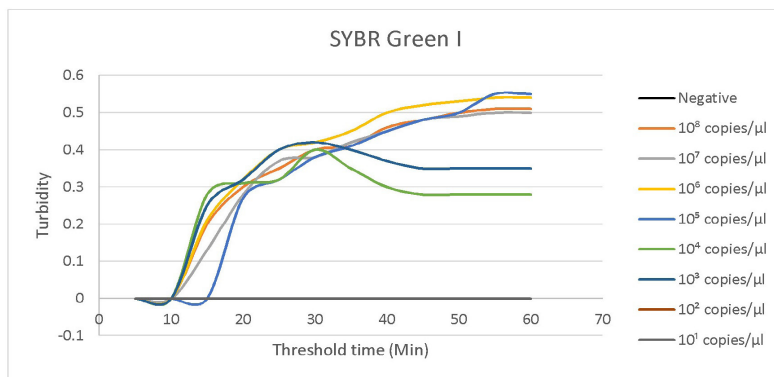


Figure 3: Real-time detection of HPV 16 using SYBR Green I dye. X-axis depicts the time of positivity and Y-axis shows the turbidity value in terms of O.D. at 650nm.

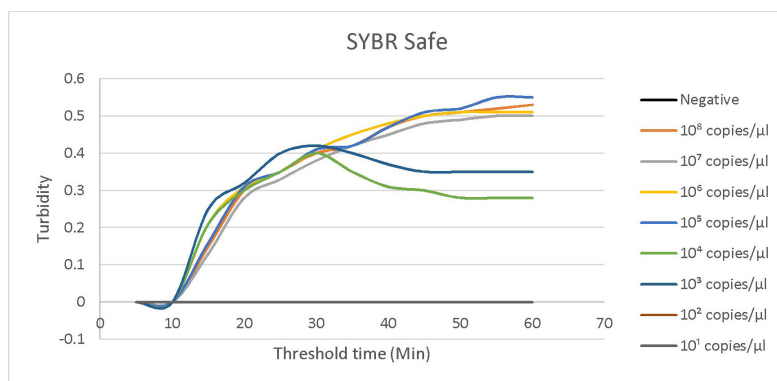


Figure 4: Real-time detection of HPV 16 using SYBR Safe dye. X-axis depicts the time of positivity and Y-axis shows the turbidity value in terms of O.D. at 650nm.

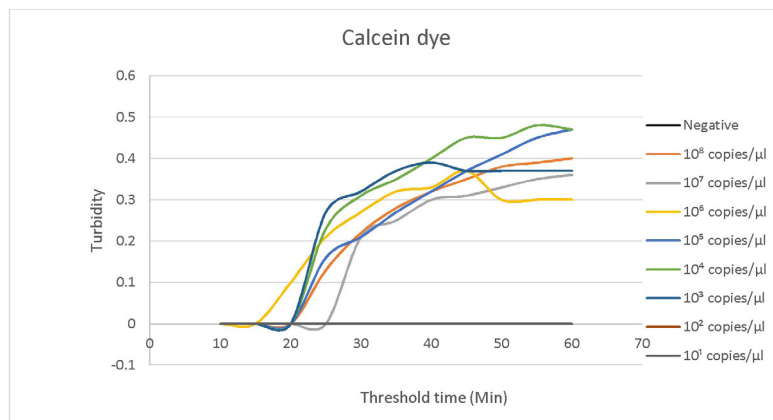


Figure 5: Real-time detection of HPV 16 using calcein dye. X-axis depicts the time of positivity and Y-axis shows the turbidity value in terms of O.D. at 650nm.

DISCUSSION

Different types of fluorescent dyes have been used for LAMP assay. Each dye has its own features which might affect the performance of the LAMP reaction. Since the selection of the dye is one of the important factors to be considered when working with a LAMP, in this study, we compared the performance of pre- and post-added fluorescent dyes by naked eye visualization, gel electrophoresis and real-time analysis of LAMP for the detection of HPV16.

The use of SYBR Green I, SYBR Safe and calcein resulted in a colour change from orange (original) to green (positive) after amplification. By naked eye visualization, the all three dyes demonstrated similar sensitivities, in which the detection limit was 10^3 copies/ μ l of HPV16. Our findings are in agreement with the previous studies, which reported no differences observed when different dyes were used for naked eye detection of LAMP products (Kwok & Kwong 2013; Wang et al. 2013). Agarose gel electrophoresis and real-time analysis demonstrated the same detection limit as naked eye detection (10^3 copies/ μ l of HPV16). In our study, the positive LAMP reaction was consistent among naked eye detection, and gelelectrophoresis and real-time analysis.

SYBR Green I has been widely used for naked eye detection of LAMP products due to its higher sensitivity (Njiru et al. 2008). Furthermore, in the presence of single-stranded DNA (ssDNA), both SYBR Green I and SYBR Safe dyes emit a weak fluorescence signal however, both dyes emit strong

signal when binding to double-stranded DNA (dsDNA). Due to these characteristics both dyes are ideal candidates for end point detection of the LAMP (Soheili & Samiei 2005; Zipper et al. 2004). Previous studies had reported the inhibitory effect of SYBR Green I on quantitative real-time LAMP (Lam et al. 2008). Therefore, in this study, SYBR Green I and SYBR Safe dyes were added after the amplification was completed which resulted in the shorter threshold time, T_t (12 mins) (Figures 3 & 4). However, the drawback is that, the addition of the dye after amplification require opening of the tube that may increase the risk of cross-contamination.

Calcein is chelating fluorescent detection reagent that initially combines with manganese ions to achieve a quenching effect. In the present study, calcein-based dye was added to a reaction mixture prior to amplification. Real time detection using pre-added calcein dye has the ability to reduce the cross-contamination risk and prevent aerosolized amplification product which could lead to false positive results as the tube was not opened for post analysis (Wastling et al. 2010). However, the use of pre-added calcein dye before the amplification process increased in T_t (22 mins) (Figure 5). Our findings are consistent with a previous study which reported the increased of T_t in real-time fluorescence detection by 10 min when calcein and manganese were used (Fischbach et al. 2015). The addition of SYBR Green I and SYBR Safe dyes after the amplification reaction gave positive results in 12

mins at the detection limit 10^3 copies/ μ l. Reaction efficiency decreases with increasing Tt.

Our findings provided valuable information that can be used in choosing an appropriate dye for endpoint and real-time detection of LAMP amplicons. However, further investigation of optimal concentration for each dye is still needed. In the present study, the effect of various concentrations of dye on Tt was not done. Studies should be further expanded in order to determine the optimal concentration for each dye which resulted in the shortest Tt and higher signal-to-noise ratio (SNR).

CONCLUSION

In conclusion, SYBR Green I, SYBR Safe and calcein dyes were found to have similar sensitivities for HPV16 in naked eye detection, gel electrophoresis and real-time LAMP. However, the use of pre-added calcein dye increased the Tt by approximately 10 mins.

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