**ORIGINAL ARTICLE**

# Sybr Green Real-Time PCR is a Useful Method in Screening for High Risk Human Papilloma Virus Types 16 and 18 in Neoplastic Cervical Cancer

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### ABSTRAK

Virus papiloma manusia (HPV) memainkan peranan penting dalam patogenesis kanser servik. Di seluruh dunia, HPV telah dikesan dalam 99.7% kanser servik. Tujuan utama kajian ini ialah untuk membangunkan kaedah SyBrGreen Real-Time PCR (Tindakbalas Berantai Polymerase-Masa Sebenar SyBrGreen) dan seterusnya menggunakan kaedah ini untuk mengenalpasti jangkitan pelbagai bagi dua jenis subtip HPV risiko tinggi. Lima puluh tujuh sampel positif dari ujian TS-PCR (Tindakbalas Berantai Polymerase-Jenis Spesifik) diuji untuk mengesan prevalen genotip HPV risiko tinggi, HPV 16 dan HPV 18. Oleh kerana wujudnya jangkitan pelbagai, keputusan menunjukkan terdapat 67 genom HPV di dalam 57 sampel positif yang dikaji. Genom HPV 16 telah dikesan pada 55/67 (82%) kes, genom HPV 18 pada 8/67 (12%) kes dan 4 kes adalah jangkitan pelbagai. HPV 16 adalah lebih prevalen, diikuti oleh HPV 18. Keputusan menunjukkan graf pencairan DNA bagi HPV 16 adalah pada suhu puncak 80.4ºC dan nilai $C_t$ (Cycle threshold) bagi produk spesifik HPV 16 adalah pada pusingan ke 20. Manakala graf pencairan DNA bagi HPV 18 adalah pada suhu puncak 79.4ºC dan nilai $C_t$ bagi produk spesifik HPV 18 adalah pada pusingan ke 22. Sebagai kesimpulan, kaedah Tindakbalas Berantai Polimerase-Masa Sebenar SyBrGreen mempunyai potensi klinikal dalam penyaringan awal, pengesanan dan pengenalpastian jangkitan oleh pelbagai-jenis HPV bagi peringkat-peringkat yang berbeza dalam pesakit-pesakit kanser servik.

*Kata kunci:* virus papiloma manusia, kanser servik, tindakbalas berantai polymerase-transcriptase berbalik, tindakbalas berantai polimerase-masa sebenar SyBrGreen

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## ABSTRACT

Human papillomavirus (HPV) plays an important role in the pathogenesis of cervical cancer. HPV has been found in 99.7% of cervical cancers worldwide. In Malaysia, it is the second most common cancer among women in all major ethnic groups. The main purpose of this study was to establish the method of SyBrGreen Real-Time PCR and apply it for identification of multiple infections of the two high risk HPV subtypes. In this study, 57 positive samples for HPV 16 and HPV 18 were used to establish a simple and sensitive method to detect and identify HPV infection in the cervical neoplasia at different stages of the disease by using real-time ABICycler SyBrGreen 1 technology. The results showed 67 HPV genomes in 57 samples. HPV 16 genome was detected in 55/67 (82%) cases while HPV 18 was detected in 8/67 (12%) cases with 4 cases showing multiple infections of HPV 16 and HPV 18. HPV 16 was the most prevalent followed by HPV 18. Using SyBr Green Real-Time PCR techniques, the results showed that DNA melting curve for HPV 16 had a peak around 80.2ºC and $C_t$ value of 20 cycles whereas the DNA melting curve for HPV 18 around 79.2ºC and $C_t$ value of 22 cycles. In conclusion, a SyBr Green Real-Time PCR method has the potential for clinical usage in detection and identification of HPV infection in cervical neoplasia at different stages of the disease.

*Key words:*    human papillomavirus, cervical cancer, Reverse Transcriptase-PCR, SyBr Green Real-Time PCR

## INTRODUCTION

Cancer of the cervix is an important public health problem worldwide and is the second most common cancer among women (Woodman et al. 2007). In Malaysia, it is the second most common cancer among women in all major ethnic groups (Ministry of Health Malaysia 2003). Human papilloma virus (HPV) has been identified as the most important viral group associated with benign and malignant neoplasia in humans; HPV DNA is estimated to be present in over 99.7% of these cancers (Walboomers et al. 1999; Lockwood & Mcintyre 2009). Using real time PCR method, Panu et al. (2002) and Bourgo et al. (2008) showed the presence of integrated HPV type 16 in cervical Intraepithelial neoplasia (CIN). Integration of viral genome into the nucleus has been proposed as the mechanism for activation, proliferation and progression

from non-invasive lesions to cervical cancers (Mitsuo et al. 1999; Fan & Chen 2004; Lockwood & Mcintyre 2009). The Hybrid Capture (HC) assay, the current diagnostic test for the screening of cervical cancer, characterizes HPV infections as low or medium/high risk but fails to identify HPV infections by type (Sonnex 1998; Monica et al. 2002 & Seaman et al. 2010). Real time PCR is an efficient technique that objectively detects and quantifies DNA target sequence in a period of two or three hours, whereas classical PCR needs at least four hours to detect the same sequence without quantifying it (Bettini et al. 2008).

Recently, two vaccines were made available for protection of infection by some HPV types, Gardasil, marketed by Merck and Cervarix, marketed by GlaxoSmithKline (Moscicki 2008). Both protect against initial infection with HPV types 16 and 18, which cause most of the

HPV associated cancers. Gardasil also protects against HPV types 6 and 11, which cause 90% of genital warts (Asif et al. 2006; Kahn 2009; WHO 2009 & Schiller et al. 2010). The vaccines provide little benefit to women who have already been infected with HPV types 16 and 18, which include most sexually active females. The World Health Organization (WHO 2009) has clear guidelines on HPV vaccination which outlines appropriate, cost-effective strategies for using HPV vaccines in public sector programs.

Cervical cancer is caused by a virus; it can be prevented in two ways: (i) by preventing HPV infection by using a potential vaccine and (ii) by screening for the presence of cervical HPV infection and managing abnormal cytologies or precancerous lesions before cancer develop. In Malaysia however, the lack of an adequate screening programme and awareness are the major factors contributing to the high incidence of cervical cancer. The main purpose of this study was to establish a simple and rapid technique to detect high risk HPV subtypes using SyBrGreen Real-Time PCR. This technique is advantageous since it eliminates the need for gel electrophoresis and ethidium bromide staining. The method of SyBr-Green Real-Time PCR can be used as a screening approach to controlling cervical cancer.

## MATERIALS AND METHODS

### Samples

Genomic DNA from 57 HPV positive samples for HPV 16 and HPV 18 identified from our Type Specific-PCR (TS-PCR) was used for this study. The samples include 37 CIN I, 12 CIN II, 15 CIN III and 3 squamous cell carcinoma (SCC). CaSki and HeLa cell lines were used as positive controls for HPV 16 and HPV 18, respectively. For negative con-

trol, blood DNA from normal women was chosen. DNA extraction was done using the QIAamp Tissue kit from QIAGEN (Germany).

### Cell culture

The vial containing cell-lines was removed from the liquid nitrogen tank and was immediately thawed into a water bath at 37ºC. The content of the vial was transferred into a centrifuge tube and 10 ml of growth medium were added. The tube was centrifuged at 1000 rpm for 10 minutes. Supernatant was removed and cells were resuspended in 5 ml of growth medium, later transferred to a culture flask and incubated in a 37ºC $CO_2$ incubator. The medium was replaced the following day. Subculture was carried out by detaching cells using tripsin. One ml of trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Flowlab) was added to the flask. The flasks were incubated in a $CO_2$ incubator for 5 minutes. The flask was then observed under the inverted microscope to check for the detachment of cells and was tapped gently to optimize cell detachment.

### Real-Time PCR with SyBr Green

Genomic DNA from 57 positive samples for HPV 16 and HPV 18 were used to prepare a triplicate reaction for each sample using Real-Time PCR. Primers from TS-PCR (Walboomers et al. 1999) were used as a primer pair for HPV type 16 and 18 in the SyBr Green Real-Time PCR. Amplification reactions were performed with a volume of 12.5µl of 2X SyBrGreen PCR Master Mix (Applied Biosystems), a total of 300nM of reverse and forward primers and 20ng of sample DNA in a 25µl final volume. The protocol was set up at 95ºC for 10 minutes to activate the Taq gold, 40 cycles at 95ºC for 15 sec and 60ºC for 15 sec, consecutively. Each reaction was accompanied

by a No Template Control (NTC) in which the template DNA was replaced by water. Amplification was performed in a 248-well PCR plate covered with optical caps in the ABI 7700HT-SDS real-time instrument (Applied Biosystem).

## RESULTS

### Optimization of SyBr Green Reaction

From our results using SyBr Green Real Time PCR, NTC was used as a negative control; CaSki and HeLa DNA were used as a positive control for HPV 16 and HPV 18 respectively. Dissociation curve was generated to show the absence of non-specific amplification. The dissociation curve showed only a single peak at higher temperature and two peaks if primer-dimer occurred. According to Lamarcq et al. (2002), Cycle threshold ($C_t$) value was considered significant when it was at least four cycles lower than $C_t$ value of the corresponding NTC. Another

study by Szuhai et al. (2001), reported that a $C_t$ value is significant when it was lower than 40 cycle's number. Figure (1a) shows the amplification plot of HPV 16 with negative control NTC, Ct value at 20 cycles. Primer-dimer of NTC was observed with a $C_t$ value at 25 cycles. The $C_t$ value of HPV 16 was significant because the differences between HPV 16 and NTC were five cycles. Figure (1b) shows dissociation curve of HPV 16. Two peaks were observed at 80.7ºC for melting temperature $T_m$ of specific HPV 16 and at 74ºC for a NTC. Figure (2a) shows the amplification plot of HPV 18 with NTC and $C_t$ value for HPV 18 was 21 cycles. Primer-dimer of NTC was observed with a $C_t$ value at 28 cycles. The $C_t$ value of HPV 18 was significant because the differences between HPV 18 and NTC were seven cycles. Figure (2b) shows dissociation curve of HPV 18. Two peaks were observed at 79.3ºC for melting temperature $T_m$ of specific HPV 18 and at 70ºC for a NTC.
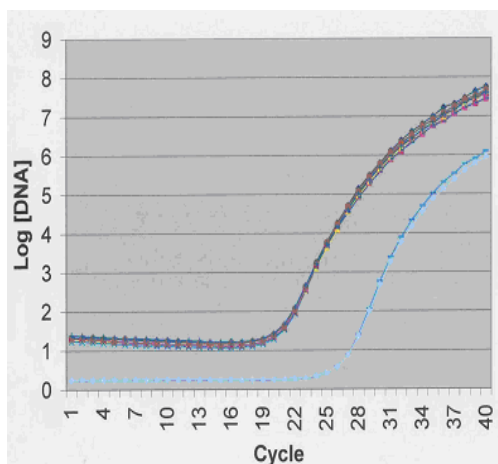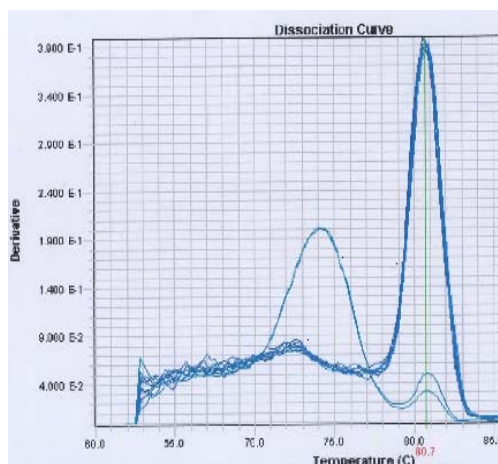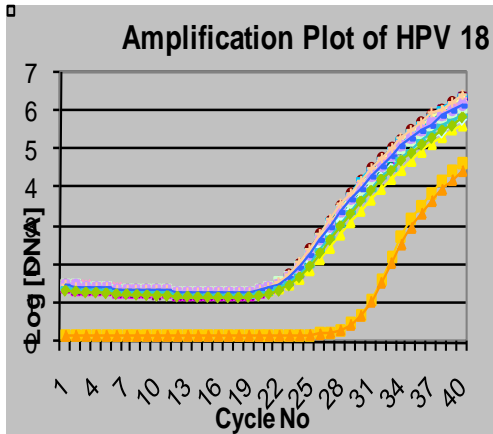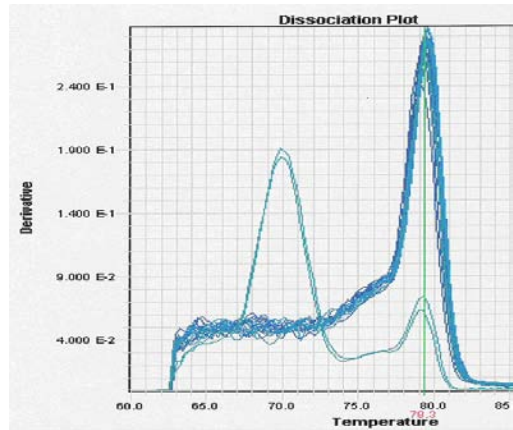


(a)                                    (b)

Figure 1: (a) Amplification plot of HPV 16 with a $C_t$ value at 20 cycles. No template control (NTC) was observed with a $C_t$ value at 25 cycles. (b): Dissociation curve of melting temperature $T_m$ for specific HPV 16. Two peaks were observed at 80.7ºC for specific HPV 16 and 74ºC for a NTC.

(a)                                                                                    (b)

Figure 2: (a) Amplification plots of HPV 18 with $C_t$ value at 21 cycles, whereas $C_t$ value for NTC is at 28 cycles.  The different cycle between HPV 18 and NTC was seven cycles.  (b) Dissociation curve of HPV 18, melting temperature $T_m$ for HPV 18 was 79.3ºC while NTC have a lower $T_m$ at 70ºC. Gene-specific product of HPV 18 did not overlap with the NTC peak.
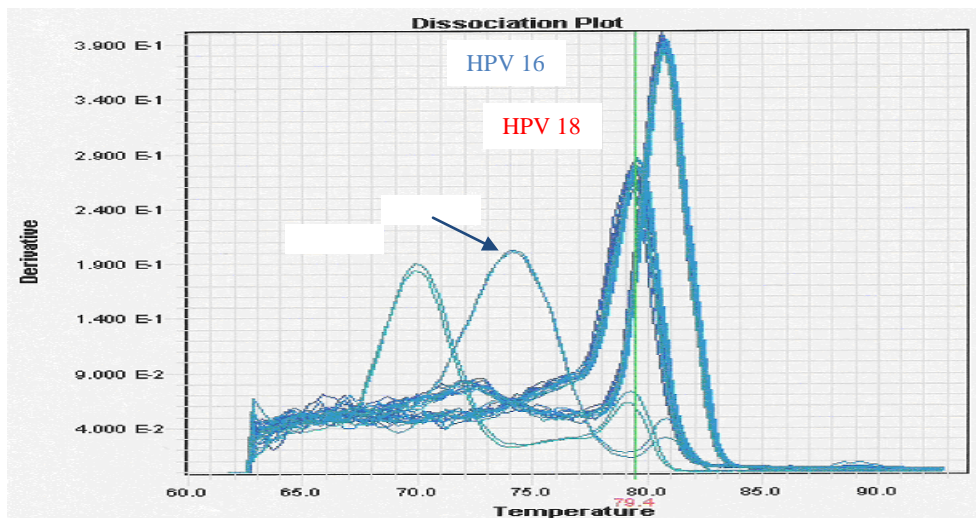


Figure 3:  Dissociation plot of multiple infections of HPV 16 and 18, showing melting temperature of HPV 16 at 80.6ºC, and melting temperature $T_m$ of NTC at 74ºC.  Whereas melting temperature $T_m$ of HPV 18 was 79.4 ºC and melting temperature $T_m$ of NTC was 70ºC.

Table 1: The $C_t$ value and $T_m$ for HPV 16 and HPV 18.

| HPV type | $C_t$ value (cycle) | $C_t$ NTC (cycle) | $\Delta C_t$ (cycle) | Melting temperature $T_m$ (°C) | $T_m$ NTC (°C) | $\Delta T_m$ (°C) |
|---|---|---|---|---|---|---|
| HPV 16 | 20 ± 1 | 26 ± 1 | 6 | 80.7 ± 0.2 | 74 ± 0.2 | 6.7 |
| HPV 18 | 22 ± 1 | 28 ± 1 | 6 | 79.3± 0.2 | 70 ± 0.2 | 9.3 |

*Identification of multiple infections of HPV types*

Results from our previous TS-PCR, showed six samples were positive for multiple infections by HPV 16 and HPV 18. Figure 3 showed a dissociation curve of HPV 16 and HPV 18. Four peaks were displayed; one peak for HPV 16, one peak for HPV 18 and two peaks for NTC. Melting temperature $T_m$ for HPV 16 was 80.7°C and for NTC was 74°C, whereas for HPV 18, melting temperature $T_m$ was 79.3°C and for NTC was 70°C. SyBr Green Real Time PCR method showed the $T_m$ for HPV 16 and HPV 18 were different although the PCR products using TS-PCR for HPV 16 and HPV 18 produced a single band as a 100 bp. The dissociation curves using SyBr Green Real Time PCR method showed that the $T_m$ for HPV 16 was 80.6°C whereas $T_m$ for HPV 18 was 79.4°C. Studies by Szuhai et al. (2001) and Kleinle et al. (2002) suggested the $T_m$ of an amplification product is based on amplicon size and GC content; fragments of different length and G+C content can be distinguished by melting curve analysis. Our study found that the $T_m$ of HPV 16 was higher than $T_m$ of HPV 18.

Table 1 showed the $C_t$ value of HPV 16 and HPV 18 were below 40 cycles and also differs from NTC by six cycles so that the $C_t$ values were significant based on studies by Szuhai et al. (2001) and Lamarcq et al. (2002). This table also showed the differences of $T_m$ between specific product of HPV 16 and NTC were 6.7°C whereas the differences of $T_m$ between specific product of HPV 18 and NTC were 9.3°C. Study by Kleinle et al. (2002), suggested the $T_m$ of the gene specific product differs from primer-dimers by at least 10°C, the peak of the gene-specific product did not overlap with the primer-dimer peak and thus the accuracy of the quantification was validated.

**DISCUSSION**

*Amplification plot of HPV 16 and HPV 18*

In this study, minimum primers for HPV 16 and 18 were 300:300nM for forward and reverse primers and DNA concentration at 100 ƞg. All graphs from Figure 1-3 showed $C_t$ value for HPV 16 was 20±1 cycles and dissociation curve of HPV 16 with melting curve $T_m$ was 80.7±0.2ºC whereas melting curve $T_m$ for NTC was 73.5±0.2ºC. Amplification plot of HPV 18 showed presence of two curves with one specific for HPV 18 and another one for NTC. $C_t$ value for HPV 18 was 21±1cycles and $C_t$ value for NTC was 28±1 cycles. Table 1 showed the $C_t$ value of HPV 16 and 18 were significant because, the difference between specific product and NTC was five and seven cycles (Kleinle 2002). For melting temperature, $T_m$ shows the differences of $T_m$ between HPV 16 and NTC were below 10°C such as 7.2°C whereas for HPV 18 the differences between specific HPV 18 and NTC were 9.4°C. Kleinle et al. (2002), suggested the $T_m$ of the gene specific product differs from primer-

dimers or NTC by at least 10°C, the peak of the gene-specific product did not overlap with the primer-dimer peak and thus the accuracy of the quantification was validated. However, this study found the differences of $T_m$ for HPV 16 and HPV 18 were below 10°C, this pheno-mena happened because the amplicon size of HPV 16 and 18 were 100bp and the size of NTC range from 50-60bp. The close-up of amplicon size between spe-cific product and NTC will affect the $T_m$. Although the amplicon size between spe-cific products and NTC was very close, the graph shows gene-specific product of HPV 16 and HPV 18 did not overlap with the NTC peak so the accuracy of the qu-alification was validated.

*Identification of multiple infections of HPV types*

From our previous sequencing results of PCR products of E7 gene for HPV 16 (RID 1055387141-08795-2060) and HPV 18 (RID 1056435350-019738-16228) we observed that, GC content for HPV 16 was higher than the GC content of HPV 18, and the PCR product of HPV 16 had high $T_m$ compared to $T_m$ of HPV 18. The differences in melting temperature can be used as a method to identify and diffe-rentiate multiple infections between HPV16 and HPV 18. Kleinle et al. (2002) and Lamarcq et al. (2002) reported that multiple specific products can be sepa-rated from each other due to different $T_m$ in most cases. This method is advanta-geous since it eliminates the need for gel electrophoresis. This finding shows, SyBr Green can be used to differentiate and identify HPV types. Table 1 shows a summary of the $C_t$ values and $T_m$ for HPV 16 and HPV 18. The $C_t$ value of HPV 16 and HPV 18 were below 40 cycles and also differ from NTC by six cycles, indicating that the $C_t$ values were significant. The findings were supported by studies by Szuhai et al. (2001) and

Lamarcq et al. (2002). Table 1 also showed the differences of $T_m$ between HPV 16 and HPV 18 with the NTCs being 6.7°C and 9.3°C respectively. The peak of the gene-specific product did not overlap with the primer-dimer peak and thus the accuracy of the quantification was validated. This SyBr Green Real-Time PCR method offers the possibility of substantially increasing the throughput of sample analysis, of analyzing different samples for the presence of any of these gene mutations and/or determining the individual gene mutation pattern. In terms of the rapidity, flexibility and the subsequent economic advantages, this approach is suitable for use in clinical and routine laboratory applications (Cubie et al. 2001; Szuhai et al. 2001; Kleinle et al. 2002; Villa et al. 2006).

## CONCLUSION

Using SyBr Green Real-Time PCR tech-nique, minimum forward and reverse primers for each HPV type 16 and 18 were required. DNA concentration for each samples were fixed at 100ng. SyBr Green Real-Time PCR method has the advantage of it being able to be used di-rectly to detect and identify the HPV types based on amplification plot and melting temperature graphs. This SyBr Green Real-Time PCR method has the potential to be used as a method for de-tection and identification of HPV types in CINs and cervical cancer patients in Ma-laysia. In addition it has the advantage of detecting multiple infections of HPVs which adds to its economic value.

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