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Optics Communications 266 (2006) 744–750
OPTICS COMMUNICATIONS
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A novel fluorescence quantification method for polymerase chain reaction system

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Received 3 March 2006; received in revised form 19 May 2006; accepted 22 May 2006

Abstract

Due to the increasing demand of simultaneous DNA detection, known as multiplexing detection, in a single sample, the current real-time polymerase chain reaction (PCR) platforms are equipped with discrete optics and detectors for different fluorescence wavelength. However, to go beyond four DNA labeling dyes, the optical loss will lower the performance on fluorescence detection. The proposed fluorescence detection system, composing of an ultra-sensitive spectrometer, can provide continuous wavelength detection and can be employed for multiple DNA quantification. The results show that this prototype provides comparable sensitivity and amplification efficiency as Roche Light-Cycler for DNA quantification and similar reproducibility within five intra assay samples with the DNA quantification method proposed in this study.

Keywords: Real-Time PCR; Miniature spectrometer; Continuous fluorescence wavelength detection; DNA quantification method

1. Introduction

The PCR process was first introduced [1–3] to replicate the specified DNA fragments in vitro. Since then, the PCR process has become one of the major tools in genomic studies. By integrating a fluorimeter with the thermal cycler, a Real-Time PCR machine [4–7] was first introduced. This machine allows detection of DNA amplification through the detection of the fluorescence labeling dye in the PCR master mix during the early phase of reaction. In addition, the concentration of target DNA template in the PCR mix before thermal cycling can be obtained from the time recorded history of fluorescence intensity. The Real-Time PCR machine has high sensitivity and consumes less time when compared to the traditional PCR machine.

The current commercial Real-Time PCR machine from Roche Molecular Diagnostics (Roche Light-Cycler) is a six-channel system with discrete optics and detectors for different fluorescence wavelength [8,9]. However, to go beyond four DNA labeling dyes, the optical loss will lower the performance on fluorescence detection. The PCR process employed to detect different viruses in one reaction is called Multiplexing Real-Time PCR. The photo demultiplexer, which was announced by Roche in 2003, is one of the novel approaches proposed to separate six optical wavelengths from one fluorescence input towards the corresponding photodiode detector. Although this solution provides multi-channel detection capability and the commercial product is announced to be available, the reliability of this kind of Real-Time PCR machine is still under evaluation due to the fact that the photo demultiplexer is not a mature product yet. Recently Lee et al. employed a miniature spectrometer to set up a PCR prototype [10] and utilized an analytical model to predict fluorescence intensity of continuous wavelength detection [11]. The proposed prototype can provide continuous wavelength detection and can be employed

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0030-4018/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.optcom.2006.05.043

for multiplexing DNA quantification. The test results in this research show the same degree of sensitivity for DNA quantification and reproducibility within five intra assay samples as compared with a commercial one.

Based on the Lee's research, the prototype proposed in this study was modified. Since the high-power xenon light source generates heat and leads to system noise, an optical fiber was employed to provide excitation light into the sample chamber, while the bulb and the power unit will be assembled as far away from the system as possible to avoid noise influence. With the modification, this system provides detection capability with low fluorescence intensity dye such as LC-Red 705 and LC-Red 640. A new DNA quantification method was developed in this study based on all fluorescence wavelength distribution records and the feature extraction by statistical method. The partial HBV DNA of 350 base pairs with LC-Red 705 dye and the partial HCV cDNA of 175 base pairs with LC-Red 640 dye were employed for DNA quantification experiments. The experiment reproducibility was compared with that of the traditional machine to verify the new system performance and the usefulness of the quantification method.

2. Experimental apparatus

A schematic view of the Real-Time PCR machine is shown in Fig. 1. The Real-Time PCR machine was consisted of a light source, an optical system for measuring Fluorescence, and a thermal cycling system. A fiber light source (PX-2 Pulsed Xenon light source, Ocean Optics Inc.) that could provide 220–750 nm broadband light as the light source for DNA quantification experiments. The broadband light was filtered through a 470 interference filter to a 5 nm FWHM light for exciting DNA labeling dye. The optical system for Fluorescence was consisted of a miniature spectrometer, an optic fiber, a collimating lens, and a computer. A miniature spectrometer (USB Fiber Optic Spectrometer, Ocean Optics Inc.) was employed to measure spectra of emission fluorescent dyes. A Fluorescence lens focused the emitted fluorescence light into the spectrometer through the transmission of a 200 μm optic fiber. Inside the spectrometer, one collimating mirror reflects the light as a collimated beam towards a cylindrical optical grating. The cylindrical grating dispersed the beam with an efficiency of 30% to a linear CCD (ILX 511, Sony) that had the sensitivity of 200 V/(lxs). This spectrometer measured the spectral wavelength ranging from 200 to 1000 nm with a 1.5 nm FWHM resolution. Measured spectral responses at different thermal cycles were transmitted into a computer for later analysis.

The DNA sample capillary tube was inserted in a circular carousel and placed into a cylindrical temperature control chamber. The temperature variation of the chamber was controlled by an air flow through a wind duct with a

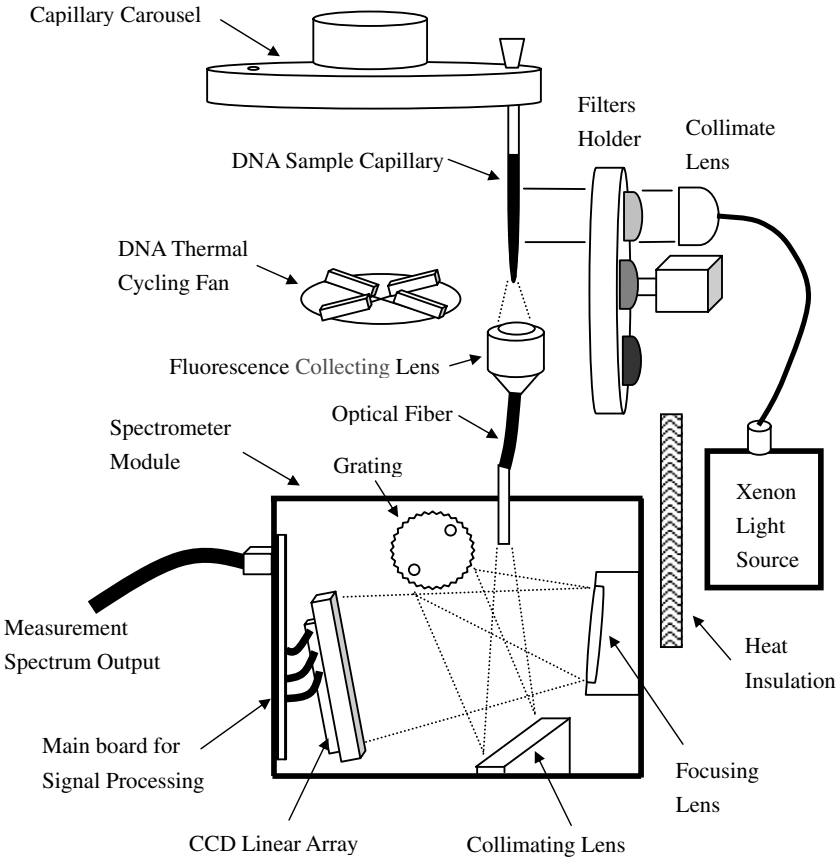

Fig. 1. A schematic view of the present Real-Time PCR machine.

heater. The maximum power provided by the heater was 300 W. A fan that was located at the entrance of the wind duct supplied an air stream through the chamber. The fan had a rated rotational speed ranging from 2200 to 6000 rpm at a rated current ranging from 0.05 to 0.75 A and a DC rated voltage ranging from 12 to 48 V. A temperature ramping rate of 20 °C/s can be achieved by forced air heating and cooling approaches. Another fan that was located at the bottom of the thermal cycling chamber run at a constant speed of 1800 rpm. It was used to stir the air stream in order to achieve a uniform temperature among 32 DNA sample capillary tubes in the thermal cycling chamber. This fan had a rated rotational speed ranging from 1600 to 1800 rpm, a rated current ranging from 0.13 to 0.15 A, and an AC rated voltage of 110 V.

The reagents of the master mix used in this assay are listed in Table 1. The partial HBV DNA of 350 base pairs and the partial HCV cDNA of 175 base pairs, mixed with primers were prepared in one capillary tube. HBV and HCV of 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 initial copies/mL and negative control, which were diluted from the original 10^9 copies/mL, were added to capillary tubes separately. Sensor and Anchor of 3 μM were probes hybridized to HBV and HCV. After processing, LC-Red 705 and LC-Red 640 were labeled on the HBV DNA and on the HCV cDNA respectively. The PCR mix was to be loaded in the capillary tubes to perform Real-Time PCR for DNA quantification. It required a 10-minute incubation at 95 °C. After the incubation, each thermal cycle underwent 3 s of 95 °C-denaturation, 10 s of 53 °C-annealing, and 16 s of 72 °C elongation to amplify the DNA fragment. The cycling condition is as described in Table 2.

The agarose gel electrophoresis was also carried out in experiments to verify the successful DNA amplification. PCR products were separated with a 2% agrose gel (GX 04004, GenAgarose, Genaxis™ Biotechnology). Electrophoresis of amplified products was performed using a Mini Gel Migration Trough (Mupid-2, COSMO BIO Co., LTD.)

Table 1
The reagents employed in this study

Reagent	Reagent concentration	HCV sample (μL)	HBV sample (μL)	Negative control (μL)
H_2O	–		1.6	
$MgCl_2$	25 mM		2	
Enzyme	–		2.4	
Primer HBV 2F	4 μM		0.5	
Primer HBV 2R	20 μM		0.5	
Primer HCV LL	20 μM		0.5	
Primer HCV 2F	10 μM		0.5	
Anchor HBV	3 μM		1	
Sensor HBV	3 μM		1	
Anchor HCV	3 μM		1	
Sensor HCV	3 μM		1	
HCV	10^3–10^9/mL	8	–	–
HBV	10^3–10^9/mL		8	–
H_2O	–	–	–	8
Total (μL)	–		20	

Table 2
The cycling condition employed in this study

Segment number	Temperature target (°C)	Hold time (s)	Slope (°C/s)	Acquisition mode
Program: Denature				*Cycles: 1*
1	95	600	20	None
Program: Amplification				*Cycles: 60*
1	95	3	20	None
2	52	10	20	Single
3	72	16	3	None

at an applied voltage of 100 V/cm for 40 min in 1× TAE buffer (Ultra Pure Grade, Amresco® Inc., USA). The PCR products were visualized with ethidium bromide staining. The staining dye was contained in 6× gel-loading buffers: 0.25% bromophenol blue and 40% (w/v) sucrose in DI water. Through electrophoresis, the observation of DNA fragments was conducted by using a short wavelength UV transilluminator (Mighty Bright, UVTM, Hoefer).

3. Fluorescence modeling

The most general approximation of the DNA labeling dye emission fluorescence modeling is described below

$$\Delta F = I_\lambda(0) \times \int_0^L \int_0^{2\pi} \int_{\varphi_1(x,\theta,\varphi)}^{\varphi_2(x,\theta,\varphi)} \sin\varphi \sum_{i=1}^{N} \varphi_i \cdot a_{i,\lambda} \cdot C_i\left(\frac{I_\lambda(x)}{I_\lambda(0)}\right)\left(\frac{I_{\lambda'}(L)}{I_{\lambda'}(0)}\right) \mathrm{d}\varphi\,\mathrm{d}\theta\,\mathrm{d}x, \quad (1)$$

where ΔF means the fluorescence signal intensity, ϕ_i is the quantum yield of the labeling dye, $a_{i\lambda}$ is the molar extinction coefficient and c_i is the DNA template concentration within each PCR cycle i. $\left(\frac{I_\lambda(x)}{I_\lambda(0)}\right)$ and $\left(\frac{I_{\lambda'(L)}}{I_{\lambda'(0)}}\right)$ denote the excitation light decay due to absorption of labeling dye, and emission fluorescence decay as light passes through the DNA sample capillary tube of length L respectively. θ and φ denote the core angle and the solid angle respectively. N represents the number of DNA labeling dyes in the tubes.

The collimated excitation light directly excites the capillary with PCR mix through the side, together with the focusing effect by the capillary wall and the short pass length, it can yield the simple estimation of $I_\lambda(0) = I_\lambda(d)$, where d denotes the diameter of the capillary.

The transition parameter S, which can be treated as constant, was employed for evaluating the integral. It is expressed as

$$S = -\left\{\frac{\log\left(1 - \frac{x}{\sqrt{x^2+R^2}}\right)}{x}\right\}, \quad (2)$$

$$\Delta F = 2 \cdot \pi \cdot I_\lambda(0) \cdot \phi_i \cdot a_{i,\lambda} \cdot \mathrm{e}^{-\frac{L}{r}} \int_0^L \left(1 - \frac{x}{\sqrt{x^2+R^2}}\right) \cdot c_i \mathrm{d}x, \quad (3)$$

Eq. (3) becomes

$$\frac{\Delta F}{I_\lambda(0)} = 2 \cdot \pi \cdot \phi_i \cdot a_{i,\lambda} \cdot e^{-\frac{L}{r}} \frac{1}{S}(1 + e^{-SL}) \cdot c_i. \quad (4)$$

By the formula, the fluorescence intensity with respect to the excitation light for different initial DNA template copies and different PCR amplification efficiencies can be predicted. Based on the modeling, the quantification method was developed.

4. DNA quantification method

Fig. 2 shows the fluorescence intensity in each PCR cycle with respect to different DNA initial copies at specific averaged DNA amplification efficiency, and that to different DNA amplification efficiencies as the number of DNA initial copies was kept constant. Since the amplification process is a nonlinear response, the estimated efficiency can only be represented in average. Moreover, different DNA initial copies can shift the predicted curve while the DNA amplification efficiency changes the trend of the curve. In addition, before the curve fitting method is applied, the signals should be preprocessed by a low pass filter because there is system noise in the fluorescence data directly obtained from the spectrometer. The LP (low pass) filter is described as follows:

$$a[i] = \sum_{r=0}^{60} b_r u[i-r], \quad (5)$$

$$A_{10} = B^{10} U, \quad (6)$$

where $a[i]$ denotes the output of this LP filter B and $u[i]$ denotes the input signals respectively. Furthermore, A represents the z-transformed form of $a[i]$. The LP filter B is described as follows:

$$B = \begin{bmatrix} 1 & & & & & \\ 1/2 & 1/2 & & & 0 & \\ 1/4 & 1/4 & 1/2 & & & \\ 1/8 & 1/8 & 1/4 & 1/2 & & \\ \vdots & & & & & \\ 1/2^{59} & \cdots & \cdots & \cdots & \cdots & 1/2 \end{bmatrix}. \quad (7)$$

After the preprocess was applied on the fluorescence signals, Curve fitting method was then employed to calculate DNA initial copies by minimizing the error between the fluorescence data with positive slope of fluorescence

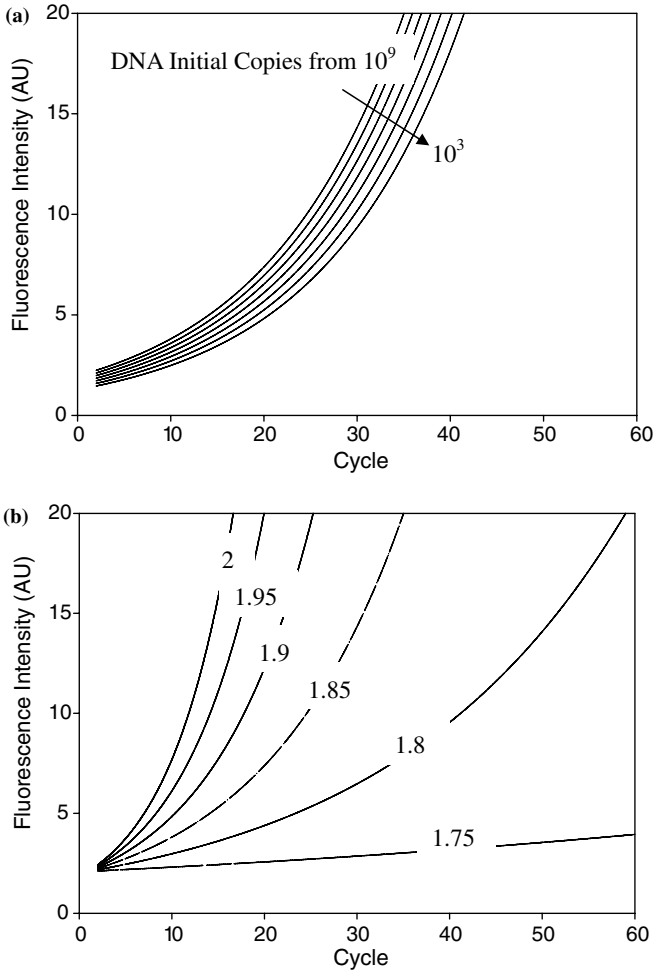

Fig. 2. The simulated fluorescence intensity corresponding to each PCR cycle (a) DNA amplification efficiency = 1.85, DNA initial titers range from 10^9/mL to 10^3/mL (b) DNA initial titer = 10^9/mL, DNA amplification efficiency from 2 to 1.75.

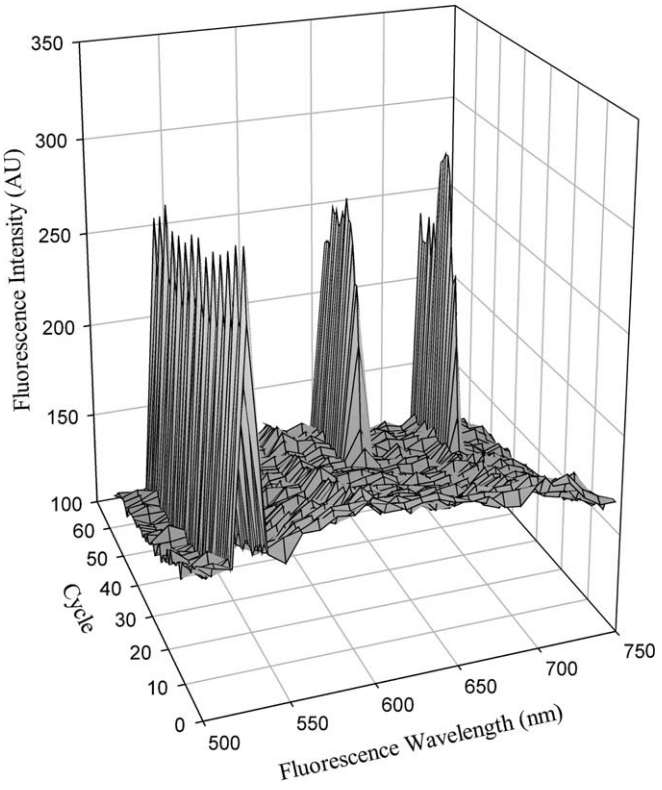

Fig. 3. The emission fluorescence spectra of LC-Red 705 with HBV and LC-Red 640 with HCV with respect to different PCR cycle. The increasing peak near 650 nm indicates LC-Red 640 dye and the other indicates LC-Red 705 one. The constant noise peak around 550 nm is due to the excitation light source.

intensity variation and the predicted line on the curve with respect to PCR cycle with different DNA initial copies and DNA amplification efficiencies.

The method includes most of the fluorescence intensity variation information within PCR cycles in determining the DNA initial copies. The analysis should base on the proper amplification of PCR runs. Subsequently the minimum DNA amplification efficiency will be set to 1.75.

5. Results

The emission fluorescence spectra of the labeling dyes LC-Red 705 and LC-Red 640 are shown in Fig. 3. For HBV DNA template and HCV cDNA template, the peak values occur in 705 nm and 640 nm respectively. Quantification results of the samples with different DNA initial copies ranging from 10^9 to 10^3 are shown in Fig. 4 and the quantification results are also listed in the figure.

To prove the ability for multiplexing Real-Time PCR, the HBV DNA and the HCV cDNA each labeled by LC-Red 705 and LC-Red 640 in one sample tube were performed quantification experiments on the proposed prototype. With the different maximum absorbance and emission of the two dyes, the prediction curve fitting coefficients should be modified to fit LC-Red 640 dye. Fig. 5 shows the DNA quantification results and the fitting curves.

Fig. 6 shows the agarose gel of the PCR products from the present prototype and the commercial Real-Time PCR machine with the same thermal cycling conditions described above. The gel photo shows that there is low interference between the primers and probes of HBV and HCV during the amplification processed. The bands in the photo demonstrate that the proposed prototype and Roche Light-Cycler have close amplification efficiency.

The reproducibility of the Real-Time PCR machine is also a key factor of the system. Coefficient of Variation (CV) with unit in percentage is usually employed as an index and it is defined by

$$\mathrm{CV} = \frac{\log_{10}\left(\frac{\sum C_{\mathrm{M}}}{n}\right) - \log_{10}(C_{\mathrm{s}})}{\log_{10}(C_{\mathrm{s}})} \times 100\%, \tag{8}$$

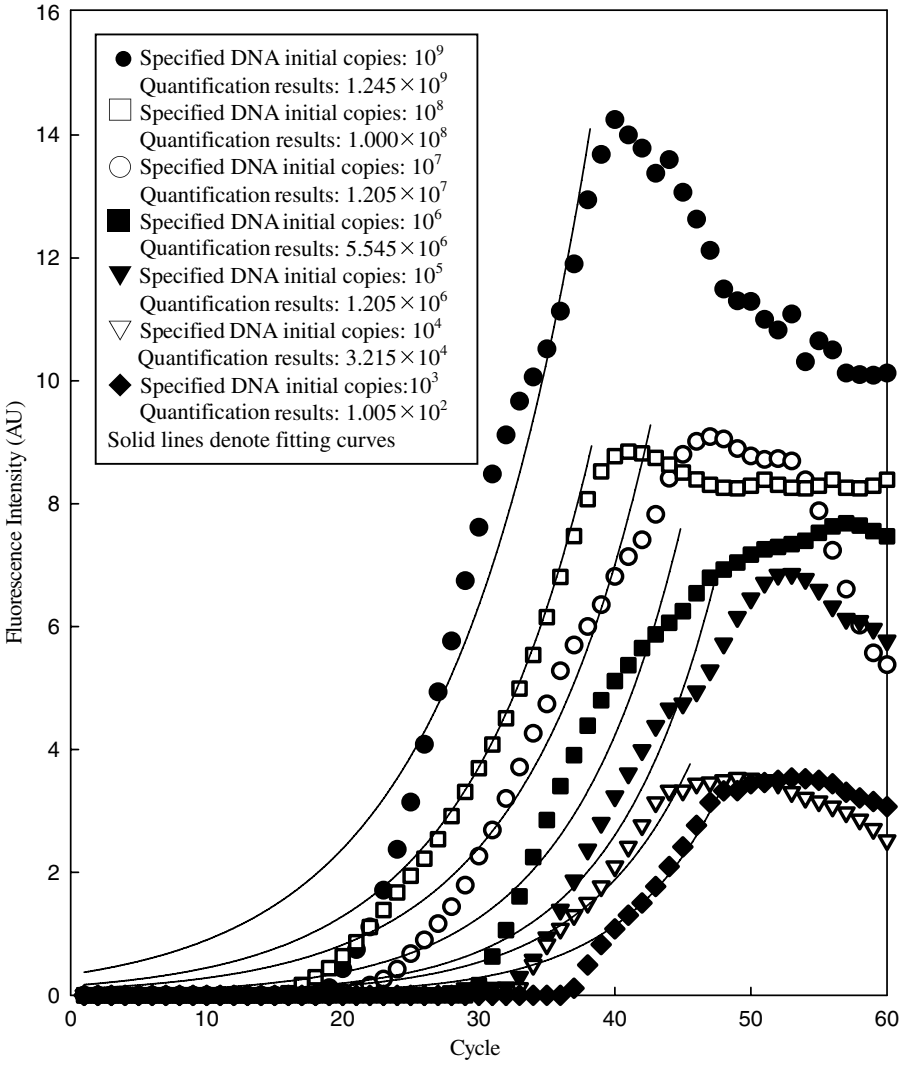

Fig. 4. DNA quantification results were obtained by curve fitting method for the samples of the different DNA initial titer.

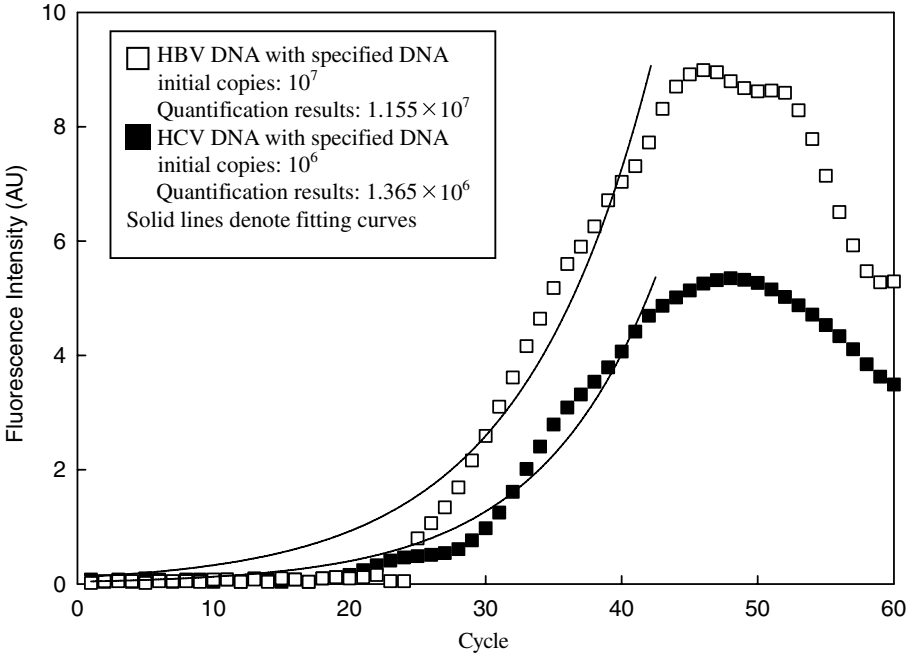

Fig. 5. DNA quantification results with two different DNA templates were performed in one sample capillary tube by the present prototype.

where C_M denotes the measured DNA initial copies in the test samples, C_s is the specified DNA initial copies and n is test sample number. The CV value indicates discrepancy between the average of several quantification results and the specified DNA copies value. A low CV value means the system has high reproducibility and measurement errors induced by all factors are minimized. Fig. 7 shows the CV value tested by intra-assay with triplicate for different initial DNA copies in the present prototype and the commercial Real-Time PCR machine. The results show that the reproducibility of the present prototype is close to that of the commercial Real-Time PCR machine which is used in this study.

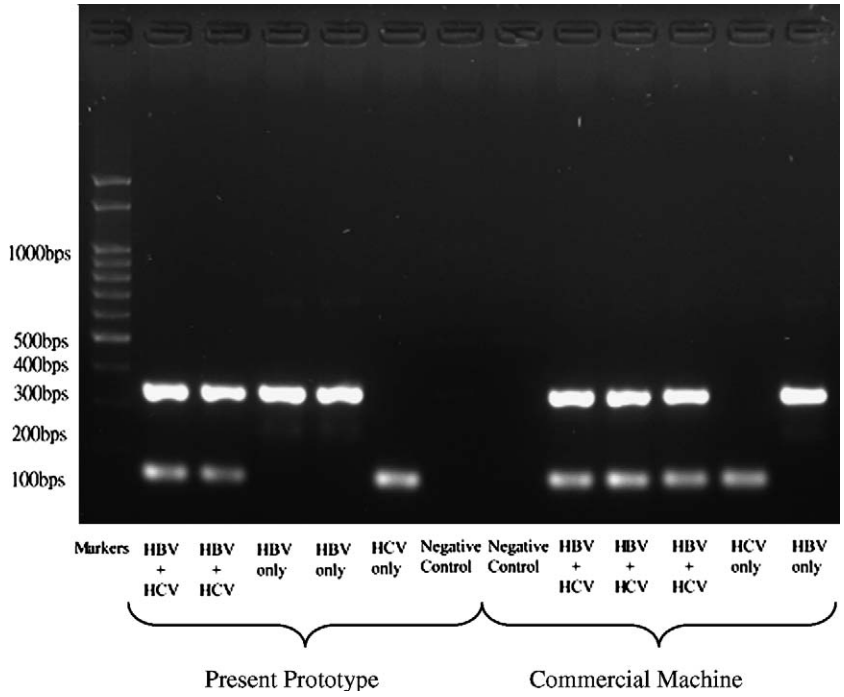

Fig. 6. The agarose gel of PCR final products by the present prototype and the commercial Real-Time PCR machine, where HBV denotes the partial DNA of the HBV virus and HCV denotes the partial HCV cDNA of the HCV virus.

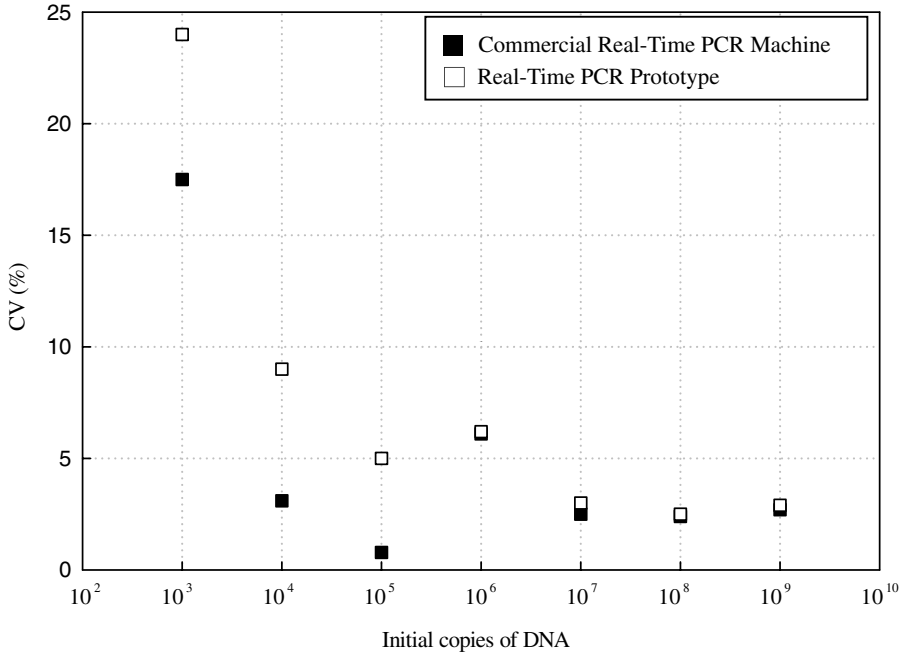

Fig. 7. The reproducibility of the Real-Time PCR machine was tested by intra-assay with three replicates for different initial DNA titers in the present prototype and the commercial Real-Time PCR machine.

6. Conclusion

The Real-Time PCR machine for DNA quantification using a miniature spectrometer for fluorescence detection was developed in this study. An analytical model was proposed to predict the fluorescence intensity within PCR cycles and the results show that the fluorescence accumulation is proportional to the DNA products concentration in the sample tube. The curve fitting method was developed to determine DNA initial copies in the samples based on the model. the partial HBV DNA of 350 base pairs with LC-Red 705 dye and the partial HCV cDNA of 175 base pairs with LC-Red 640 dye in one sample tube were performed quantification experiments on the proposed prototype to prove the ability for multiplexing Real-Time PCR. The system can provide wide spectral spans from 350 nm to 1100 nm for multiplex DNA quantification and has no fluorescence detection channel limitation as present in the commercial Real-Time PCR machine. It was proved that the proposed prototype with the curve fitting quantification method could provide the same degree of sensitivity and similar reproducibility of DNA quantification as compared with the commercial PCR machine when the prototype is utilized to do multiplex HBV and HCV PCR. Furthermore, the agarose gel of final products show that the amplification efficiency is close to that of the commercial machine under the same thermal cycling conditions.

Acknowledgement

This work was financially supported by research project No. 93-2212-E-027 of the National Science Council, Taiwan, ROC.

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