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A real-time convective PCR machine in a capillary tube instrumented with a CCD-based fluorometer

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A B S T R A C T

This research reports the design, analysis, integration, and test of a prototype of a real-time convective polymerase chain reaction (RT-cPCR) machine that uses a color charged coupled device (CCD) for detecting the emission of fluorescence intensity from an RT-cPCR mix in a microliter volume glass capillary. Because of its simple mechanism, DNA amplification involves employing the cPCR technique with no need for thermostating control. The flow pattern and temperature distribution can greatly affect the cPCR process in the capillary tube, a computational fluid dynamics (CFD) simulation was conducted in this study for the first time to estimate the required period of an RT-cPCR cycle. This study also tested the PCR mix containing hepatitis B virus (HBV) plasmid samples by using SYBR Green I fluorescence labeling dye to assess the prototype performance. The measured results from the image-processing scheme indicate that the RT-cPCR prototype with a CCD-based fluorometer can achieve similar DNA quantification reproducibility compared to commercial machines, even when the initial DNA concentration in the test PCR mix is reduced to 10 copies/μL.

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1. Introduction

Mullis et al. developed the polymerase chain reaction (PCR) in 1986 to clone specific deoxyribonucleic acid (DNA) fragments in vitro by using temperature cycling [1]. A real-time PCR (RT-PCR) machine was introduced in 1997 by integrating a fluorometer with a temperature cycler [2,3]. This RT-PCR system identifies DNA amplification by detecting the fluorescence-labeling dye in the PCR mix during the early phase of reaction. In addition, the recorded time history of fluorescence intensity can be used to determine the concentration of target DNA fragments in the PCR mix before thermal cycling. However, an RT-PCR machine requires using a complex thermal controller for repetitively heating and cooling samples, usually for 35–50 cycles. Restricted by the large size, costliness, and long amplification time, using traditional PCR thermocyclers have been limited to principal hospitals and laboratories. Recently, new platforms have been advanced by simplifying the equipment structure and reducing the amplification times.

A promising solution is to induce natural convection with temperature gradients, thereby enhancing the amplification of DNA [4]. When the reagents in a sample circulate repeatedly among temperature zones because of the natural convection effect, the sample is anticipated to undergo three steps of a PCR cycle (i.e., nucleic acid denaturation, annealing, and extension) in a single circulation. Therefore, convective PCR can substantially shorten the amplification time from multiple hours to 30–40 min [5,6]. By using this mechanism, a dedicated thermocycler, the most expensive module of the conventional PCR, is not required. The real-time convective PCR (RT-cPCR) apparatus could also be devised by combining fluorescence detectors. However, the current RT-cPCR systems adopt two or even more individual temperature controllers and elaborate designs necessitating a specific shape of tubing or additional chambers to circulate the sample fluids thoroughly [7–11]. Current RT-cPCR systems also require skillful manipulation for certain operations such as loading and unloading reagents from thin tubing as well as sealing both tube ends without trapping air bubbles in the tube, thus causing detection errors.

Chou et al. designed and fabricated a novel capillary convective PCR (ccPCR) platform with a capillary tube mounted on a heater at a constant temperature of 95°C [12–14]. When using...
this platform, no complex microfluidic chip design, special tubing, or vessels are required. The constantly heated capillary base drives the lowest part of the sample fluids and raises them by convection while denaturing the template simultaneously. During the ascending of the sample, its temperature falls because of cooling from the surrounding air. As the sample reaches the cool temperature zone near the top of the tube, it undergoes annealing and extension, after which the DNA template descends and is heated again. Thus, PCR cycles are achieved by natural convection. This study adopted a concept similar to that of Chou et al. to form a real-time convective PCR machine in a capillary tube instrumented with a CCD-based fluorometer. In such a simple platform without costly and delicate thermocyclers and additional complex hardware, this study demonstrates that correct primer and amplicon design are essential to perform DNA amplification successfully. Moreover, the computer-aided analysis was implemented using the computational fluid dynamics (CFD) simulations to resolve the flow pattern in the capillary of an RT-cPCR machine and to determine the time required for completing an RT-cPCR cycle. To assess the performance of the prototype, a single DNA template, HBV 122 base pairs, with known concentrations and a single labeling dye, SYBR Green I, was used in the PCR mixes undergoing the same thermal cycling in both the prototype and commercial RT-PCR machines for comparing their measured and predicted fluorescence intensities emitted from the glass capillaries. We claim that the current RT-cPCR machine can offer a new DNA amplification method with ease of operation and low cost that is well suited for point-of-care applications in less-developed countries.

2. Experimental apparatus

Fig. 1 shows (a) a schematic view and (b) a photograph of the RT-cPCR machine. The RT-cPCR machine comprised a light source, an optical system for measuring fluorescence intensity, and a controllable thermal system for maintaining a constant temperature of 95°C. A blue LED (UNICE E-O Services Inc., Part No. RL5-B2430, TWN) was used to provide 450–520 nm of broadband light as the light source for DNA quantification experiments. The broadband light was filtered using a 480 interference filter to 5-nm full width at half maximum (FWHM) light for exciting the DNA labeling dye. A monochromatic CCD camera (Watec Inc., VAT-902H, JPN) was also employed to measure a wide spectral response of fluorescence intensity reflected from the PCR mix through the transmission of a 530-nm optic filter. The proposed RT-cPCR machine contains a thermoelectric (TE) cooler and a fan for respectively raising and lowering the case temperature. The TE cooler was placed between a Cu holder substrate and a sponge to form a heating element. A thermocouple was inserted into the TE cooler to determine the temperature of the Cu holder substrate for the feedback control to maintain a constant temperature of 95°C. Referring to the published results [12–14], the sample container was a commercial off-the-shelf glass tube (Light-Cycler Capillaries 100 μL, Roche) with a closed bottom. The dimensions of the capillary tube were 32 mm in length with inner and outer diameters of 2.3 mm and 3.2 mm, respectively. The test tube, heated from the bottom at a fixed temperature of 95°C, can form a temperature gradient within the reagents and generate natural convection to induce spontaneous circulation of the reagents in a glass capillary for developing the DNA amplification process, including denaturation, annealing, and elongation.

This research employed the hepatitis B virus (HBV) as the template DNA for amplification. The HBV DNA of 122 bp was mixed with the primers prepared in a capillary tube. Table 1 lists the reagents of the master mix used in this assay. HBVs of $10^3$ initial copies/μL, $10^4$ initial copies/μL, $10^5$ initial copies/μL, $10^6$ initial copies/μL, and a negative control, diluted from the original $10^6$ copies/μL, were systematically added to the capillary tubes with the primer sets designed using the existing software (Light Cycler Probe Design Software 2.0, Roche). Primers (HBV F: 5'- CCTAGACGCTTGTGTTGTCGCACCG-3'; HBV R: 5'- TCCAGTGGCGCAGCACCTGC-3') were then synthesized by Mission Biotech, Taiwan. In the experiments, we selected a sample tube with a height-to-diameter ratio of 7.7 and a fixed volume of 75 μL to maintain a single-circulation environment for stable DNA amplification.

In determining the fluorescence intensity, a numerical model was developed to predict the fluorescence signal that was the function of DNA concentration in each PCR cycle in comparison with the RT-cPCR machine [15]. The model prediction values were then

![Fig. 1. Illustration of (a) a schematic view and (b) a photograph of the RT-cPCR machine.](image)

| Reagent     | Reagent concentration | HBV sample (μL) | Negative control (μL) |
|-------------|-----------------------|----------------|------------------------|
| H2O         | –                     | 50.5           | 55.5                   |
| MgCl2       | 25 mM                 | 9              | 9                      |
| Enzyme      | –                     | 7.5            | 7.5                    |
| Primer HBV 2F | 10 μM              | 1.5            | 1.5                    |
| Primer HBV 2F | 10 μM              | 1.5            | 1.5                    |
| HBV         | $10^3–10^6$ copies/μL | 5              | –                      |
| Total       | (μL)                  | –              | 75                     |


compared with the commercial discrete detection channel reading to verify the validity. This research employed this model as a basis for analyzing the fluorescence signal measured by the system prototype to attain precise RT-cPCR quantification.

The most general approximation of the fluorescent signal can be expressed as follows:

$$\Delta F = 2L\int_0^1 \int_0^{2\pi} \int_0^\pi \sin \theta \times \left( \frac{I(x)}{I_0} \right) \left( \frac{I(L)}{I_{L_0}} \right) \phi d\phi d\theta dx,$$

where $\Delta F$ means the fluorescent signal intensity and is proportional to $I_0$, the excitation light intensity, times the labeling dye quantum yield $\phi$, the molar extinction coefficient $\alpha$, and the DNA template concentration $c_i$ within each PCR cycle $i$. The two terms $(I(x)/I_0)$ and $(I(L)/I_{L_0})$ denote the excitation light decay caused by the absorption of labeling dye and emission fluorescence decay as light passes through the DNA sample capillary tube of length $L$. The integration is along the DNA sample capillary tube length and around the whole core angle $\theta$ from 0 to $3\pi/4$ and the solid angle $\phi$ from $0$ to $\pi$, which is determined according to the different optical engine arrangements. The core angle, the solid angle, and the integration length of the RT-cPCR prototype with a continuous fluorescent wavelength sensing system can be referred to in Fig. 1a. The collimated excitation light directly excites the capillary with the PCR mix through the side. The focusing effect caused by the capillary wall and the short pass length can yield the simple estimation of $I_0 = I_d(a)$, where $d$ denotes the diameter of the capillary. The $(I(x)/I_0)$ term can be determined as a constant and the emissive fluorescence decay term $(I(L)/I_{L_0})$ can be estimated by the following:

$$\left( \frac{I(L)}{I_{L_0}} \right) = \exp (0.00334d).$$

The integration can be expressed as:

$$\frac{\Delta F}{I_0} = 2\pi L \alpha d c_i \cos^\theta \int_0^{2\pi} \phi d\phi d\theta dx,$$

Use of the formula can accurately predict the fluorescent intensity with respect to the excitation light for the sample with different initial DNA template copies.

3. Computational analysis

When a fluid layer is heated underneath, a fluid particle in the bottom layer is relatively warmer and lighter than that in the top layer. Consequently, the fluid flow has a tendency to ascend, whereas the viscous force effect can slow the upward travel of the particle. This process is known as the Rayleigh–Bénard instability, leading to the generation of convective movements.

Considering the buoyancy and viscous forces involved in this mechanism, the instability development is controlled by the Rayleigh number, which is defined as follows:

$$Ra = \frac{g \beta (T_2 - T_1) h^3}{\nu \alpha},$$

when the Rayleigh number exceeds a critical value, convection occurs. Here, the variables $g$, $\beta$, $T_1$, $T_2$, $h$, $v$, and $\alpha$ are the gravity acceleration, thermal expansion coefficient, temperature at the top and bottom of the fluid, height of the fluid layer, thermal diffusivity, and kinematic viscosity, respectively. Furthermore, $Ra$ is a crucial parameter affecting the stream function, velocity, and temperature distributions inside a convection cavity. Another parameter available to control the thermo-fluid behavior is the aspect ratio $h/d$, where $d$ is the cavity diameter. In the PCR situation, the required reaction efficiency constrains the reaction solution and the temperature difference; hence, $Ra$ can only be changed by varying the cavity height, leaving the geometry as the primary parameter for flow control.

The current physical model considers the buoyancy-driven flow in a capillary. Numerical calculations using the CFD software ANSYS/Fluent® were conducted to explore the thermo-flow characteristics, which are mainly characterized according to the $Ra$ number, for appraising the effectiveness of the RT-cPCR process. The theoretical formulation was based on the steady 3D conservation equations of mass, momentum, and energy for incompressible laminar flows. The fluid physical properties were assumed to be constants with the Boussinesq approximation used to allow for the buoyancy effect, whereas the viscous dissipation and Joule heating were negligibly small. Table 2 lists the thermophysical properties of the test materials in computations. The water thermal expansion coefficient was $2.83 \times 10^{-3} /K$. In treating the boundary conditions, the top and bottom surfaces were the steady temperatures of $338$ K and $368$ K, respectively, with the adiabatic vertical walls involved. The heat transfer coefficient was $5 \, W/m^2 K$, and the room temperature was $300$ K.

### 4. Results and discussion

Numerical simulations were conducted using the CFD software ANSYS/Fluent® to predict the flow and temperature fields in a capillary tube. The average cell length was approximately 0.074 cm with the least spacing of 12.6 $\mu$m to resolve the steep variations of velocity and temperature near the surfaces. Computations were conducted on the total grids of 145472, 183913, and 229086 points at $CFL = 0.5$ and 0.25. During the transient calculations, the normalized residual errors of the flow variables ($u$, $v$, $w$, and $T$) converged to $10^{-4}$ with the mass conservation check within 0.5% for each time step. The calculated velocity and temperature profiles along the tube centerline at different grids and CFL values indicated that satisfactory grid independence could be achieved using a mesh setup of 183913 grids with $CFL = 0.5$. A complete simulation for developing a stable thermo-flowfield (with the greatest variations of the centerline velocity and temperature predictions below 0.5%) requires approximately 240 h of central processing unit (CPU) time on an Intel®-Core 2® 990X-3.47GHz work station. Fig. 2 shows (a) the simulated temperature fields controlled by Rayleigh–Bénard (RB) convection, as well as a comparison of (b) the visualized particle path images with (c) the predicted time sequences of streaklines and velocity contours of flow pattern (with an animation, $T = 30$ s). The temperature of 95°C was chosen so that a double-stranded DNA could be melted into two strands in the bottom of the capillary tube. Each strand was further duplicated into the double-stranded DNA along the axial tube length with the support of a polymerase enzyme under the effect of natural convection. To view the internal flow structure, the deionized water flow was seeded with 50-$\mu$m-diameter polystyrene polyme particles (density $\rho_p = 1005$ kg/m$^3$). This study also used the graphic software Tecplot 360® to generate streaklines by releasing a sequence of particles from the discharge points and integrating the solved velocity field to locate their positions in the flow. The calculated streaklines may be further animated to a file. Considering the kinematic impermeability and no-slip conditions satisfied by the real

| Reagent       | Density, $\rho$ (kg/m$^3$) | Specific heat, $CP$ (J/kg K) | Thermal conductivity, $K$ (W/m K) |
|---------------|----------------------------|------------------------------|----------------------------------|
| Mineral oil   | 884.1                      | 4182                         | 0.6                              |
| Glass capillary | 2225                     | 835                          | 14                               |
fluid on the walls, the velocity contours demonstrated the swirling flow pattern in which fluid elements on the exterior streamlines circulated inside the RB convection chamber of the capillary tube at a slower rate than those on the interior streamlines. The particle path images from flow visualizations and the predicted time development of streaklines and velocity contours obviously showed a single-roll flow pattern with a mean revolving speed of approximately 1.3 mm/s, corresponding to a 30-s period for finishing a rolling cycle. Overall, the predicted single-roll flow pattern agreed favorably with the observed images. In the capillary, the rolling motion of the fluid allowed the sample to endure annealing and extension effects, and the DNA template fell and was heated once again to achieve the PCR cycles. Both the simulated and experimental results suggest the required time of approximately 30 s to realize a single PCR cycle. Because it normally requires 55–60 cycles to complete a single PCR process, the total reaction time is predicted to be approximately 28 min to complete DNA amplification.

This study adopted the target DNA fragment, HBV 122 bp fragment, for the DNA quantification measurements with the number of initial copies of target DNA ranging from $10^1$ copies/μL to $10^5$ copies/μL. The test samples were prepared using the LightCycler-Fast Start DNA master (with Cat. No. 03 003 230 001, Roche) and SYBR Green I labeling dye in all of the runs. The quantification experiments were performed on both the commercial RT-PCR instrument and the prototype RT-cPCR machine. The
measured results were then compared with the predicted fluorescence intensity computed using the proposed models. To enhance the fluorescence signal quality for the prototype machine, an adaptive signal preprocessing approach was developed based on the lower–upper–middle (LUM) filter scheme [16]. A LUM filter algorithm is based on the comparison between lower order statistics, middle order statistics and upper order statistics to determine the signal intensity output. If optimal parameters can be chosen, the LUM algorithm is good at eliminating noise while keeping the useful information of the fluorescence image. Furthermore, the reproducibility of DNA quantification for both machines was compared using intra-assay and inter-assay methods. This comparison can verify whether the present prototype machine can attain high-quality performance that is comparable with the commercial RT-PCR instrument. Fig. 3 shows (a) a comparison of the fluorescence signal gain plot processed by applying the filtering scheme to the captured fluorescence images with the predictions from the fluorescence model, plus (b) the electrophoresis gel test results. With increasing template, the product appeared earlier to check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon) for qualitative analysis. In general, the model predictions are in favorable agreement with the measured data from the RT-cPCR prototype machine after treating the fluorescence images. The average error against different initial DNA copies is less than 8%, indicating that adaptive signal processing is an effective approach to eliminate noise and achieve fidelity for accurate DNA quantification. For the electrophoresis gel experiments, lane 1, 100-bp ladder, the initial DNA copy numbers from $10^5$, $10^4$, $10^3$, $10^2$, $10^1$ to 0 were amplified by the RT-cPCR platform (lanes 2–7) with a total reaction time of approximately 28 min, which is consistent with the simulated results. This research established an effective simulation model applicable to predict the suitable temperature distribution and flow pattern in the tube for determining the feasibility of the PCR.
reaction procedure, as well as the required time for accomplishing a PCR revolving cycle.

Fig. 4 shows the influence of the adaptive signal processing on fluorescence images from 14 to 23 min at initial DNA copies of \(10^5\) copies/µL to resolve DNA quantification. This study compared the images of raw data with the processed images and noticed the removal of fluorescence signal noise by implementing the adaptive signal processing. The reproducibility of the RT-cPCR machine is primarily affected by the accuracy of the fluorescence detection system. The coefficient of variation (CV) with the percentage unit is commonly adopted as an index and is defined as follows [14]:

\[
CV = \left( \frac{\log_{10}(\sum C_M) - \log_{10}(C_S)}{\log_{10}(C_S)} \right) \times 100\%
\]  

(5)

where \(C_s\) means the specified initial DNA copies, \(C_M\) represents the measured initial DNA copies, and \(n\) denoted the different experimental data on different days. The CV value stands for the discrepancy between the average of several quantification results and the number of specified DNA copies, whereas a relatively low CV value indicates high reproducibility of the system with all induced measurement errors minimized. Fig. 5 shows the CV values tested by using different initial DNA copies from the present prototype machine and the commercial RT-PCR instrument. The values of correlation coefficient \(R^2\) indicate the accuracy of the measured results. When the \(R^2\) values are close to 1, the accuracy approaches perfection. The values can be determined by:

\[
R^2 = \frac{n(\sum C_M) - (\sum C_i)(\sum C_M)}{\left[n(\sum C_i^2) - (\sum C_i)^2\right] \times \left[n(\sum C_M^2) - (\sum C_M)^2\right]}
\]  

(6)

The \(R^2\) values of the commercial RT-PCR instrument and this RT-cPCR platform were 0.993 and 0.988, respectively. The reproducibility of the prototype machine was significantly enhanced by implementing the filtering scheme to the captured images for reaching similar CV values, compared to those obtained from the commercial RT-PCR instrument. This study demonstrates that the developed adaptive image-processing scheme can adequately filter the captured fluorescence images for achieving good reproducibility of the proposed low-cost CCD-based fluorescence detection system in the DNA quantification process. Table 3 presents the advantages of the proposed prototype machine over the conventional RT-PCR instrument. The proposed RT-cPCR prototype machine basically adopts a simple design concept by employing single temperature control in the heating system and a capillary as the reagent holder with a weight of less than 1 kg, and the reaction time of the prototype RT-cPCR platform can be retained to less than 30 min. The estimated price of the RT-cPCR prototype machine can be reduced to US$1000 for easier access to medical applications in less-developed countries.

### Table 3

|                          | Traditional RT-PCR machine | RT-cPCR machine |
|--------------------------|----------------------------|-----------------|
| Heating                  | Three temperature cycling  | Single temperature control |
| Reagent holder           | PVC tube (Eppendorf)       | Capillary       |
| Weight                   | More than 5 kg            | Less than 1 kg  |
| Reaction time            | More than 90 min          | Less than 30 min|
| Cost                     | $80000–$30,000            | Less than $1000 |

5. Conclusion

This study investigated the real-time cPCR processes for two different RT-PCR machines by performing numerical computations and experimental measurements. The major results are summarized as follows:

(1) This study performed an assessment of accuracy between the RT-cPCR prototype machine with the CCD-based fluorometer and the conventional RT-PCR instrument to determine the initial copies of HBV 122 bp DNA in the PCR mix. The measured results showed that both machines demonstrate similar accuracy in determining the initial copies of target DNA in the PCR mix for the number of copies reduced to \(10^4\) copies/µL in 28 min of reaction time.

(2) The CFD simulations and flow visualizations clearly revealed a single-roll flow pattern with a 30-s period for attaining a single PCR cycle. A CFD-based simulation model was established successfully to predict the appropriate temperature distribution and flow pattern in the capillary for determining the feasibility of the PCR reaction procedure and the required time for accomplishing a PCR revolving cycle.

(3) An adaptive image-processing algorithm based on the LUM filter was developed and integrated into the cPCR prototype machine. The test results showed a substantial reduction of CCD detector noise after the filtering treatment for the captured images of excited fluorescence from the PCR mix.

(4) The advantages of the RT-cPCR prototype machine over the commercial RT-PCR instrument include low cost, light weight, and short reaction time. Therefore, a portable DNA testing device is possible for personal detection.
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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.snb.2013.04.003.

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Biographies

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Pei-Jer Chen received PhD degree from the University of Pennsylvania in 1987. Afterwards, he joined the Graduate Institute of Clinical Medicine of National Taiwan University as a professor. His main research areas include animal models of hepatitis B and D infection and replication, liver tumor related gene mutation, development and clinical trials of novel treatments for hepatitis B and C and New strategies for liver tumor management, including combination therapy, chemo-, immune, and gene therapy.

An-Shik Yang received his Bachelor of Science (1982) and Master of Science (1984) degrees from the National Tsing Hua University in Taiwan, and PhD (1991) degree from the Pennsylvania State University in USA. He joined the Department of Energy and Refrigerating Air-Conditioning Engineering at National Taipei University of Technology (NTUT) as an associate professor in fall 2007, and was promoted to full professor in spring 2010. Dr. Yang is an Associate Fellow of American Institute of Aeronautics and Astronautics (AIAA). His research interest is in the areas of micro-fluidic design, multiphase fluid dynamics, and heat transfer.