Nucleic Acids Research, 2005, Vol. 33, No. 18 ***e156***
doi:10.1093/nar/gni157

Miniature RT–PCR system for diagnosis of RNA-based viruses

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Received March 31, 2005; Revised July 23, 2005; Accepted September 22, 2005

ABSTRACT

This paper presents an innovative portable chip-based RT–PCR system for amplification of specific nucleic acid and detection of RNA-based viruses. The miniature RT–PCR chip is fabricated using MEMS (Micro-electro-mechanical-system) techniques, and comprises a micro temperature control module and a PDMS (polydimethylsiloxane)-based microfluidic control module. The heating and sensing elements of temperature control module are both made of platinum and are located within the reaction chambers in order to generate a rapid and uniform thermal cycling. The microfluidic control module is capable of automating testing process with minimum human intervention. In this paper, the proposed miniature RT–PCR system is used to amplify and detect two RNA-based viruses, namely dengue virus type-2 and enterovirus 71 (EV 71). The experimental data confirm the ability of the system to perform a two-step RT–PCR process. The developed miniature system provides a crucial tool for the diagnosis of RNA-based viruses.

INTRODUCTION

The past decade has witnessed many significant advances in molecular biology and nucleic acid analysis technology, particularly in the genomics and diagnosis fields. PCR and RT–PCR are essentially primer extension reactions for amplifying specific gene fragments. PCR related techniques are crucial for the detection, quantification and sequencing of DNA molecules. Recently, the continuous development of MEMS (Micro-electro-mechanical-system) technology and microfabrication techniques have facilitated many advances in the execution of chemical and biochemical reactions on a microchip. The concept of performing chemical and biochemical analyzes using a micro total analysis system (μ-TAS), in which pretreatment, transportation, reaction, separation and detection of samples are integrated on a single microchip, can now be tested (1–3). Micromachined analytical devices and systems have a number of significant advantages, including high throughputs, disposability, low consumption of reagents and samples, portability, low power consumption, low cost and the potential for automation and integration.

Previous researchers have employed MEMS fabrication techniques to develop a range of micro systems for DNA amplification (4). These devices have demonstrated considerable potential. For example, micro-PCR chips have been reported comprising silicon substrates with micro heaters and temperature sensors (5,6). Microfabricated silicon-based micro-PCR chip was reported by Northrup *et al*. (5). Boron-doped polysilicon resistors were located outside the PCR chamber as heating elements. Wooley *et al*. successfully integrated PCR and capillary electrophoresis system by bonding two pieces of Si wafer (6). It was shown that β-globin has been successfully amplified and separated by a cycling rate of 10°C/s heating and 2.5°C/s cooling. Although the low thermal inertia of these devices resulted in a rapid DNA amplification process, the temperature sensors and heaters were generally located outside the reaction chamber, and hence it was difficult to obtain an accurate measurement of the temperature distribution within the PCR chamber. Following, the use of platinum (Pt) resistors as sensing and heating elements in microchips fabricated on glass substrates has also been reported (7,8). However, the developed micro devices suffered problems of temperature non-uniformity, low thermal response and high power consumption. Various other MEMS-based PCR chips are also reported in the literature (9–11). Recently, miniature devices for RNA-based analysis have been reported by Obeid *et al*. (12). In their study, a monolithic micro device integrating continuous-flow RT and PCR processes

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was adapted in their system. However, several problems were reported, most notably the requirement for a constant time ratio of 4:4:9 when performing the thermal steps in DNA amplification, the fixed numbers of cycles selection, the need for exterior heaters, sensors and pumps and a high power consumption.

Microfluidic control systems show potential in a diverse array of biological applications. These micro pumps and valves make the control of fluids inside microchannels feasible. For example, Fu *et al*. (13) reports an integrated microfabricated cell sorter consisting of peristaltic pumps, dampers, switch valves and input/output wells to perform automatic cell sorting. An elastic polymer fabrication process coupled with external compressed air source to cause thin film distortion for micro-pumps and micro-valves was also reported (14).

The present study develops a miniature two-step RT–PCR system for amplifying RNA-based molecules such as RNA virus or mRNA. The proposed miniature system comprises two major components, namely a micro temperature control module and a microfluidic control module. In the micro temperature control system, the heating and temperature sensing elements are fabricated of the same material Pt and are located within the reaction chambers (Figure 1b) to ensure a uniform temperature distribution, a low power consumption and high heating and temperature sensing rates. Meanwhile, the biocompatible PDMS (polydimethylsiloxane) microfluidic control module utilizes the movement of individual membranes to realize micro pumps and micro valves with which to control the fluid flow within the device. As shown in Figure 1a, the microfluidic device incorporates two reservoirs to store the RT and PCR reagents, respectively, before the corresponding reaction processes. Having placed the RNA templates in the RT reaction chamber, the RT–PCR chip performs two reaction processes: (i) a RT reaction to synthesize the complementary DNA (cDNA) from the RNA molecules and (ii) a PCR to further amplify the specified region of the synthesized cDNA template in order to identify the target virus. In the developed device, the micro pumps deliver the RT and PCR reagents and the synthesized cDNA templates through the microchannels peristaltically, while the micro valves isolate the RT and PCR. To our best of the current authors' knowledge, the proposed design represents the first attempt to utilize on-chip micro pumps/valves and two identical micro temperature control elements to conduct an automatic detection of RNA viruses. The developed device provides researchers with the ability to control the thermal cycles precisely and to arbitrarily adjust the time ratios and sample volumes. As such, the proposed miniature RT–PCR system provides an innovative tool for clinical diagnosis applications.

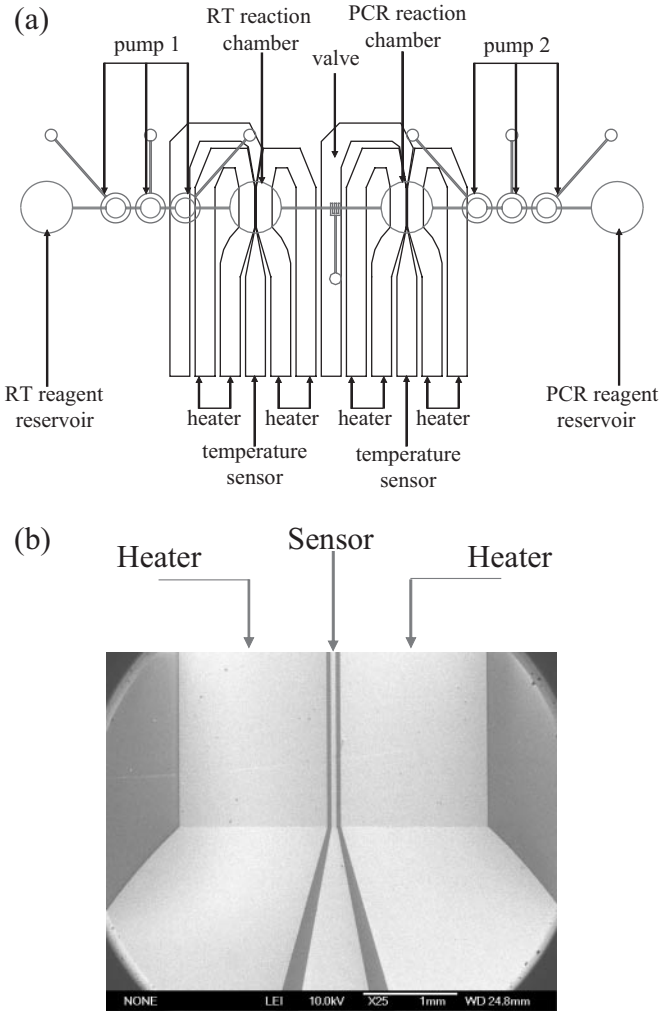

Figure 1. (**a**) Schematic diagram of two-step micro RT–PCR chip. Micro pumps and valves are integrated with the chip to control bio-sample transportation. (**b**) SEM image of on-chip temperature control system. The two micro heaters are located within the reaction chamber in order to improve the uniformity of the temperature field.

MATERIALS AND METHODS

Micro temperature control module

The micro temperature control module comprises three major components, namely micro heaters, micro temperature sensors (Figure 1b), and an application specific integrated circuit (ASIC) controller (16). The micro heaters are used to heat the sample, while the temperature sensors are used to detect the temperature distributions inside the RT and PCR chambers. Finally, the ASIC controller is used to adjust the heater output power in order to establish and maintain the required temperature field inside the reaction chambers. In the present study, the heating and sensing elements are in the form of Pt resistors. Pt is an ideal material for these elements since its resistance varies linearly with temperature. Furthermore, the use of the same material for both the heaters and the sensors simplifies the fabrication process greatly. The controller implements a neural network (NN) control scheme to prevent a non-linear heating effect caused by variations in the heating element resistance as the temperature increases. The experimental data confirm that the developed micro temperature control system provides a stable temperature control and consumes minimum power.

Microfluidic control module

The microfluidic control module comprises a micro valve, two micro pumps, microchannels, two reaction chambers and two reagent storage chambers (Figure 1a). A PDMS casting

process is employed to provide a straightforward means of fabricating these components using an SU-8 mold (16). The microfluidic control module was treated with oxygen plasma to coagulate the siloxane and bond with the glass-based temperature control module. Cavities with well-defined volumes ~30 µl are formed using a mold of thick-film photoresist (SU-8, MicroChem Corp., USA) and are used as chambers to store the RT and PCR reagents. Microchannels connected to these chambers transport the RT and PCR reagents to their respective reaction chambers and carry the sample through the miniature RT–PCR system. During the two-step RT–PCR process, the reagent and sample flow is controlled automatically by means of two micro pumps and a micro valve. The micro pumps each comprises three individual PDMS membranes, and are connected to electromagnetic valves which operate under the control of a microprocessor (17). Varying the frequency of the electromagnetic valve, and applying a sequential control to the individual membranes, establishes a peristaltic effect, which causes the reagents and sample to be driven through the microfluidic device. The active micro valve also incorporates three PDMS membranes. This valve is designed to block the flow in the microchannel between the two reaction chambers, thereby isolating the RT and PCR. As in the case of the two micro pumps, the micro valve is activated by external compressed air supplied under the control of the microprocessor.

Control system

The proposed miniature RT–PCR system is controlled by a predictive control scheme (18) based on NN theory (19). A schematic diagram of the ASIC control system is shown in Figure 2. In the control system, an ATMEGA8535 microcontroller (ATMEL Corp.) acts as a 10-bit analog to digital converter (ADC) and an 8-bit pulse-width-modulation (PWM) module controls the micro sensing and heating elements. The function of the ADC is to convert the signal received from the detection circuit using an 8 kHz sampling rate. Meanwhile, the PWM module heats the RNA/DNA samples at a heating rate which is governed by the specified duty cycle value. The output of the PWM is connected to a MOSFET (metal-oxide-semiconductor field-effect transistor), which acts as a switch to control the flow of current through the heating resistors, thereby providing a means to control the heating efficiency of the micro thermocycler. As described above, the microcontroller provides digital signals to regulate electromagnetic valves (EMV, SMC Inc., S070M-5BG-32, Japan), which in turn cause the thin PDMS membranes to deflect pneumatically under the influence of compressed air.

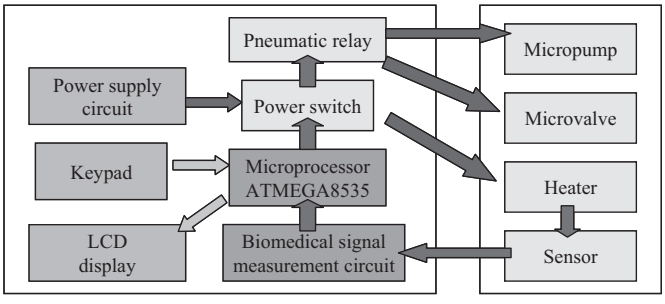

Figure 2. Schematic diagram of ASIC control system comprising controller circuit, electromagnetic valves and micro RT–PCR chip. Note that the control system is operated by a commercially available 9 V battery.

Importantly, the complete control module, comprising the microcontroller, the peripheral control circuits, the electromagnetic valves and an air compressor (Ever Motor Electronic Co., P05, Japan), is assembled as a portable system measuring just 12 cm × 21 cm × 8.5 cm and can be powered by a commercially available 9 V battery.

The control system uses a microprocessor to perform a feedback control. With signals from the temperature sensor, a PWM control scheme could be used to control the micro heaters. A backward linear prediction scheme (16) programmed in the microprocessor could reduce the noise and perform appropriate duty cycles of the PWM module. The predictor of order is 32, and the delay time between two taps to obtain a stable condition is 300 µs. In the stable condition, the temperature variance on the thermocycler is <0.2°C.

Virus strain

In the present study, the developed miniature RT–PCR system was utilized to perform the rapid identification of two RNA-based viruses, namely dengue virus type-2 and enterovirus 71 (EV 71). These particular viruses were chosen since they both caused the epidemics which have caused high fatalities throughout the Asia-Pacific region in recent years (20,21). Table 1 lists the primer sequences of these genes and also presents the appropriate annealing temperature for each PCR procedure. The PCR products are reasonably spaced, and each primer set has a very high specificity, which facilitates a high diagnostic accuracy. Viral RNA was extracted from dengue virus type-2 (New Guinea C strain) infected mosquito *Aedes pseudoscutellaris* (C6/36) cells (22). AD4 anti-sense cDNA primer commencing from the 3′ end of the RNA template was used to initiate cDNA synthesis. The primer set (AD3-AD4) specifically amplified a 419 bp fragment of the dengue virus NS1 region since this fragment has been widely used for the detection of dengue viruses (15). EV 71 was also tested using the proposed miniature RT–PCR system. EV 71 is a neurotropic virus which has caused morbidity and mortality in children worldwide in recent years. The EV 71 virus was obtained from the spinal cord fluid of an 8-year-old child autopsy specimen who died during the 1998 EV 71 outbreak in Taiwan. The 331 bp fragment of the EV 71

Table 1. Primers of RNA-based dengue-2 virus and EV 71

Strain	Gene/annealing temperature	Primer (5′ → 3′)
Dengue-2 Virus	AD3-AD4 (419 bp)/52°C	F: CTG ATT TCC ATC CCG TA
		R: UAT ATG GGT TAT TGG GA
EV 71	EV2449–EV2780 (331 bp)/53°C	F: GTG GCA GAT GTG ATT GAG AG
		R: GTT ATG TCT ATG TCC CAG TT

The PCR products are reasonably spaced. Each primer set has a very high specificity, ensuring a high diagnostic accuracy (15,20–22).

VP1 region was utilized for PCR detection of the virus using the primer set EV2449–EV2780.

RT and PCR reagents

In the current experiments, the synthesis of cDNA was carried out in the RT reaction chamber (Figure 1a) with 10 μl of reaction mixture containing 1 μg of RNA, 0.5 μl of 10 mM dNTP, 2 μl of 5× reaction buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$], 0.5 μl of 10 μM primer, 1 μl of 0.1 M DTT and 0.5 μl of moloney murine leukemia virus RT (200 U/ml, BRL, USA) at 43°C for 30 min and at 65°C for 10 min to prevent non-specific binding before the *Taq* DNA polymerase addition. Following the RT of the RNA template, the microfluidic control module automatically transported 2 μl of the synthesized cDNA to the PCR chamber to further amplify the specific region. The PCR mixture contained: 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10× PCR buffer [15 mM $MgCl_2$, 500 nM KCl, 1.5 M and Tris–HCl (pH 8.7)], 200 nM of the appropriate paired primers and 1 U of *Taq* DNA polymerase (Amersham, UK). The PCR was conducted at 94°C for 10 s, 52°C for 20 s and 72°C for 20 s for 25 cycles, followed by an additional 72°C 1 min for elongation in the final cycle. Finally, the RT–PCR product was analyzed by gel electrophoresis in a 1.5% agarose gel, stained by ethidium bromide (Sigma Chemical, USA) and then visualized under UV (ultra-violet) light.

RT–PCR

Thanks to the on-chip microfluidic control module, the RT–PCR operation processes can be performed automatically. RNA reagents/templates were first loaded in the open reaction chambers by using pipettes. To form the microfluidic control module, the proposed design requires an upper PDMS plate to be bonded on top of the micro temperature control chip. PDMS is known to be an excellent biocompatible material for biological applications. Moreover, the cheap and easy PDMS casting fabrication allows disposal of the reaction chamber preventing cross contamination. After loading the reagents/templates in the corresponding reservoirs and setting the thermal cycling condition, amplification process could be achieved within 1 h. The micro RT–PCR operation processes are described as follows:

Step 1. Turn on the micro system.
Step 2. Clean the microchip with 70% alcohol.
Step 3. Bond the PDMS microfluidic control module.
Step 4. Load the RT reagent, PCR reagent and RNA template in RT reagent reservoir, PCR reagent reservoir and the RT reaction chamber, respectively (Figure 1a).
Step 5. Pump 10 μl RT reagent from the RT reagent reservoir to the RT reaction chamber.
Step 6. Wait for 30 min for cDNA synthesis (RT reaction).
Step 7. Pump 2 μl cDNA from RT reaction chamber to the PCR chamber.
Step 8. Pump 8 μl PCR reagent from the PCR reagent reservoir to the PCR chamber.
Step 9. Wait for 15 min for PCR amplification.
Step 10. Use a pipette to extract the remaining cDNA and RT–PCR products.
Step 11. Perform slab-gel electrophoresis.
Step 12. Clean the microchip with 70% alcohol.

RESULTS AND DISCUSSION

In the developed miniature RT–PCR system, the heating and cooling rates, temperature uniformity, fluid pumping rate and power consumption are of particular concern. The resistances of the sensors and heaters were designed to be 350 and 30 ohm, respectively. Due to the low thermal inertia of the micro system, the heating and cooling rates were found to be 20 and 10°C/s, respectively, which are much faster than the heating and cooling rates of a conventional PCR machine (Thermo Hybaid Sprint, UK) 3 and 2°C/s, respectively. The temperature variation at each set point was found to be less than ±0.2°C. The on-chip micro temperature sensor provides the means to detect the temperature inside the reaction chamber on a real-time basis. For repeated PCR thermal cycles, the rapid heating and sensing rates of the developed miniature RT–PCR system yield a reduction of 60% of the reaction time compared to the conventional PCR machine, i.e. 70 s as opposed to 176 s. It is noteworthy to know that within the 95°C for 20 s, 52°C for 20 s, 72°C for 20 s thermal cycle, the micro system saves >100 s for temperature variation.

Figure 3a illustrates the linear relationship between the sensor resistance and the measured temperature. It can be

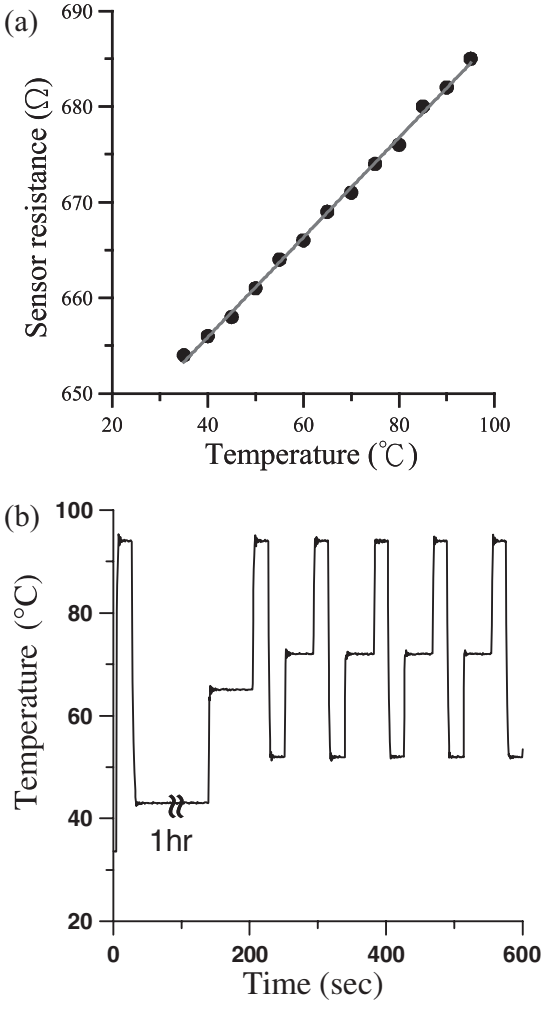

Figure 3. (**a**) TCR (temperature coefficient resistance) of micro temperature sensor. (**b**) Typical thermal cycle generated by miniature RT–PCR chip.

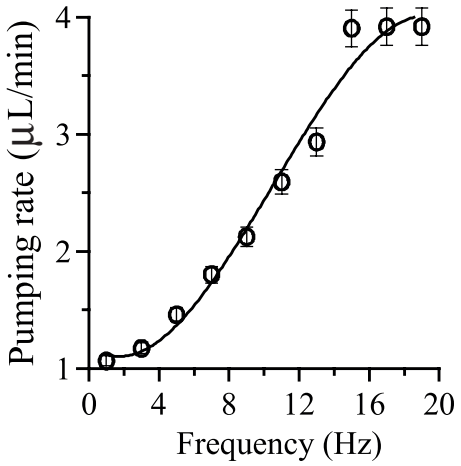

Figure 4. Relationship between driving frequency and pumping rate.

shown that the temperature coefficient of resistance is 0.0032/°C. Meanwhile, Figure 3b shows a typical RT–PCR thermal cycle generated by the RT–PCR chip. Clearly, we demonstrated that the proposed system is capable of providing a rapid and precise temperature control.

The on-chip pneumatic pumps and valves enable the miniature RT–PCR chip to perform the RT and PCR two-step process is automatically using the ASIC controller. As shown in Figure 4, the pumping rate can be modulated by changing the driving frequency of the electromagnetic valves. It is clear that increasing the activation frequency increases the pumping rate of the peristaltic micro pumps significantly. The pumping rate attains a maximum value of 4 μl/min at a driving frequency of 15 Hz. The fluid flow could be successfully interrupted when the micro pneumatic valve is closed. The experimental data indicate that a pressure of 10 psi is sufficient to cause the micro valve to shut off the flow of a fluid moving at ~30 μl/min. Therefore, the micro valve is capable of isolating the two stages of the RT–PCR process completely. The average power consumption during the RT and PCR was found to be 1.18 W (compared to 250 W for a conventional PCR machine). Therefore, a 9 V Ni-MH rechargeable battery is sufficient to operate the system for >15 h.

As shown in Figure 5, successful amplifications of the cDNA synthesis from the 10 723 base dengue virus type-2 template and the 7500 base EV 71 template were obtained using the developed miniature RT–PCR system. In Figure 5a, the fluorescence signals in the first lane (L) represent the 100 bp ladder DNA markers (Yeastern Biotech Corp., Taiwan). Meanwhile, the second lane signals (C1) correspond to the 419 bp RT–PCR products of the dengue virus type-2 obtained using the developed system. The 419 bp sequence contains two diagnostic restriction endonuclease cutting sites, namely HindIII and BstII. These sites were used in this study to verify that the miniature RT–PCR chip was capable of successfully amplifying the 419 bp fragment. The experimental results shown in the third and final lanes (Hind and Bst) correspond to the digested DNA fragments of 193/226 and 187/232 bp, respectively. An observation of the results in Figure 5b confirms that the 331 bp sequence of EV 71 can also be successfully amplified by the miniature RT–PCR chip (C2). Note that

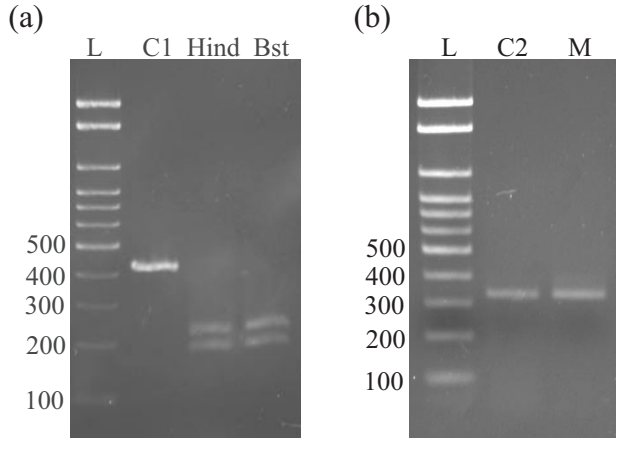

Figure 5. Micro RT–PCR products of (**a**) dengue virus type-2 and (**b**) EV 71 in a 1.5% agarose gel stained with ethidium bromide. Lane L: DNA marker (100 bp ladder). Lane C1: dengue virus type-2 RT to synthesize cDNA. PCR primers AD3-AD4 were used for PCR amplification. Corresponding fragment size 419 bp. Lane Hind: HindIII enzyme digests 419 bp into 193 and 226 bp. Lane Bst: BstII enzyme digests 419 bp into 187 and 232 bp. Lane C2: EV 71 RT to synthesize cDNA. PCR primers EV2449–EV2780 used for PCR amplification. Corresponding fragment size 331 bp. Lane M: conventional machine RT–PCR product for EV 71.

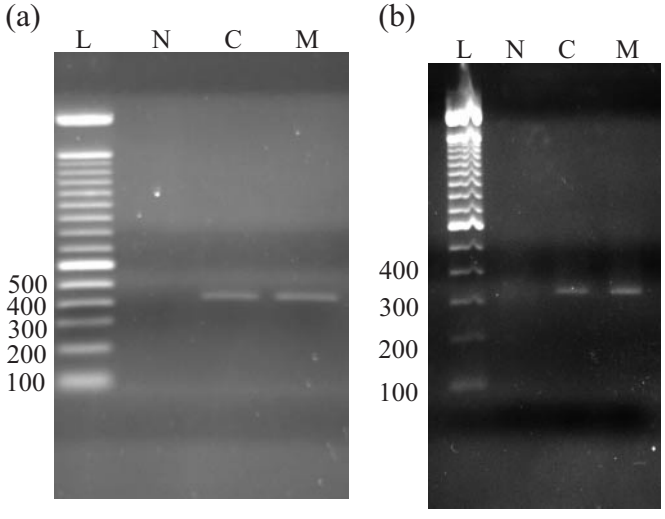

Figure 6. Slab-gel electropherograms for: (**a**) dengue virus type-2 and (**b**) EV 71. Note: (L): DNA marker, (N): negative control, (C): micro RT–PCR product and (M): traditional PCR machine positive control.

the signals are comparable with the signal obtained using the conventional machine (M).

A cheap and easily fabricated microfluidic control module enables disposability to prevent cross contamination. Figure 6 illustrates results about negative control of the system. The first lane (L) is the 100 bp DNA marker. The second lane (N) is the negative control generated by the microsystem. In this approach, RT and 30 cycles of amplification were performed without the RNA template. It is clearly seen that no contamination occurs in the microsystem. The third lane (C) is the signals amplified by the micro RT–PCR system. The last lane (M) is the positive control generated by the traditional PCR machine. Figure 6a and b are denoted for dengue virus and EV 71, respectively. The experimental data reveal that the PCR

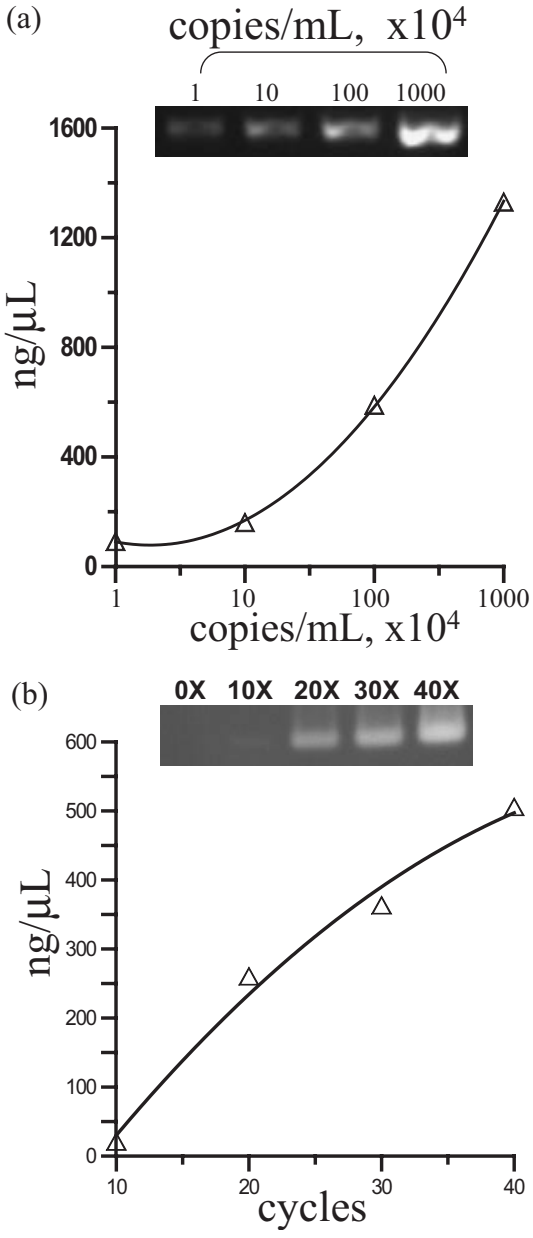

Figure 7. Effects of (**a**) input concentration and (**b**) number of cycles on amount of amplification product for developed miniature RT–PCR chip.

requires just 15 min when programmed for 25 thermal cycles. Moreover, the complete RT and PCR can be carried out within 1 h. Furthermore, the proposed system can perform the two-step process automatically by the on-chip microfluidic control module with minimum human intervention. It demonstrates that the performance of the developed miniature RT–PCR system is superior to that of the conventional PCR machine in a rapid and automatic way.

This study also explored the correlation between the amount of amplified product and the concentration of the input DNA template. In this approach, the dengue virus type-2 cDNA template was synthesized by the developed micro system and then the optical density (OD) value was detected to determine the concentration of cDNA. After serial dilution, samples with concentrations ranging from 1×10^4 to 1000×10^4 copies/ml were amplified for a constant 20 thermal cycles. The corresponding slab-gel electropherograms and a plot of the products as a function of the input concentration are presented in Figure 7a. It can be seen that the PCR products increase with increasing input concentration of the template. The relationship between the number of thermal cycles and the amount of amplified product was also investigated. The DNA products were amplified at 0, 10, 20, 30 and 40 cycles, respectively, with a constant initial cDNA template concentration of 4.25×10^5 copies/ml. As shown in Figure 7b, the amount of amplified DNA product increases with the numbers of cycles.

Experimental data indicate that the micro system has a possibility of 30% to successfully amplify the mRNA templates with a minimum initial concentration of 0.6 pg/μl. However, the micro system can reach a high reproducibility up to 96% if the initial template concentration is >6 pg/μl. The statistic results are generated after testing >100 mRNA templates. Therefore, the micro system detection limit is concluded to be 6 pg/μl.

CONCLUSION

This study has demonstrated a portable RT–PCR system. Two types of infectious RNA viruses, namely dengue virus type-2 and EV 71, have been successfully amplified and detected in an automatic two-step RT–PCR process. The novel combination of the micro temperature control module and the microfluidic control module renders the proposed miniature RT–PCR system an idea candidate for the rapid diagnosis of RNA-based viruses. The miniature RT–PCR chip not only overcomes the limitations of temperature field non-uniformity and high power consumption reported in previous studies, but also utilizes on-chip micro pumps and valves to automate the diagnosis process. Moreover, the RT–PCR device is a portable battery-operated system. The deliberate choice of PDMS and glass as biocompatible substrate materials ensures a cheap, straightforward and reliable fabrication process, and enables the current device to be readily integrated with other microfluidic devices such as capillary electrophoresis or pre-PCR process chips. The effects of the input concentration and number of cycles on the amplification products have been explored. The miniature RT–PCR system presented in this study provides an invaluable tool for the rapid diagnosis of RNA-based viruses.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support provided to this study by the National Science Council of Taiwan (NSC 92-3112-B-006-004) and the MOE Program for Promoting Academic Excellence of Universities (EX-91-E-FA09-5-4). The authors would also like to express their gratitude to the Center for Micro/Nano Technology Research, National Cheng Kung University, Tainan, Taiwan for permitting access to major fabrication equipment. Finally, the authors would like to thank Mr. Ying-Ray Lee for their assistance in preparing clinical samples. Funding to pay the Open Access publication charges for this article was provided by National Science Council of Taiwan.

Conflict of interest statement. None declared.

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