Advances in Biological Sciences Research (ABSR), volume 4
2nd International Conference on Biomedical and Biological Engineering 2017 (BBE 2017)

Miniaturized System with a Facile Isothermal Amplification Microfluidic Chip for Rapid Detection of Zika Viruses

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Keywords: Microfluidic chips, Loop-mediated is other mal amplification (LAMP), Formatting, Miniaturized System.

Abstract. A real-time fluorescence detection biomedical miniaturized system with a disposable microfluidic chip was developed for loop-mediated isothermal amplification (LAMP) reactions. The miniaturized system was developed consisting of a mini heating plate, a temperature sensor, a temperature controller and an adjustable mechanical stage. Based on experimental result, the temperature of the heating plate is evenly distributed, and the error of the temperature is less than 1.3%. The microfluidic chip was designed and fabricated by MEMS technology and thin-casting method. The volume of the LAMP reaction was changed from 25 μL to 2 μL. The volume of the LAMP reaction was optimized. The nucleic acid of Zika viruses was amplified and detected in real-time mode with our miniatureized system by using above microfluidic chip. From the experiment results, the detection range was from 100 copies to 10^6 copies. This biomedical system has the potential for point-of-care diagnostics (POCT) with the many advantages, such as not high cost, short analysis and detection time, not much reagent and sample consumption and so on.

Introduction

Recently isothermal amplification methods, not requiring any thermal cycling, have been reported and application [1, 2]. When compared to Polymerase chain reaction (PCR) methods, loop-mediated isothermal amplification (LAMP) method is a novel gene amplification method under isothermal conditions (60-65 ℃) without the need to accurately toggle the reaction mixture between different temperatures. With the development of microfluidics technology, many efforts have been focused on lab-on-a-chip or micrototal analysis systems. Lab-on-a-chip or micrototal analysis systems have some advantages like high throughput, short analysis time, small volumes and high sensitivities [3, 4], which can be capable of ready measurement of disease biomarkers in physiological fluids [5, 6].

Combination of LAMP and microfluidic technology will miniaturize the LAMP detection system and facilitate the realization of point-of-care (POC) pathogen detection. Uddin *et. al.* [7] reported a portable automatic end-point detection system for LAMP reactions on Microfluidic Compact Disk Platform. This system allows a rapid and automatic endpoint detection which could lead to the development of a point-of-care diagnosis device for foodborne pathogens detection in a resource-limited environment.Fang *et. al.* [8] reported a LAMP integrated microfluidic chip with optical fibers for detection of pathogens using absorbance detection, which was an octopus-like

multiplex microfluidic loop-mediated isothermal amplification assay. However the fabrication process was complicated.

In this study, we develop a miniaturized system with a facile isothermal amplification microfluidic chip for rapid detection of Zika viruses (ZIKV). A dramatic increasing ZIKV infection in the Americas was reported in 2015. The World Health Organization (WHO) called for fast-tracked development of Zika virus diagnostics [9]. Confronting with the Zika virus epidemics, the development of rapid, scalable diagnostic devices and method is needed. Thus we develop a biomedical system with a microfluidic chip for real-time fluorescence detection of various viruses, which can be carried out in multiple point-of-care (POC) molecular diagnostic applications. ZIKV-LAMP reaction on our microfluidic chip with high sensitivity, specificity, and rapidity, which potentially enable LAMP assays to be highly portable for on-site analysis.

Experimental Section

Materials and Reagents

Sylgard 184 (PDMS) was purchased from Dow Corning (Midland, MI, USA). Liquid paraffin and low temperature paraffin were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. DNA Amplification Kit and Fluorescent Detection Reagent was obtained from Eiken China Co., Ltd.

According to the bioinformatic analysis, a conservative fragment of the ZIKV Envelop gene were synthesized and inserted in a pUC plasmid to establish the amplification target. Among the fragment, six primers of LAMP assay were designed according to the gene sequences of the reference virus using a PrimerExplorer V4 program. Details of the final primers are shown in Supplementary Table 1. All the primers were synthesized by Sunbiotech (Beijing, China).

Microchip Fabrication

Microchips were designed and fabricated with several microcells by using PDMS. Silicon positive moulds were prepared by MEMS technologies. After the silicon positive moulds were accomplished, PDMS layers with different structures were fabricated by using the thin-casting method. After oxygen plasma treatment, the glass and the PDMS layer were aligned and bonded together. The microfluidic chips were finished and ready to be used, shown in Fig 1.

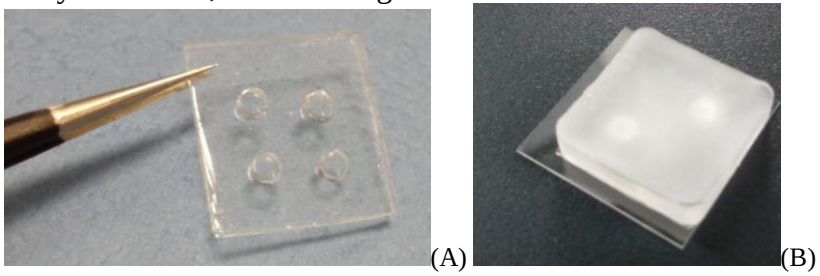

(A) (B)

Figure 1. Photograph of the microfluidic chip with four microcells for LAMP reaction and with the low temperature paraffin as a cover.

ZIKV-LAMP Reaction

Routine LAMP assay was carried out with a Isothermal Master Mix (Optigene, the United Kingdom) in a final volume of 25 ul containing 0.125 nM each of the outer primers F3 and B3, 1.0 nM each of the primers FIP and BIP, and 0.5 nM each of the

primers LF and LB. The reaction mixture containing distilled water was used as negative controls.

Miniaturized System

The miniaturized system was developed consisting of a mini heating plate, a Pt temperature sensor, a PID temperature controller, a adjustable mechanical stage and a portable confocal fluorescent detector (ESE LOG ESML 10-MB-3007, Germany) shown in Fig. 2. This system with a microfluidic chip was used to implement the ZIKV-LAMP reaction and the real-time fluorescent detection of the amplification products. The results of LAMP procedure was output from the computer.

Results and Discussion

Miniaturized System Setup

The schematic diagram of the miniaturized system is shown in Fig 1. The real-time fluorescence detection was implemented by using the detection mode of single measurement for one hour. The interval between measurements was set at 1s ~ 60 s.

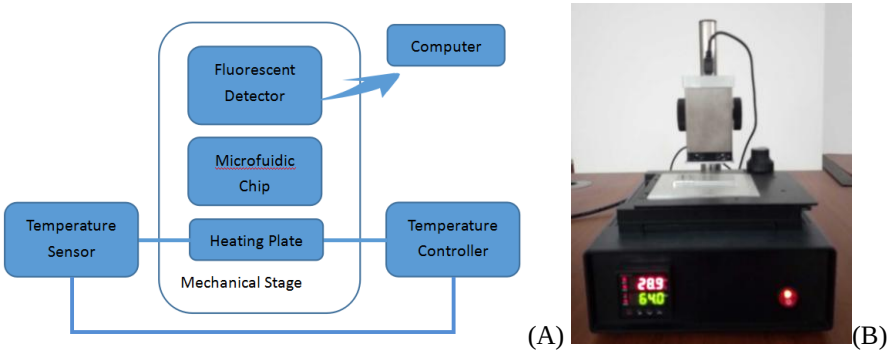


Figure 2. Schematic (A) and photograph (B) of the miniaturized system for real-time detection of ZIKV-LAMP reaction on a microfluidic chip.

Table 1 Temperature distribution at different locations at the rectangular heating plate.

Test Position	Test 1(℃)	Test 2 (℃)	Test 3 (℃)	Average Temperature (℃)
A	62.3	62.9	63.8	63
B	62.8	61.8	63.9	62.8
C	63.2	63	63.3	63.2
D*	62.6	62.8	63.4	62.92
E	62.7	62.8	62.5	62.7
F	62.1	61.4	63.3	62.3
G	61.5	61.7	61.2	61.5

*D represents the center position of the rectangular heating plate. The other six points, which are three rows and two columns, evenly distributes at the rectangular heating plate.

The temperature provided by the heating plate of the miniaturized system was carefully studied and the temperature was measured at different locations. The test results are shown in the table 1. The size of the rectangular heating plate is 30mm×70mm. Based on experimental result, the error of the temperature is less than 1.3% at different locations of the rectangular heating plate. The temperature difference varies from 0.1 to 0.8 degree Celsius. The temperature of the heating plate is evenly distributed, which can be used for heating three microfluidic chips with the size of 20mm×20mm.

Optimization of Experimental Conditions

Miniaturized system with a facile microfluidic Chip has been developed for the rapid LAMP reactions and the real-time fluorescence detection. LAMP reaction mixture was prepared in a centrifuge tube and then dropped in the microcell of the microfluidic chip. As shown in Fig 1, the size of the microfluidic chips is 20 mm ×20 mm and the diameter of the microcells is about 3 mm and the depth is 2.5mm. Therefore the maximum volume of the microcells is about 17.7 μL. 10 μL, 5 μL and 2 μL of LAMP reaction mixture were introduced into microfluidic chips, respectively, and then several microliters of liquid paraffin and low temperature paraffin were added to avoid evaporation during heating process. The amplification reactions in microchips were performed at 60 ℃ - 65 ℃ in our miniaturized system for 1 h (See Fig 2).

The microchip with the LAMP reaction mixture was placed on the mechanical stage, and the x-y axis of the moving frame in the mechanical stage was adjusted to ensure that the fluorescence detector was aligned with the microcell. Then z axis of the moving frame in the mechanical stage was adjusted to ensure to obtain moderate fluorescence intensity. After determining the optimal position, the microchip was removed. The temperature switch of the heating plate was open and the temperature of heating plate was raise to the reaction temperature by using the temperature sensor and controller. After the temperature is stable, the chip was re-placed on the mechanical stage, while the fluorescence detector was open to start real-time testing. The experimental results are shown in Fig 3A. From the experimental results, dynamic curve of 2 μl LAMP reaction is similar with that of the negative control, while dynamic curves of 5 μl and 10 μl LAMP reaction is obviously different from the negative control. 10 μl of the LAMP reaction volume has been used for the following experiments because of high FI.

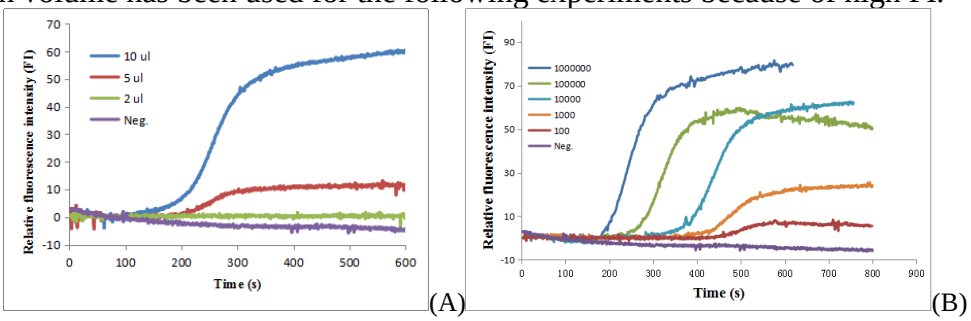


Fig 3. Dynamic curves of the real-time fluorescent detections of the amplification products of ZIKV-LAMP reactions on microfluidic chips by using our miniaturized system. Curves "10 μl", "5 μl "and "2 μl" in (A) represent the optimization results with the volume of 10 μl, 5 μl and 2 μl, respectively. Curves "1000000" - "100 " in (B) represent the optimization results of 1000000 copies - 100 copies in each ZIKV-LAMP reaction, respectively. Curve "Neg." represents the negative control.

Real-time Fluorescence Detection of ZIKV

The amplification products of LAMP reactions in microfluidic chips were directly real-time detected in our miniaturized system, which was also used to assess the method of the real-time fluorescence detection and our miniaturized system. Utilizing the above optimized condition, ZIKV-LAMP reactions were detected with serial ten-fold standard plasmids on the disposable microchips.

The dynamic cures of the real-time fluorescence detection of the ZIKV-LAMP reaction and the negative control on microfluidic chips by using our miniaturized system are shown in Fig. 3B. From Fig 3B, we found the difference between the positive ZIKV-LAMP reaction and the negative control is very various, which indicates that our real-time fluorescence detection miniaturized system with a microfluidic chip is successfully used for LAMP reaction.

Also from the amplification results with serial ten-fold standard plasmids on the disposable microchips, the peak time is increased with the increasing template concentration of standard plasmids. Moreover dynamic curves of the real-time fluorescent detections of the 10^5-10^3 template plasmid copies expressesd a dose-dependent positive amplification signals, similar with the detection results by Genie II instrument (shown in Supplementary Fig. 1). But the detection limit is lower than Genie II instrument, as the amplification signals can hardly be observed when the amplification templates are lower than 10^2 copies/ reaction.

Summary

A miniaturized system with a disposable microfluidic chip was developed for ZIKV-LAMP reaction and real-time detection. The function of the system miniaturized has been verified by the LAMP experiments, which can provide the stable temperature. The error of the temperature was less than 1.3%. Tthe volume of the reaction system is reduced from 25 microliters to 5 microliters because of using microfluidic chips. It is successfully used for IKV-LAMP reaction and real-time detection. The detection range was from 100 to 106 copies/reaction. This miniaturized system would be possible for on-site, low-cost POCT.

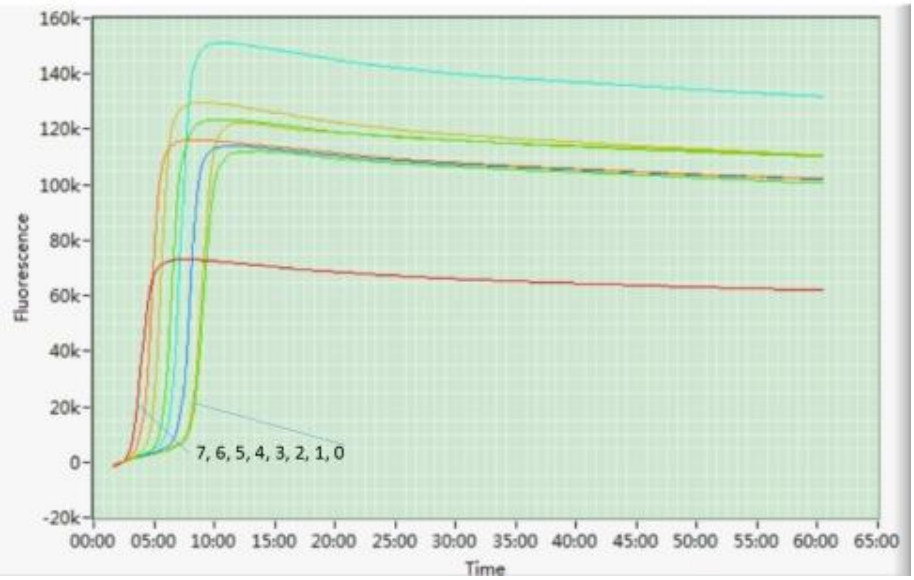
Acknowledgement

This research was financially supported by the National Science Foundation of China (61372055, 31571007, 61571420 and 8137171) and the National Key Scientific Instrument and Equipment Development Project (2012YQ090197).

Supplementary Results

Supplementary Table 1. Details of primers used for ZIKV-LAMP assay.

Primer name	Genome position	Sequence (5'-3')
F3	1495-1512	AAGCACTGGCTGGTTCAC
B3	1674-1691	TCCAGAGCTCCAGCAAGG
FIP(F1c+F2)	(1555-1574) - (1513-1533)	GTGGAGTTCCGGTGTCTGCCAAGGAGTGGTTCCACGACAT
BIP(B1c+B2)	(1599-1621) - (1644-1663)	AGAGTTCAAGGACGCACATGCCTGCTCCTTCTTGACTCCCTA
LF	(1534-1553)	CAGCGTGCCAAGGTAATGGA
LB	(1622-1641)	AAAAGGCAAACTGTCGTGGT



Supplementary Fig 1. Real time fluorescent LAMP assays of serial ten-fold standard plasmids. Digits of 7-0 indicate 10^7 to 10^0 copies/reaction as the reaction templates. 25 μL LAMP reaction mixture was added in centrifugal tubes. The amplification reactions in centrifugal tubes were carried out for 60 min using a real-time fluorescent Genie II instrument (Optigene, the United Kingdom) at 60 ℃ - 65 ℃, which was proved that the ZIKV-LAMP reaction can proceed normally.

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