Rapid DNA Amplification in Bouyancy Driven Closed Loop Microfluidic Systems

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ABSTRACT

Bouyancy driven polymerase chain reaction (PCR) is emerging as a simplified version of thermally driven bio-analysis systems. In this paper, we have designed, fabricated and modeled a simplified closed loop thermocycler (CLTC) that can perform PCR in as little as 10 mins. Robustness and generality of the CLTC was demonstrated by performing PCR amplification of single as well as multiplex systems containing 5 different human respiratory infection associated virus targets. We present a quantitative analysis of PCR yields and observe consistent performance over several orders of magnitude of initial template loading dilution. We also demonstrate the use of convective flows in a variety of closed loop systems designed to create extensional flow fields and to execute tunable thermocycling and pumping operations in a format suitable for integration into miniaturized chemical and biochemical analysis systems.

1 INTRODUCTION

Many modern genomic and molecular biology assays involve selective amplification of specific regions of interest within a larger DNA template to extremely high concentration levels, followed by extraction and purification of the amplified products for subsequent analysis. The polymerase chain reaction (PCR) is typically used to perform this amplification, and is a key component in a wide variety of assays encompassing genomic analysis and sequencing, detection of infectious disease agents (e.g., influenza, E. coli, influenza, SARS), analysis of forensic samples, and medical diagnostic tests. In each of these applications, the current inability to perform PCR amplification in a rapid and inexpensive format continues to pose significant challenges to efforts aimed at moving DNA analysis technology out of the laboratory and directly into settings where the information is most needed.

The underlying mechanics associated with PCR are fundamentally simple. A typical protocol involves first pipeting a PCR reagent cocktail (template DNA, primers, dNTPs, thermostable *Taq* polymerase enzyme, etc.) into plastic reaction tubes or multiwell plates. These tubes or plates are then inserted into a programmable thermocycling machine that repeatedly heats and cools the static reaction volume to temperatures corresponding to *denaturation* of the double-stranded target DNA (95 °C), *annealing* of primers to complimentary locations on the denatured single-stranded fragments (50 – 60 °C), and enzyme catalyzed *extension* to synthesize the complimentary strands (72 °C). Under ideal conditions, this scheme yields a doubling of dsDNA (double-stranded DNA) copies of the target region upon completion of each cycle ($Y = (1 + x)^n$; where Y is the copy yield, n is the number of cycles, and x is the mean efficiency per cycle). The total time required to complete one cycle of amplification includes the time over which the temperature of the reaction mixture must be held constant at each temperature step ('hold time'), as well as the time required to heat and cool the reaction mixture between successive steps ('ramping time'). In conventional benchtop thermocyclers, cycling times are primarily limited by the rate at which inactive hardware elements (e.g. metal blocks) can be heated and cooled, rather than the kinetics of the reaction itself. Consequently, it is not uncommon for a 30 – 40 cycle amplification reaction to require 2 to 3 hours of total time to complete, thereby limiting achievable throughput. Because this often imposes a serious bottleneck in many genomic analysis applications, the development of novel strategies to reduce the thermocycling time associated with the PCR process continues to be an active area of research.

Convective flow-based thermocycling systems offer a novel and highly effective approach toward satisfying these demanding requirements[1-5]. In addition to all the benefits of scaling down (e.g. faster heating and cooling rates, reduced reagent consumption), these devices completely eliminate the electronic control component necessary to perform dynamic temperature cycling. Though PCR microchips have achieved a significant reduction in process time compared to their macroscopic counterparts, their speed of operation is still limited by the heating and cooling rates of inactive hardware components. Convection-based thermocyclers, on the other hand, do not suffer from these constraints, thereby providing the opportunity to perform PCR at speeds limited solely by the kinetics of the reaction. These flows occur throughout nature and play a central role in a wide variety of transport processes occurring in the Earth's atmosphere and oceans, and in industrial processes involving chemical reactions, heat transfer, and crystal growth[6, 7]

Bouyancy driven convection can be induced in several formats, either by applying a temperature gradient parallel or orthogonal to gravity. The vertical gradient case is often studied in the context of toroidal flow loops (thermosyphons) in which the upper half of the loop is cooled at a constant temperature while the lower half is heated with a constant flux. Here, the onset of convective motion generally occurs in the vicinity of $1 < Ra_{crit} < 100$, depending on the geometry and heat transfer characteristics of the loop. Flow direction is randomly determined by the overall dominating forces for unique conditions. Fluid flow in such toroidal geometries occurs solely due to the Rayleigh Bernard convective forces[8]. On the other hand,

a controlled unidirectional fluid motion can be induced by applying horizontal temperature gradients.[3, 4, 9]. Consequently, a new length scale is introduced into the system where, Ra_{crit} depends on both the difference in thermal conductivity between the fluid and bounding sidewalls and the spacing between the vertical arms and corresponds to a value of 36 or less[9]. Rayleigh number

$$Ra = \frac{g\alpha(\Delta T/L)r^4}{\nu\kappa}$$

can be defined in terms of the channel radius r and horizontal temperature gradient ($\Delta T/L$). This critical value decreases as the side wall conductivity decreases and the spacing between vertical arms increases.

The inherent structure and ability of horizontally controlled closed loop geometry to circulate fluid between various temperature zones makes it ideally suited to perform temperature sensitive biochemical reactions like PCR. The self-actuated simplified mechanism of this system offers a complete passive thermocycling scheme. To optimize the reaction kinetics and the time spent by the fluid elements at various temperature zones, flow rates can be precisely tuned by controlling the independent variables such as channel radius (r), temperature gradient (ΔT) and spacing between the vertical arms (L). Another key advantage of such systems is that all fluid elements follow similar flow trajectories and are exposed to the various temperature zones for identical time frames thereby enhancing the amplification efficiency.

In this paper, we extend our studies of closed loop thermocyclers and investigate harnessing convective flows to perform tunable pumping and thermocycling operations potentially suitable for integrated lab-on-chip devices. In each of these applications, the flow can be easily and precisely manipulated by adjusting the magnitude of the applied temperature gradient and/or the fluidic network geometry. The inherent simplicity of these components makes them ideally suited for construction of inexpensive portable chemical and biochemical analysis systems.

2 EXPERIMENTAL METHODS

A simple Closed Loop Thermocycler (CLTC) was constructed using fluorinated ethylene propylene tubing (FEP; Zeus Industrial Products Inc.) and two independently controlled Peltier heaters (Melcor Corp.) assembled in a triangular configuration supported by a hollow aluminum block as shown in Figure 1. The choice of FEP tubing was

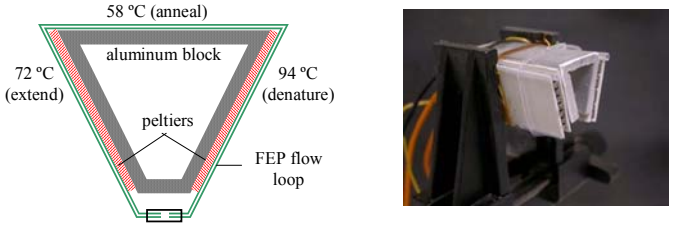


Figure 1. Schematic and actual picture of CLTC

motivated by its attractive combination of properties including low cost, mechanical flexibility, optical transparency, and biocompatibility. Consequently, no surface treatments were necessary in order to perform PCR. FEP tubing of three different diameters (24-gauge, 26-gauge and 28-gauge) was used to perform flow characterization and PCR amplification. A 9 cm tubing segment of each diameter was used to form the CLTC reactor. Tubing was filled with PCR reagents and the two open ends of the segment were joined together using a short segment of lower gauge PVC tubing. Slightly overfilling the tube before sealing largely alleviated bubble formation within the channel. The total volume of the closed loops was approximately 10 µL, 20 µL and 32 µL for 28-gauge, 26-gauge and 24-gauge tubings respectively. Aluminum adhesive tape was used to affix the tubing directly to the Peltier surfaces to ensure good thermal contact. Temperatures were monitored using flat thermocouple probes affixed to the Peltier surfaces. The heaters were independently maintained at 95 °C (denaturing) and 72 °C (extension) while the average temperature in the third arm of the loop was passively maintained at 58°C. A small window was cut in the aluminum tape at the top location of the triangular setup for flow imaging purpose.

2.1 PCR Conditions

Several reaction systems were tested in order to evaluate PCR performance. All reagents were supplied in kits from Maxim Biotech, Inc. Reactions involving amplification of the following targets were studied: a) 191-base target associated with the membrane channel proteins M1 and M2 of the influenza-A virus, b) 242-base target associated with the human L32 gene sequence, c) 474-base target associated with the human β-Actin gene sequence, and d) a multiplex system containing 5 different respiratory infection associated virus targets (264-base respiratory syncytial virus (RSV), 315-base corona virus, 390-base influenza virus, 484-base adenovirus, and 547-base rhino virus). Standard 50 µL reaction mixes contained 30 µL of optimized buffer/dNTP mix, 10 µL of primer mix, 8.75 µL of ddH_2O, 1 µL of template DNA and 0.25 µL of 5 units/µL AmpliTaq polymerase. After the reactions were complete, products were aspirated from the reaction chambers, run on a 2% agarose gel at 60 V for 1 hour, and stained with 100x SYBR-Green I. Product yields were measured from purified PCR products (QIAquick PCR Purification Kit; Qiagen) using a NanoDrop ND-1000 spectrophotometer.

3 RESULTS AND DISCUSSION

3.1 Flow Characterization and Modeling

To demonstrate the capabilities and advantages of the self-actuated convective PCR system, 2-D flow visualization in CLTC was carried out by filling the reactor with a suspension of 6 µm diameter fluorescent latex spheres. FEP reactors of three different diameters and a

fixed loop length of 9 cm were simultaneously loaded into the CLTC. Initially, temperature of one of the two heaters was raised gradually, after which the second heater was also activated to maintain PCR conditions on all the three sides. Due to the existing parabolic flow profile through out the loop, spheres close to the wall were observed to be moving at lower velocities as compared to those near the center line of the tubing along the x-axis. However, some of the spheres along the center line also moved slowly presumably due to their close vicinity towards the wall in z-direction. Average velocities were calculated by monitoring the sphere motion over a fixed distance throughout the crossection for successive steady state temperature gradients.

As depicted by the low value of the critical Rayleigh number, the onset of fluid motion was observed at a very low temperature gradients on the order of 1ºC. Under PCR conditions, flow velocities of the order of 1.14 mm/sec, 1.74 mm/sec and 2.67 mm/sec were obtained corresponding to 28, 26 and 24-gauge tubings respectively. It was also observed that increase in temperature gradient increases the flow rate in a manner that scales with Ra/Pr, where Pr is the Prandtl number (ν/κ). Using this scaling, flow rates obtained in channels of different diameter collapse onto a master curve that can further be generalized to predict flow characteristics for any similar system.

3.2 PCR in closed loop systems

The feasibility of performing PCR in a convective loop format was illustrated using similar reactors used for flow characterization in closed flow circuits. Flow velocities up to 2.67 mm/s were achieved under these conditions, corresponding to a cycle time of approximately 34 seconds. When the flow loop was loaded with PCR reagents, a successful amplification of the 191 base pair fragment (3.9 kb template) could be performed in as little as 10 minutes (Figure 2a.) Simultaneous amplification of a mixture containing primers and template for the 191-bp influenza-A virus and 242-bp human L32 gene targets was also achieved in 50 minutes (Figure 2b). We have also demonstrated the amplification of a 295-bp β-Actin target and a multiplex mixture of targets associated with 5 different respiratory viruses in 26-gauge tubing within 50 minutes of reaction time (Figure 2c). It is notable that no modifications to the reagent mixtures used in conventional thermocycler-based protocols were necessary in the loop-based PCR device presented here. Further optimization and scale-down of the reactor volume are likely to yield improved performance.

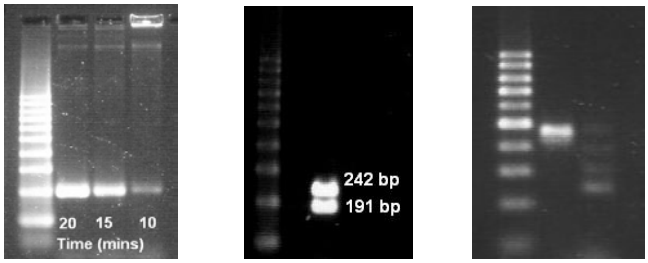


Figure 2. a) PCR of 191 bp in 24 AWG reactor
b) 2-Plex PCR C) Multiplex PCR

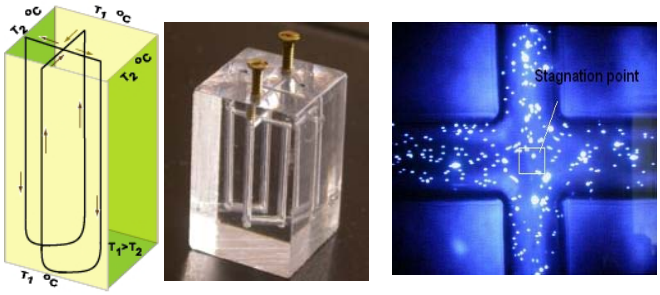
3.3 Quantification of CLTC Product Yields

In order to quantitatively verify PCR performance in the CLTC, product purification was carried out to remove unused primers followed by spectrophotometric measurement of the final nucleotide concentration. Table 1 summarizes the yields obtained in the three reactors for different reaction times. It is clearly demonstrated that the CLTC efficiency is over 5 times better than the conventional benchtop thermocyclers both in terms of yield as well as reaction times.

Table 1.	24 AWG (µg/mL)	26 AWG (µg/mL)	28 AWG (µg/mL)	Benchtop thermocycler (µg/mL)
Time/Cycle	33.75 sec	51.75 sec	78.75 sec	300 sec
10 mins	3.96	-	-	2.1
15 mins	4.8	-	-	1.8
20 mins	7.37	5.03	-	1.9
25 mins	-	6.97	-	2.03
30 mins	-	8.57	6.97	2.13
40 mins	-	-	8	2.03
50 mins	-	-	8.9	2.8
2.5 hrs	-	-	11.07	12

4 OTHER CONVECTIVE SYSTEMS AND APPLICATIONS

In addition to performing biochemical reactions, these devices can also be used to generate extensional flow fields in a cross-slot geometry or to perform on chip pumping and thermocycling operations when integrated with microfluidic systems. Figure below shows a closed cross-slot pattern



fabricated in a Plexiglas block which, when heated from any two opposite sides while keeping the other two sides at a lower temperature, can generate extensional flow patterns at the stagnation point of the cross-slot. Flow visualization using fluorescent spheres confirmed that microspheres become trapped at the stagnation point for 4-5 seconds experiencing elongational forces in the opposite directions. These non-pulsatile continuous flow fields can be harnessed to stretch DNA/polymers and to study single molecule behavior.

4.1 Convectively Driven Micropumps

We have also investigated the use of these flow loops to pump fluids in the horizontal plane by constructing devices where the lower horizontal segment of the loop is replaced by a longer segment creating a 3-D flow path. Similar flow rates are observed in these devices, indicating that sufficient pumping capacity exists to drive flows in a variety of microfluidic channel networks.

A few general guidelines for the scale-down of convective flow systems in miniaturized loops can be derived. We can use the flow rate data in Figure 5b to extract a rough estimate of the minimum channel diameter for flow to occur by extrapolating to the conditions corresponding to zero flow rate. This analysis indicates that channel diameters can be scaled down to at least 180 μm at this tubing diameter (28 gauge) for a particular loop length, maintaining a temperature difference of 35 °C between annealing and denaturing temperatures. This minimum diameter can be further reduced through the use of shorter flow loops because the corresponding decrease in hydrodynamic resistance allows higher flow rates to be achieved under the same thermal driving force.

5 CONCLUSIONS

Exploiting convective flow fields to perform PCR amplification of DNA is an emerging area of research with the potential to offer a simple low-cost alternative to conventional thermocycling equipment. Moreover, convective flow systems are capable of achieving rapid cycling times, even approaching those attainable in microfluidic systems, while still operating within a convenient format. Natural convection systems can also serve as a useful platform to perform a variety of thermally activated biochemical reactions where temperature cycling is required (e.g., real time PCR, cycle sequencing of DNA). Devices can be easily constructed using optically transparent substrates to provide *in situ* fluorescence detection capability. Elimination of external electronic controls and pumping equipments considerably reduces power consumption making them ideally suited for developing battery operated portable bio-analysis systems.

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