Towards Rapid Prototyped Convective Microfluidic DNA Amplification Platform

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ABSTRACT

Today, Polymerase Chain Reaction (PCR) based DNA amplification plays an indispensable role in the field of biomedical research. Its inherent ability to exponentially amplify sample DNA has proven useful for the identification of virulent pathogens like those causing Multiple Drug-Resistant Tuberculosis (MDR-TB). The intervention of Microfluidics technology has revolutionized the concept of PCR from being a laborious and time consuming process into one that is faster, easily portable and capable of being multifunctional. The Microfluidics based PCR outweighs its traditional counterpart in terms of flexibility of varying reaction rate, operation simplicity, need of a fraction of volume and capability of being integrated with other functional elements. The scope of the present work involves the development of a real-time continuous flow microfluidic device, fabricated by 3D printing-governed rapid prototyping method, eventually leading to an automated and robust platform to process multiple DNA samples for detection of MDRTB-associated mutations. The thermal gradient characteristic to the PCR process is produced using peltier units appropriate to the microfluidic environment fully monitored and controlled by a low cost controller driven by a Data Acquisition System. The process efficiency achieved in the microfluidic environment in terms of output per cycle is expected to be on par with the traditional PCR and capable of earning the additional advantages of being faster and minimizing the handling.

Keywords: Polymerase Chain Reaction (PCR), Microfluidics, Convective-Flow, Peltier, Micro-heater, Arduino

1. INTRODUCTION

The revolutionary concept of miniaturization derives its motivation from the Apollo mission to the moon to the development of artificial organs, which required all electronic components to be shrunk to save on space and money. This historical event not only led to the advent of the Microelectronics industry but also served as a harbinger to a whole new field of science called Microelectromechanical Systems (MEMS). Microfluidics, a niche area of application of MEMS devices, was developed in the 1980s with the realization of inkjet print-heads and DNA chips. It involved the study of fluid flow in geometry confined to submillimeter scale. The uniqueness of fluid flow in microfluidic systems is that the flow is laminar. While Micro-analytical methods like gas-phase chromatography, high pressure liquid chromatography and capillary electrophoresis which employed small quantities of the sample served as an early motivation to the advent of microfluidic technology, the real necessity arose from the explosion of genomics which required high throughput DNA sequencing [1]. Since then, a lot of cutting edge research work has been undertaken in this area with the sole purpose of saving on space, cost and human resources in an eclectic range of applications: one of which is the Polymerase Chain Reaction (PCR) [2].

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First introduced by Karl Mullis in 1986, PCR involves replication or amplification of a target DNA employed in a wide range of applications from identification of disease causing pathogens, cloning, sequence-tagged sites, to phylogenic analysis of DNA from ancient sources [3][4]. Today, PCR plays an indispensable role in the field of biomedical research and its inherent ability to exponentially amplify small volumes of DNA has proved inevitable to medical research especially in identifying several diseases including multiple drug-resistant tuberculosis (MDR-TB), a major world-wide concern.

PCR is typically performed on a benchtop machine called a thermocycler in the laboratory. However, they are tied down by disadvantages like being bulky, requirement of external human intervention and inability to serve as a point of care device deployable to remote areas and villages. These disadvantages are overcome by the microfluidic devices better known as lab-on-chip PCR devices that ensure reduced thermal mass of the system. These on chip devices can be Continuous Flow PCR reactors which in turn maybe may be circulatory as in serpentine channels([5]), oscillatory[23] or closed loop [6]. Lagrangian model of transport phenomenon supports a closed loop system for PCR because DNA molecule cannot undergo molecular diffusion or fail to go through the PCR cycle and can be precisely tracked through the three stages of denaturation, annealing and extension, the advantage being that the dwell time of the DNA in each temperature zone can be controlled slightly better than in the case of the cylindrical cavity cells [7].Circulatory systems require forced convection involving the use of an external pump to circulate the fluid throughout the device [8]. However, natural convection eliminates the need for an external pump and such units are easily integrated into a microfluidic device with electrophoresis on the same chip[9].

The first real time PCR process that involved integration of a fluorimeter with a temperature cycler requiring repetitive heating and cooling for 35-50 cycles and restricted by high costs, large size and long amplification times [10]. However, simple natural convection based designs involve performing PCR in a capillary tube which when heated from below causes the sample to rise due to buoyant forces set up in opposition to the gravitational forces, one of the early pioneering work being that of Krishnan et.al [11] where it was demonstrated that the temperatures required for PCR could be achieved by natural convection by choosing an aspect ratio (height of capillary/diameter of capillary) of appropriate value. Several experimental works have been taken up to cary out PCR process in a capillary tube [12], [13], [13]From our experience on developing detection systems that significantly reduce the timeframe for detection, we have made efforts to develop a microfluidics based robust platform using silicon tubing as the micro-reactor chamber and peltiers as the heating elements for generating proof of principle of a natural convection based PCR unit. The peltier temperature is controlled using a simple relay based controller (Fig 3(b)) controlled by logic based control exercised through National Instruments based Data Acquisition System. The objective of the authors is to produce a cost effective working prototype based on natural convection based fluidic flow that is easily portable. We anticipate that the size of this platform would eventually be scaled down with the help of the 3D printer and be fully automated to minimize handling, provide a high throughput as well as improving the chances of identification of patients carrying diseases like MDR-TB.

2. MATERIALS AND METHODS

2.1 Methodology

The conventional thermocycler being a static system suffers the disadvantage of consuming huge quantity of power to regulate heat of its structural components because of the demand of the PCR process to raise and lower the temperature cyclically. PCR chip may be designed based on time domain approach or space domain approach [2]. In a time domain approach PCR mix is kept static in micro-wells and heated using Tungsten Lamp, Infrared radiation or hotplates to do the periodic heating and cooling of the PCR mixture ([14], [15]). These prototypes are limited by the longer time duration and are being increasingly replaced by continuous flow type PCR systems ([16]) which work on space domain approach.

In the space domain approach the PCR mix flows continuously through the microfluidic chip which has a pre-defined geometry and the temperature of the chip is a function of the position in the channel. The advantage of the latter is that sample preparation and Amplified PCR output detection can be easily integrated into this system ([17], [18]). Several research works have been undertaken based on serpentine geometry wherein each loop corresponds to 1 cycle of PCR. However this geometry is limited by the fact that only a fixed number of cycles can be obtained within the fixed dimensions of the chip, does not offer the flexibility of increasing or decreasing the number of cycles and generally requires an external pump. A more flexible and cheaper alternative is the use of natural convection to create the thermal

zones corresponding to different PCR zones naturally when one end of the cavity is heated and the other end of the cavity is relatively cooler. The fluid flow is driven by the density gradients that are created by the difference in temperature as demonstrated by Krishnan et.al [11].

Thermosiphon effect and Rayleigh Bernard Convection are the two phenomena playing an indispensable role in the convective circulation of a fluid in a cavity. While the former effect involves passive heat exchange based on natural convection causing liquid to flow from a the relatively hotter side to the relatively colder side due to a temperature gradient, the latter occurs when a fluid is heated and density gradients are created which cause the fluid to circulate within the geometry. These phenomenon are utilized to circulate the PCR reaction mix through the three temperature zones corresponding to denaturation, annealing and extension [19].The advantage of this process is that it reduces reaction time considerably and can be implemented at low cost.

2.2 Theory

Rayleigh's number exhibits a dependence on two other dimensionless quantities: Prandtl number and Grashof's number which in turn are the functions of aspect ratio as demonstrated by S. Lifshitz and Y. Zvirin [20]. Therefore, the dimension of the tube namely the inner diameter and length were chosen such that the aspect ratio ensured that the Rayleigh's number was sufficiently high to initiate convection. The dependence of Rayleigh's number on Prandtl number and Grashof's number are shown in equation (1)-(3).

$$\text{Rayleigh's number, } Ra = PrGr \tag{1}$$

$$\text{Prandtl number, } Pr = \frac{\vartheta}{\alpha} = \frac{c_p \mu}{k} \tag{2}$$

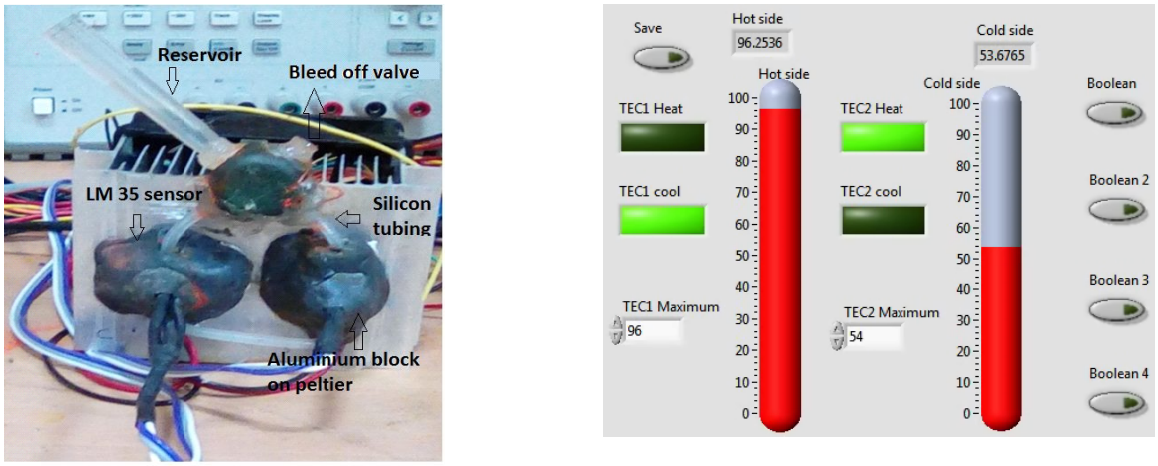
$$\text{Grashof's number, } Gr = [g\cos(90-\theta)]\beta \Delta T D^3/\vartheta k \tag{3}$$

where, gcos (90-θ) is the component of gravitational acceleration acting along the flow direction and β, ϑ, k (coefficient of volume expansion, kinematic viscosity, and thermal diffusivity, respectively) correspond to fluid property at the average temperature between the highest and lowest PCR temperature. ΔT is the difference in temperature between the two peltier heated zones and D is the inner diameter of the tube.

2.3 Experimental

The experimental setup utilizes Thermosiphon effect, which is a method of passive heat exchange based on natural convection. When a region of the loop is exposed to a higher temperature compared to other part of the loop, the working fluid absorbs thermal energy and loses density at the hot region. Lower density fluid molecules then tend to move upward leaving vacancy for the heavier fluid giving rise to a circulatory convective flow. In the experimental setup, a temperature gradient akin to the Rayleigh Bernard convection in cylindrical cavities demonstrated in the early work of Krishnan et.al [11] and Chunsun Zhang et.al [6] was set up; the difference being that the geometry was altered to that of a toroidal loop. A temperature gradient was created by placing the peltiers on diametrically opposite arms of the loop and maintaining them at two different temperatures of 94℃ and 60℃ respectively. It was observed that the fluid cycled through the entire volume by natural convection due to the temperature gradient. In the initial phase of the work, the proof of principle was generated by bending a silicon tube into a circular loop and passing it through both the aluminum block and connecting the free ends of the tube into a hub. The temperature control unit was built using two peltiers (1.5 cm x 1.5 cm) mounted on an Aluminum heat sink with fins (8.2 cm x 6.2 cm). Aluminum blocks (1.5 cm x 1.5 cm) were mounted on these peltiers and held together with conductive thermal paste. The two set of units were then covered by epoxy putty and epoxy adhesive to minimize heat loss to surroundings. LM 35 sensors were mounted atop the aluminum blocks to detect the temperature and help in the closed loop control of the peltier elements. The inner diameter and length of the tubing were chosen to be 1 mm and 13 cm respectively. A simple relay circuit with four relays and a darlington pair was built on a dotted board to help switch the polarity of the peltiers in a timely fashion so that the denaturation and annealing temperatures may be maintained at the two peltier blocks (Fig 1 (a)).

The sequential switching of the relays was done based on the logic based algorithm fed into the National Instruments Data Acquisition system with the help of LabView™ software interface such that a tight temperature control of the two peltiers is maintained at 94℃ and 60℃ respectively by changing the polarity of the peltier (making the hot side cool and vice versa) rather than turning it off observable in Fig 1.(b). The physical setup was modified to include a reservoir with the toroidal loop such that the hydrodynamic pressure maintained by the liquid head acted as a natural pump pushing the liquid through the entire length of the tubing without the buildup of air bubbles.



(a) (b)

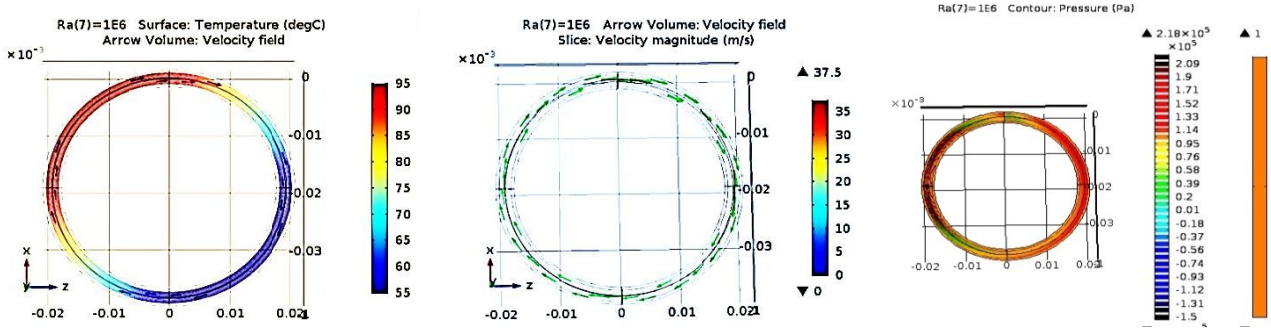
Figure (1): Experimental analysis, (a) Experimental setup with toroid loop of silicon tubing passed through two aluminum blocks mounted on the Peltier elements and sealed with a combination of epoxy putty and epoxy adhesive and (b) Temperature recorded corresponding to the denaturation and annealing zones

Another bleed off valve was facilitated in the setup (as shown in Fig 1 (a)) to let out any air bubbles that may be trapped within the tube. It was also expected that such a unit would prevent contamination of the PCR mix due to physical handling of the tube at a later stage. The flow rate was slightly reduced by keeping the tilt angle of the setup (θ) between 30 and 60 degree to ensure that the entire volume of liquid was flowing through the three zones yet sustained in each zone for sufficient time, a pre-requisite for PCR reaction to be carried out in an optimized manner in future. However, this setup had the disadvantage of requiring very high reaction volume of nearly 500 microlitres, a major drawback when using PCR for spot detection of diseases like MDR-TB. In order to tackle this problem, in the final setup the silicon tube was filled with an excess of the fluid till the other end started overflowing and the ends of the tube were sealed with heat-shrink.

3. RESULTS AND DISCUSSIONS:

3.1 Simulation

COMSOL simulation of the setup was done to determine the temperature gradient, velocity and pressure profile expected in the experimentation. The PCR mix is assumed to have properties of water at a temperature of 73 ℃ and the thermal properties[21] corresponding to silicon tubing are taken to be that of silicon (density 1500 kg /m^3, thermal conductivity of 1.3W/m K , heat capacity at constant pressure of 1200 J /kg K).



(a) (b) (c)

Figure (2): COMSOL simulaiton results, (a) Temperature distribution in the toroidal loop, (b) Velocity distribution in the toroidal loop, and (c) Pressure profile inside the toroidal loop

It is observed that the temperatures corresponding to the three sub-phases of PCR are achievable with the help of two Peltiers maintained at denaturation and annealing temperatures. The velocity profile of the fluid generated by the software indicated an average velocity of about 20 m /sec and the pressure profile predicted an average pressure of about 2.18×10^5 Pa corresponding to a Rayleigh's number of 10^6. The thermal gradient profile exhibited the three temperature zones corresponding to denaturation, annealing and extension as shown in Fig. (2) The temperatures were monitored and recorded real time with the help of a Data Acquisition System (National Instruments) (Fig 1(b)).

3.2 Experimental

Fig 3(a) shows the experimental setup for conduction of PCR. One major challenge faced in the first phase involved removal of air bubbles which if present obstructed fluid circulation due to natural convection. The first method devised to tackle the challenge involved the use of a hub that takes both ends of the tube. Though the system helped rid air bubbles during initiation of the PCR reaction, after a while they were found to reappear leading us to believe that the gas permeability property of silicon and low solubility of dissolved gas under the difference in temperature as quoted by authors in [22] are responsible for the observation. The second setup involving heat-shrink to join the end of the tube after slightly overfilling the tubing helped reduce the reaction volume and prevent air bubbles during initiation of the convective cycling. The inner diameter of the tubing was chosen to 1mm and the length of tubing was chosen to be 13cm.

(a) (b)

Figure (3): Final setup and circuit – (a) Final setup for PCR and (b) Relay based control circuit

4. CONCLUSION

The intention of the authors is to develop a protocol after identifying challenges involved in the development of an affordable point of care unit for on the spot detection of diseases like MDR-TB especially in developing countries which have poor laboratory settings and resources. The present work does not go beyond the proof of principle of convective flow for PCR reaction. The next stage of the work is expected to include real biological samples to test efficacy of the system in terms of sufficient amplification of the biological samples in a reduced time frame. One of the challenges is that Silicon tubing is inherently gas permeable making it susceptible to air bubbles which obstructs natural convection. Since some authors have successfully demonstrated PCR reaction in similar setups with the use of FEP (Fluoroethylene propylene) or PTFE (polytetrafluoroethylene)[23] tubing the authors to believe that the silicon tubing may be replaced with FEP or PTFE tubing. Alternatively, the friction to fluid flow on the inner walls of the Silicon tubing could be reduced by pushing in a chemical like Hexamethyldisilazane (HMDS) through the tubing before introducing the PCR mix. The other challenges include filling and sealing of the tube without air bubbles. This may be efficiently done by fabricating 3D printed micro-reactor with valves to ensure the tubing is filled without air bubbles.

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REFERENCES

[1] G. M. Whitesides, "The origins and the future of microfluidics.," Nature 442(7101), 368–73 (2006).

[2] C. D. Ahrberg, A. Manz, and B. G. Chung, "Polymer chain reaction in microfluidic devices," Lab Chip 16, 1995–2000 (2016).

[3] S. Park, Y. Zhang, S. Lin, T. H. Wang, and S. Yang, "Advances in microfluidic PCR for point-of-care infectious disease diagnostics," Biotechnol. Adv. 29(6), 830–839 (2011).

[4] Y. Zhang and P. Ozdemir, "Microfluidic DNA amplification-A review," Anal. Chim. Acta 638(2), 115–125 (2009).

[5] H. Tachibana, M. Saito, K. Tsuji, and K. Yamanaka, "Chemical Self-propelled continuous-flow PCR in capillary-driven microfluidic device : Microfluidic behavior and DNA amplification," Sensors Actuators B: Chem. 206, 303–310 (2015).

[6] C. Zhang and D. Xing, "Parallel DNA amplification by convective polymerase chain reaction with various annealing temperatures on a thermal gradient device," Anal. Biochem. 387(1), 102–112 (2009).

[7] J. W. Allen, M. Kenward, and K. D. Dorfman, "Coupled flow and reaction during natural convection PCR," Microfluid. Nanofluidics 6(1), 121–130 (2009).

[8] P. Nath, T. S. Maity, F. Pettersson, J. Resnick, Y. Kunde, N. Kraus, N. Castano, and J. Dunbar, "Polymerase chain reaction compatibility of adhesive transfer tape based microfluidic platforms," Microsystem Technologies 20(6), 1187-1193 (2010).

[9] K. H. Chung, Y. H. Choi, and M. Y. Jung, "Natural convection PCR in a disposable polymer chip," Proc. IEEE Sensors, 1217–1220 (2009).

[10] D. S. Lee, B. H. Chang, and P. H. Chen, "Development of a CCD-based fluorimeter for real-time PCR machine," Sensors Actuators B: Chem. 107(2), 872–881 (2005).

[11] M. Krishnan, V. M. Ugaz, and M. a Burns, "PCR in a Rayleigh-Bénard convection cell," Science 298(5594), 793-793 (2002).

[12] E. T. Lagally, C. a Emrich, and R. a Mathies, "Fully integrated PCR-capillary electrophoresis microsystem for DNA analysis.," Lab Chip, vol. 1, pp. 102–107, 2001.

[13] W. P. Chou, P. H. Chen, M. Miao, L. S. Kuo, S. H. Yeh, and P. J. Chen, "Rapid DNA amplification in a capillary tube by natural convection with a single isothermal heater," Biotechniques 50(1), 52–57 (2011).

[14] Y. Ouyang, G. R. M. Duarte, B. L. Poe, P. S. Riehl, F. M. dos Santos, C. C. G. Martin-Didonet, E. Carrilho, and J. P. Landers, "A disposable laser print-cut-laminate polyester microchip for multiplexed PCR via infra-red-mediated thermal control," Anal. Chim. Acta 901, 59–67 (2015).

[15] C. R. Phaneuf, N. Pak, D. C. Saunders, G. L. Holst, J. Birjiniuk, N. Nagpal, E. Popler, A. L. Shane, R. Jerris, C. R. Forest, C. R. Phaneuf, N. Pak, D. C. Saunders, G. L. Holst, J. Birjiniuk, N. Nagpal, S. Culpepper, E. Popler, A. L. Shane, R. Jerris, and C. R. Forest, "Thermally multiplexed polymerase chain reaction Thermally multiplexed polymerase chain reaction," Biomicrofluidics 9(4), 1–14 (2015).

[16] D. Moschou, N. Vourdas, G. Kokkoris, G. Papadakis, J. Parthenios, S. Chatzandroulis, and A. Tserepi, "All-plastic, low-power, disposable, continuous-flow PCR chip with integrated microheaters for rapid DNA amplification," Sensors Actuators B: Chem. 199, 470–478 (2014).

[17] C. Gärtner, R. Klemm, and H. Becker, "Methods and instruments for continuous-flow PCR on a chip," Proc. SPIE 6465, 646502-646509 (2007).

[18] N. Crews, C. Wittwer, and B. Gale, "Continuous-flow thermal gradient PCR," Biomedical microdevices 10(2), 187–195 (2008).

[19] P. Bergé and M. Dubois, "Rayleigh-bénard convection," Contemp. Phys. 25, 535–582 (1984).

[20] S. Lifshitz and Y. Zvirin, "Transient heat and mass transfer in a natural circulation loop," Wärme-und Stoffübertragung 380, 371–380 (1993).

[21] Online Tool, "Fluid Properties Calculator- http://www.mhtl.uwaterloo.ca/old/onlinetools/airprop/airprop.html" (2017).

[22] T. Nakayama, Y. Kurosawa, S. Furui, K. Kerman, M. Kobayashi, S. R. Rao, Y. Yonezawa, K. Nakano, A. Hino, S. Yamamura, Y. Takamura, and E. Tamiya, "Circumventing air bubbles in microfluidic systems and quantitative continuous-flow PCR applications," Anal. Bioanal. Chem. 386(5), 1327–1333 (2006).

[23] N. Agrawal, Y. A. Hassan, and V. M. Ugaz, "A pocket-sized convective PCR thermocycler," Angew. Chemie - Int. Ed. 46(23), 4316–4319 (2007).