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Plastic microfluidic chip for continuous-flow polymerase chain reaction: simulations and experiments

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Abstract

A continuous flow polymerase chain reaction (CF-PCR) device comprises a single fluidic channel that is heated differentially to create spatial temperature variations such that a sample flowing through it experiences the thermal cycling required to induce amplification. This type of device can provide an effective means to detect the presence of a small amount of nucleic acid in very small sample volumes. CF-PCR is attractive for global health applications due to its less stringent requirements for temperature control than for other designs. For mass production of inexpensive CF-PCR devices, fabrication via thermoplastic molding will likely be necessary. Here we study the optimization of a PCR assay in a polymeric CF-PCR device. Three channel designs, with varying residence time ratios for the three PCR steps (denaturation, annealing, and extension), were modeled, built and tested. A standardized assay was run on the three different chips, and the PCR yields were compared. The temperature gradient profiles of the three designs and the residence times of simulated DNA molecules flowing through each temperature zone were predicted using computational methods. PCR performance predicted by simulation corresponded to experimental results. The effects of DNA template size and cycle time on PCR yield were also studied. The experiments and simulations presented here guided the CF-PCR chip design and provide a model for predicting the performance of new CF-PCR designs prior to actual chip manufacture, resulting in faster turn around time for new device and assay design. Taken together, this framework of combined simulation and experimental development has greatly reduced assay development time for CF-PCR in our lab.

Keywords

PCR; microfluidics; thermofluidic modeling

Introduction

The polymerase chain reaction (PCR) can be used to detect pathogens from very small amounts of nucleic acid starting material, making it an extremely sensitive technique. Of the many microfluidic PCR devices developed [1–8], most take on one of two basic designs: stationary PCR and CF-PCR. Stationary PCR devices work by thermocycling the device and PCR reagents together over time, and CF-PCR devices work by directing reagents through a

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single-channel spatial temperature gradient, thereby reducing the thermal mass of the reagent and effectively shortening the PCR cycling time. By controlling the overall flow rate and the size of the channel along its length, and thus the residence times at different temperatures, the approach has the flexibility needed to meet the requirements of different assays [9]. Because of this advantage, several groups have reported success with CF-PCR and some have reported single molecular amplification using CF-PCR [10,11]. We are interested in the design and development of thermoplastic continuous flow PCR (CF-PCR) devices for applications in global health, and understanding the interplay between channel design and PCR yield in these devices is critical to this work.

The first CF-PCR device, developed by Kopp[1], used a rectangular serpentine channel with three temperature zones on a glass substrate supported by three independent copper block heaters. After the first CF-PCR device, a lot of work has been done to improve the channel design, thermal control and flow control of CF-PCR devices: radial[12], spiral[13], and straight CF-PCR channel[14,15], non-contact thermal control[2,16], and droplet-based CF-PCR[12] are examples. Considering the application in global health, the utilization of out power source (IR, laser, magnesium) need to be limited, the thermal and flow control need to be simplified and the chip fabrication need to be high throughput and low cost. Therefore, we apply two thin film heaters on a hot-embossed serpentine plastic chip to perform CF-PCR.

To achieve better CF-PCR performance, controlling the residence times for denaturation, annealing, and extension is critical. While most of the former CF-PCR chips designed do employ strategic residence time ratios based on general knowledge of PCR reaction kinetics, little systematic work has been done to optimize this ratio in chips. Further, since each new PCR assay requires one to optimize chip conditions before it can become a reliable diagnostic, an integrated simulation-experimental framework has the potential to greatly reduce assay development time. Here, we quantify the effect of the absolute and relative residence times on PCR assay performance in microfluidic CF-PCR platforms. We present three thermoplastic CF-PCR devices with different residence time ratios. Optimization of the performance of a standard PCR reaction was experimentally carried out by varying the flow rates, the fixed heater temperatures, and the channel dimensions, all resulting in changes in the absolute and relative residence times. Thermal and fluidic simulation has been used before to investigate the performance of CF-PCR devices[3,17,18]. However, to more accurately predict and explain the mechanisms of CF-PCR performance, we needed to develop analysis method to simulate the temperature histories of individual DNA molecules flowing through a CF-PCR device. To our best knowledge, it is the first time a method applying a Lagrangian thermal modeling approach has been applied to the design of a CF-PCR device.

Materials and Methods

CF-PCR device fabrication and assembly

The microfluidic PCR channel was fabricated in Zeonex 690R (Zeon Chemicals, Louisville, KY) by hot-embossing a blank 1 mm thick Zeonex plaque using an epoxy master mold and then thermally sealing the imprinted channel with a blank Zeonex cover slip [19]. The channel layout for the epoxy mold was designed in AutoCAD, and a thin film transparency mask (Fineline Imaging Co., Colorado springs, CO) was made based on it. Then, applying standard lithographic methods, an SU-8 mold with a depth of 100 μ m was fabricated. Since SU-8 molds are not robust enough for hot-embossing in our system, the structure of the SU-8 mold was transferred to a stronger epoxy mold using an intermediate PDMS mold. This mold was made from a mixture of PDMS and hardener at a ratio of 10:1 (Sylgard 184, Dow Corning, Midland, MI). The mixture was degassed for 30 min in a vacuum oven before

being poured onto the SU8 mold and cured for 2.5 hours at 80°C. Similarly, the epoxy (Rencast 4037R, Hunstman Advanced Materials, Los Angeles, CA) was mixed with hardener at a ratio of 100:13 and degassed for 30 min before being poured onto the PDMS mold and cured at room temperature for 24 hours, followed by 2.5 hours of hard baking at 273°C. After this process, the epoxy mold contained the same pattern as the original SU-8 mold, but was much more durable and could be used multiple times.

A hot-press (Heated Press 4386, Carver, Wabash, IN) was used to fabricate the PCR device. 7g of Zeonex resin pellets were put on a flat steel plate and then covered with another steel plate. The Zeonex is preheated at 198°C for 5 min and hot-pressed for another 5 min at 1000 psi to get a flat blank plaque. The blank Zeonex plaque was put on top of the epoxy mold and preheated to 157°C for 5 min, followed by hot-embossing for 10 min at 750 psi. After two holes were drilled at the designated inlet and outlet of the embossed chip, it was thermally bonded with another blank plaque at 131°C, 400 psi for 2 minutes, creating a continuous closed channel between the two sealed faces of the chip.

Nanoports (Cat. # N333-01, Upchurch Scientific, Oak Harbor, WA) were glued onto the inlet and outlet of the CF-PCR chip and flexible PTFE tubing (Cat. #35668, Alltech, Deerfield, IL) was connected the Nanoport on the inlet with a syringe, which was fixed on a syringe pump (PHD2000, Instech Laboratories, PA). The Nanoport on the outlet was connected to a back pressure regulator, which provided 15 psi back pressure to the micro-channel to minimize evaporation of the PCR reagent mixture [8].

Chip design

Three different designs were studied. In Design A (Figure 1a), the annealing and extension zones were combined, forming a two-step PCR channel with a separate denaturation zone, modeled after commonly used commercial PCR machine protocols (Applied Biosystems 7300, Foster City, CA). In Design B (Figure 1b), an extension zone was added between the annealing and denaturation zones. The extension zone was set at the region corresponding to 72°C, as determined by the computational model, which is the optimal temperature for the polymerase used here. Design C (Figure 1c) had the same extension zone as Design B, but the denaturation and annealing zones were designed to be narrower in order to reduce the residence time in these two sections.

The PCR channels each have thirty cycles in total (Figure 1d), with a uniform depth of 100 μm and widths of 200 μm and 400 μm for the narrow and wide sections respectively. In the channel lengths between the denaturation, annealing, and extension zones, the channels are narrower to reduce the time for temperature transitions [17]. The first half cycle of each of the designs is for preheating the polymerase.

PCR experiments

Stable temperature profiles were maintained by two thin film heaters (0.30" $\times$ 3.11", Cat. # HK5208R44.1L12A, Minco, Minneapolis, MN) affixed to the PCR device. The power consumption of each of the two heaters was 2.14 and 0.45 Watts. Two-sided thermal conductive tape (McMaster Carr, Elmhurst, IL) was used between each heater and the chip to ensure good thermal conductivity and minimum thermal loss (Figure 1e). Thermocouples (Cat. # 5SRTC-TT-J-40-36, Omega, Stamford, CT) were bonded inside the chip at regular intervals to monitor the temperature gradient in real time (Figure 1f).

A programmable syringe pump (PHD2000, Instech Laboratories, PA) was used to control the flow rates. The performance of the PCR reaction in chip was compared with the performance of the same assay in a tube using a commercial thermocycler. The PCR yield was measured for different PCR cycle times (43s, 32s, 21s, 16s, and 10.5s for a single

cycle), achieved by varying the inlet flow rates. λ phage DNA was used as the test template (Sigma, St. Louis, MO). Two different assays were used. 62 bp and 150 bp DNA fragments were individually amplified by a common forward primer (5'-GAG TGG AGC AAG CGT GAC AA-3') and different reverse primers (5'-CAG CGC GTA GGC TTC GAT-3' for 62 bp and, 5'-CAG CTTC TCC TTG ACG GCT TT-3' for 150 bp) (IDT, Coralville, IA). The PCR reagent mixture included 0.2ng/ μ L λ phage DNA as the template, 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), the primer pair (10 μ M for each), 0.01% v/v BSA, and 0.75% w/v PEG8000 (Omega, St. Louis, MO). The collected PCR products were analyzed with an Agilent 2100 Bioanalyzer (Foster City, CA, USA) to measure the size and concentration of the products. As a positive control, the same PCR reaction was carried out in a commercial PCR machine in a tube (ABI7300 real-time PCR, Applied Biosystems Inc, CA) using the same number of cycles. Each cycle includes 15s at 60°C and 60s at 95°C. The total cycle time for the machine is about 3 minutes, since it includes the time necessary for the machine to reach each temperature. The on-chip CF-PCR products were compared to the PCR products from the commercial machine to measure the yield of the CF-PCR chip. As a negative control, the same PCR reagents without template were pumped through the PCR channel to confirm lack of contamination.

Fluid and thermo-particle computational modeling

Temperature profiles of the CF-PCR devices and movement of reagents through the channel were simulated with a commercial computational fluid dynamics (CFD) software package (STAR-CD V4.10, CD-Adpaco), which is a finite-volume code that allows for unstructured meshes of tetrahedral and prism types. Computational models consisting of unstructured grids were generated using a commercial auto-meshing package (PRO-AM, CD-Adapco) and optimized for better convergence of temperature and flow field solutions; CAD-based models were rapidly built and simulated, and the detailed method is found in the literature [20]. A SIMPLE (Semi-Implicit Method for Pressure Linked Equation) algorithm with tolerance of 1.0×10^{-7} was applied to solve the momentum and continuity equations. Conjugate heat transfer method was applied to solve the energy equations of both liquid solid phases. The resulting coupled non-linear equations were linearized using the upwind scheme and applying the algebraic multigrid technique. Additionally, a flat velocity profile was applied at the inlet with a uniform flow rate of 1.2 μ L/min (corresponding to a 43 s cycle time) and the temperature value was cyclically updated every iteration using the mean temperature value at the outlet region.

To simulate the temperature history of a moving individual DNA molecule through the thermo-fluidic channel in the CF-PCR device, one-way coupled Lagrangian thermal simulations were carried out using a recently developed LCD (Lab-chip Designer) program [20]. In fluid mechanics, the Lagrangian frame of reference is where the observer follows an individual fluid parcel as it moves through space and time. The LCD program consists of three main parts; importing pre-computed flow and temperature field data, calculating particle motion and energy equations, and post-processing spherical coiled DNA trajectories and temperature histories. Additionally, DNA residence times in the three reactive zones were calculated.

Results and Discussion

Simulation results

Simulations were run for one PCR-cycle of each of the three designs. By using cyclic boundary conditions between the inlet and outlet regions, the single cycle model reduced computational time effectively and allowed us to test new designs more rapidly. Temperature contour plots for each of the three designs are shown in Figure 2a; here, the

upper and lower boxes in each picture represent the denaturation (95°C) and annealing (60°C) zones where two distinct heaters set at constant temperatures are placed. Similar trends were observed in each of the three designs, with temperature gradually changing between 95°C and 60°C over a cycle. Of note, the temperature gradients are high in the proximity of the upper heater in all cases. Figure 2b shows representative temperature histories for a moving single DNA molecule through the channel for each of the three designs. To enable quantitative comparisons, for each design, the simulated DNA molecules were loaded at identical positions close to the inlet, and PCR cycle times were controlled to be the same. In Design A, the DNA is in the annealing temperature region for the longest time, and then is heated rapidly to the denaturation temperature, with a short duration at the extension temperature. In Design B, the DNA experiences a longer extension time, because the channel is wider in that region. Similar results have been reported by other group [15, 17]. Design C has a similar extension time to Design B. However, the annealing and denaturation times are shorter than design B, because of the narrower channel at these two regions. This data is numerically summarized in Table 1. Due to the decision to use only two fixed heaters (instead of three), each design has a transition zone between the different temperature zones. These times are included in the residence time analysis and are called “transition zones.”

In theory, both annealing and denaturation take less than 1s once DNA reaches the required temperature and chemical conditions [21]. The extension step requires more time than other two steps, because of the limitation of polymerase reaction rate [21]. Taq DNA polymerase has an predicted extension rate of 60–100 nucleotides per second at 72°C [22]. Therefore, ideally, a single molecule of DNA would require a residence time ratio of 1:1:1.5 seconds in each cycle to amplify a 150 bp template. However, the PCR environment on the chip is quite different than in a tube, so the duration time for each step will likely need to be longer than the condition in tube [23].

From the simulations, the following predictions were made. Design A had a time ratio of 1.1:8.5:0.4 seconds. The short extension time was expected to limit PCR yield, since the polymerase likely would not have enough time to complete extension. The long annealing time in Design A was thought to be unnecessary for PCR, and removing it would reduce the overall cycle time. Design B had a time ratio of 1.69:1.35:1.7 seconds. Since the polymerase was likely able to perform some extension in the first transition zone, the extension time of design B was expected to be sufficient. Design C had a time ratio of 0.8:0.8:1.9. Although annealing and denaturation instantaneously occur in ideal conditions [21], we predicted it would be unlikely for all DNA to complete these two reactions in 0.8 s. Therefore, Design C was expected to have a slightly lower PCR yield than Design B, but higher than Design A.

Experimental results

This section compares the experimental results to the predictions about the different designs made using the simulations. Both the size and concentration of PCR products were measured with a Bioanalyzer (Agilent 2100). PCR yields obtained from the chip were plotted for each design and for each of the two amplicons (Figure 3).

Effects of chip design

Figure 3 shows the PCR yields of three designs, A, B and C, for the two amplicon sizes. Each experiment was repeated three times. The average yields and standard deviations are indicated in the figures. Confirming our predictions from the simulations, Design B had the best PCR performance, followed by Design C, while Design A had the worst PCR performance. This held true for both the 62bp and 150bp amplicons. T tests were done to determine the statistical significance of differences in the performance of the three designs at

the same PCR cycle time and same template size. In both Figure 3a and Figure 3b, the p values between the different flow rates for each design are $p < 0.05$. For differences between Designs A, B and C in both plots, the p values are all $p < 0.01$. The only exception is for the difference between Design B and C at 21.5 seconds cycle time for the 150 bp amplicon, which is not statistically significant ($p = 0.1$).

Effect of flow rate

For CF-PCR devices, as flow rate is increased, the overall PCR cycle time is reduced. At faster cycle times, the time available for the PCR reagent to reach equilibrium in each temperature zone is reduced, likely decreasing the PCR yield [3]. This effect is seen for each of the three designs (Figure 3). For example, the PCR yield of Design B (62bp) was reduced from 101.3 nmol/L to 24.2 nmol/L when cycle time was reduced from 43s to 10.5s (Figure 3a).

For the shorter template (62 bp), the effect of cycle time on PCR yield in Design A was larger than that in Designs B and C. For instance, Design A 10.5 s was only 6.17% as efficient 43s, while Design B was 23.8% as efficient under the same conditions. This result suggests that the extension time limitation of Design A is more influential at shorter cycle times. However, this trend was less significant with the longer amplicon (150 bp). In general, it takes more time for the polymerase to complete a longer amplicon. Designs B and C also exhibited lower yield at shorter cycle times for the larger product.

Effect of product size

The effect of amplicon size on the PCR yield for Designs A, B, and C is also shown in Figure 3. While Design A was limited by the short extension time at all five cycle times, the PCR yield was higher for the 62 bp template than for 150 bp template at all cycle times, since the extension time for small size template (62 bp) is less than the large size (150 bp) ($p < 0.05$). For Design B, the yield difference between 150 and 62 bp was not significant at the 43 s cycle time ($p = 0.5$), indicating that the longest extension time was sufficient for extending both the large and small templates. For Design C, the difference in extension efficiency between the two different amplicons is not significant until the cycle time is reduced to the 16 s ($p < 0.01$).

In addition to optimization of the DNA residence times at the denaturation, annealing, and extension temperatures, reduction of the transition time between reaction zones is another way to optimize CF-PCR. Reducing the corresponded channel width, increasing the fixed heater temperatures or areas of coverage both decrease the transition times. Cooling rates can also be increased through active heat transfer. Implementation of these methods increases the complexity of the device, which is not ideal for applications in global health, where simplicity has major advantages. A shorter template is preferred for fast detection since a faster cycle can be applied without extension time limitations affecting yield. Shorter templates, however, may decrease the sensitivity of a particular PCR assay and must be evaluated for each new assay design during diagnostic development.

Limit of detection on chip

A series of diluted templates were amplified on the PCR chip using the most efficient design (Design B) and in a commercial PCR machine (ABI 7300), to measure the sensitivity of each assay. Although the on-chip PCR yield overall was lower than the commercial PCR machine assay, both yielded detectable PCR products at the lowest template concentration at 1×10^{-4} ng/ μ l (Figure 4).

Lagrangian thermal modeling

We hypothesize that optimal performance of CF-PCR devices can be achieved more quickly if suitable computational tools are employed at the design stage. To date, most commercial computational tools allow for thermo-fluid simulations that predict the temperature and velocity fields of fluid phase along the overall device, and the low and high temperature gradients in three reactive regions of microchannel [24]. The Lagrangian thermal modeling approach used here in the CF-PCR microfluidic device is less commonly applied due to a lack of suitable computational tools. An exception is one interesting study of residence times in CF-PCR devices using simple energy equations in a one dimensional model along the axial coordinate [25]. The current dynamic DNA simulation is a new way to evaluate the performance of CF-PCR microfluidic devices in terms of DNA residence by simulating temperature histories of large numbers of molecules as they pass through the high and low temperature regions. Additionally, it is important to note that the residence time of a DNA molecule can vary widely due to the parabolic velocity profile in a rectangular microchannel. For example, a DNA molecule located closer to the wall will have a longer residence time because of the no-slip condition of fluid on the wall. Therefore, the stochastic method used here (simulation of a random distribution of many DNA molecules in a section) was chosen to more closely model the behavior of molecules in a real system.

Conclusions

To improve the practicality of CF-PCR devices for global health applications requiring high volume manufacturing of devices, a thermoplastic chip (Zeonex 690) and simple, fixed two heater temperature control strategy were used to make disposable CF-PCR devices. The performances of three different designs were studied for different PCR cycle times (43s, 32s, 21s, 16s, and 10.5s). While all three designs demonstrated a better performance at longer cycle times, one design (Design B) performed best overall. Fluidic and thermal particle simulations were applied to predict the amount of time each DNA molecule spent in each of the temperature zones. The simulation results were used to predict the PCR performance of different designs before experiments were performed. Predictions of PCR performance agreed with the experimental results. Two different-sized templates (150 bp and 62 bp) were used to study the effect of template size on PCR yield. In the same experimental conditions, the smaller template had better PCR yield than larger template. The results presented here show that simple on-chip CF-PCR assays can be realized with inexpensive materials amenable to high throughput manufacturing by using computational models of proposed devices before fabrication to reduce the number of design iterations.

List of abbreviations

PCR	polymerase chain reaction
CF-PCR	Continuous flow polymerase chain reaction
DNA	deoxyribonucleic acid

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Figure 1.

(a) Design A, single cycle; (b) Design B, single cycle; (c) Design C, single cycle; (d) Layout of a 30-cycle CF-PCR channel (design B). The channel is 100 μm in depth and varies from 200–400 μm in width. (e) CF-PCR device. (f) Schematic side view of the cross section of PCR chip at *.

Figure 2.

(a) Thermal gradient maps of single PCR channels for Designs A, B, and C. The rectangles on the two ends of channel mark the heater positions. (b) Temperature histories of one typical simulated single DNA molecule flowing through Designs A, B, and C.

Figure 3.

PCR yields of three designs at different PCR cycle times (43, 32, 21, 16, and 10.5 s/cycle) for (a) 62 bp (b) 150 bp amplicons. In both (a) and (b), the p values between the different flow rates for each design are $p < 0.05$. For differences between designs A, B and C in both plots, the p values are all $p < 0.01$. The only exception is for the difference between Design B and C at 21.5 seconds cycle time in plot (b). The p value is 0.1.

Figure 4. PCR sensitivities of the 62 bp assay in a commercial PCR machine (ABI 7300) and PCR chip (Design B).

Residence and transition times in seconds of a simulated DNA molecule in each of the three designs for a constant cycle time (43 s).

Table 1

Time (s)	Annealing zone (59.8–60°C)	Transition zone 1 (60–70°C)	Extension zone (70–74°C)	Transition zone 2 (74–94.9°C)	Denaturation zone (94.9–95.1°C)	Transition zone 3 (94.9–59.8°C)
Design A	8.5	4.45	0.4	5.8	1.1	22.75
Design B	1.35	6.31	1.7	7.1	1.69	24.85
Design C	0.8	6.61	1.9	7.4	0.8	25.49