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Titer-plate formatted continuous flow thermal reactors: Design and performance of a nanoliter reactor

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Abstract

Arrays of continuous flow thermal reactors were designed, configured, and fabricated in a 96-device (12 × 8) titer-plate format with overall dimensions of 120 mm × 96 mm, with each reactor confined to a 8 mm × 8 mm footprint. To demonstrate the potential, individual 20-cycle (740 nL) and 25-cycle (990 nL) reactors were used to perform the continuous flow polymerase chain reaction (CFPCR) for amplification of DNA fragments of different lengths. Since thermal isolation of the required temperature zones was essential for optimal biochemical reactions, three finite element models, executed with ANSYS (v. 11.0, Canonsburg, PA), were used to characterize the thermal performance and guide system design: (1) a single device to determine the dimensions of the thermal management structures; (2) a single CFPCR device within an 8 mm × 8 mm area to evaluate the integrity of the thermostatic zones; and (3) a single, straight microchannel representing a single loop of the spiral CFPCR device, accounting for all of the heat transfer modes, to determine whether the PCR cocktail was exposed to the proper temperature cycling. In prior work on larger footprint devices, simple grooves between temperature zones provided sufficient thermal resistance between zones. For the small footprint reactor array, 0.4 mm wide and 1.2 mm high fins were necessary within the groove to cool the PCR cocktail efficiently, with a temperature gradient of 15.8°C/mm, as it flowed from the denaturation zone to the renaturation zone. With temperature tolerance bands of ±2°C defined about the nominal temperatures, more than 72.5% of the microchannel length was located within the desired temperature bands. The residence time of the PCR cocktail in each temperature zone decreased and the transition times between zones increased at higher PCR cocktail flow velocities, leading to less time for the amplification reactions. Experiments demonstrated the performance of the CFPCR devices as a function of flow velocity, fragment length, and copy number. A 99 bp DNA fragment was successfully amplified at flow velocities from 1 mm/s to 3 mm/s, requiring from 8.16 minutes for 20 cycles (24.48 s/cycle) to 2.72 minutes for 20 cycles (8.16 s/cycle), respectively. Yield compared to the same amplification sequence performed using a bench top thermal cycler decreased nonlinearly from 73% (at 1 mm/s) to 13% (at 3 mm/s) with shorter residence time at the optimal temperatures for the reactions due to increased flow rate primarily responsible. Six

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different DNA fragments with lengths between 99 bp and 997 bp were successfully amplified at 1 mm/s. Repeatable, successful amplification of a 99 bp fragment was achieved with a minimum of 8000 copies of the DNA template. This is the first demonstration and characterization of continuous flow thermal reactors within the 8 mm × 8 mm footprint of a 96-well micro-titer plate and is the smallest continuous flow PCR to date.

Keywords

CFPCR; 96-well PCR; Nanofluidic device; High Throughput PCR; Thermal Reactor

1. Introduction

The development of nucleic acid technology and micro/nano-fabrication techniques over the past two decades has led to many pioneering devices with the potential to improve diagnosis and treatment of diseases. One typical example is the micro-polymerase chain reactor (μ PCR) for the amplification of nucleic acids. Micro PCRs contribute rapid amplification, high accuracy, and the potential for integration with other functional components to form micro total analysis systems (μ TAS) [1]. In parallel, high throughput reactors configured in titer plate format (96-1572 wells), loaded and manipulated using robotics, were used for large-scale biochemical analyses like the Human Genome Project (HGP) and in commercial clinical laboratories. The benefits included minimized contamination, reduced labor and material costs, and improved accuracy [2]. Combining the concepts of μ TAS and a parallelized high throughput approach could maximize the power of nucleic acid analysis and lead to accelerated detection and more appropriate treatment for patients.

To precisely diagnose a disease using nucleic acid analysis, several steps are mandatory, including: cell capture, cell lysis, nucleic acid purification, nucleic acid amplification, and detection/identification [3]. Amplification, typically using PCR, is a key step in the analysis [1]. PCR is based on thermally-driven reactions at three temperatures: 90–95°C for denaturation, 50–70°C for renaturation, and 70–75°C for extension [4]. By repeating the thermal cycles from 20–40 times, billions of copies of the target molecules can be produced from even a single starting copy. Successful amplification relies on the correct chemical composition of the reagent mixture and precise control of the temperatures of the cycling unit at each stage. Thermally-driven reactions are used for other steps in the analysis sequence, including the ligase detection reaction for identification of single –point mutations [5,6].

The continuous flow polymerase chain reactor (CFPCR) [7–13] is an alternative to chamber-type PCRs, which are miniaturized versions of bench-top instruments, for thermal cycling [14–18]. The CFPCR uses a continuous flow of reagents in a microchannel passing through three thermostatic zones. The advantages of CFPCR are less thermal capacitance and higher heat transfer rates to the PCR cocktail. This can result in rapid amplification and the potential to approach the theoretical limit of the biochemical reaction, which is determined by the kinetics of polymerase extension [7].

Several arrays of PCR devices have been reported [19–22], but all were chamber-type thermal reactors. A PCR array with picoliter volume was fabricated on a silicon substrate with different size cavities, ranging from 1.3 pl to 32 μ l in volume to examine the minimum volume required for a successful μ PCR [19,20]. The device was repeatedly moved manually between three constant temperature heat blocks by moving an attached weight. The times used for the experiment were 5 s for denaturation, 5 s for renaturation, and 10 s for extension. The total time for 40 cycles was 18 minutes with a heating/cooling rate of 16°C/s.

Fluorescence detection was used to monitor the fluorescence intensity, then converted to an amplicon concentration. The PCR cocktail was mixed with 0.2 w/v (%) BSA to minimize the enzyme adsorption on the walls of the silicon microchambers. A 200 bp DNA fragment was successfully amplified in an 85 μ l microchamber.

A multi-chamber thermal cycler was described by Zou, et al. [21,22]. This device had 16 silicon heating components, arranged in a 4×4 pattern, that were mounted on a printed circuit board (PCB) by flip chip bonding to make 16 individual, isolated heater blocks. The microchamber PCR array was mounted on the heater blocks for repeated thermal cycles. The temperature sensors and heaters were aluminum, vapor deposited on the PCB board, and connected to proportional-integral-derivative (PID) controllers. The heating and cooling rates were 34–50 $^{\circ}\text{C/s}$ and 23–31 $^{\circ}\text{C/s}$. The heat transfer between each heater block or microchamber was reduced by the low thermal conductivity of the PCB board and the plastic microchamber device. The temperature distribution of the fluid in the microchamber was estimated using finite element analysis. A 320 bp DNA fragment in 20 μL of DNA cocktail was successfully amplified for 30 cycles in 15 minutes.

In addition to the typical microchamber-type PCR arrays, high throughput PCR was realized in a continuous flow environment by generating multiple analyte droplets in a polycarbonate microchannel [23]. Two inlets were connected to the device, one for the PCR cocktail and the other for a carrier oil, either a light mineral oil or sunflower oil. Flow through each inlet was controlled independently to allow selection of the size of the droplets. A 60 bp DNA fragment was amplified in 32 cycles using two different temperature zones, 95 $^{\circ}\text{C}$ for denaturation and 72 $^{\circ}$ for annealing/extension.

Several techniques have been used to estimate the temperature distribution in microfluidic devices to ensure that the correct temperatures for reactions and desired performance were achieved. Those techniques have included numerical simulations [7,9,24–27], analytical modeling [28], thermochromic liquid crystals [29–31], infrared cameras [9,32], and temperature-dependent fluorescent dyes [22,33].

Previous studies of the temperature distribution in μPCR devices have demonstrated that precise temperature control was critical for successful amplification using a μPCR [9]. Three key components necessary to achieve distinct, uniform temperature zones were: high thermal conductivity copper plates to supply an uniform temperature boundary condition to each temperature zone; increased lateral thermal resistance in the form of grooves in the backside of the device, taking advantage of the lower thermal conductivity of air, to reduce the heat transfer between temperature zones; and a thinner polycarbonate substrate to minimize the vertical temperature gradient between the heating elements and the microchannels and reduce the thermal capacitance.

As an investigation of the potential for closely packing continuous flow (CF) thermal reactors, a CF thermal reactor array with 96 devices was designed and fabricated on a polycarbonate substrate [34], with its dimensions, 120 mm long and 96 mm wide, and device positioning conforming to the titer plate standard [35]. Thermal management techniques introduced for single reactors [9] were adapted to the realization of both a nanoliter CF thermal reactor and an array of 96 CF thermal reactors to achieve three distinct and uniform temperature zones. Finite element analysis was used to guide the design and estimate the final temperature distribution. A set of three PCR experiments were performed to elucidate the factors limiting the amplification performance of the nanoliter CF thermal reactors.

2. Chip Design and Thermal Modelling

2.1. Configuration of the multi-well nanoliter CFPCR chip

A 96 CFPCR array polycarbonate chip is shown in Figure 1a together with a standard titer plate. Each nanoliter CFPCR unit of the 96 CFPCR array, was confined to an 8 mm $\times$ 8 mm area, as shown in Figure 1b. The spiral microchannels were 20 μ m wide in the denaturation and renaturation zones, and 40 μ m wide in the extension zone, with a constant depth of 40 μ m to obtain a nominal time ratio of 1:1:4 for denaturation, renaturation, and extension in the innermost microchannel at all flow velocities.

2.2 Thermal modelling methods

Continuous flow PCR requires three distinct, uniform temperature zones. To achieve the desired thermal performance, an understanding of the temperature distribution in a CFPCR device is important. Finite element analysis with ANSYS (v. 11.0, ANSYS, Inc., Canonsburg, PA) was used to parametrically determine the dimensions of the thermal reactors and to estimate the temperature distribution. First, thermofluidic simulations were used to design the thermal resistances between the 95°C of the denaturation zone and the 55°C of the renaturation zone. Second, a thermal model was used to understand the temperature distribution across 20 microchannels packed in an 8 mm $\times$ 8 mm area. Third, a series of thermofluidic simulations were used to estimate the temperature distribution of the PCR cocktail along a single microchannel as a function of the flow velocity.

Two standard ANSYS mesh elements were used in the simulations: a 3-D thermofluid element - an 8-node brick; and a 3-D thermal solid element – a tetrahedron with 10 nodes. The 3-D thermofluid element was used to mesh the PCR cocktail in the microchannel and the 3-D thermal solid element was used for the polymer substrate and the copper plates of the heating unit. Due to the large differences in the thermal properties of the copper plates and the polymer, the model was an ill-conditioned conjugate heat transfer problem. A robust solver, using the Preconditioned Generalized Minimum Residual method, was used to generate a more reliable solution [36]. Grid sensitivity was evaluated using three different element sizes to ensure that the simulated results were independent of element size. The natural convection coefficient for the three models was assumed to be 15 W/m² K on the surfaces exposed to the ambient environment.

2.3 Design of the thermal resistance between the denaturation and renaturation zones

For simplicity, the preferred cooling mechanism for the 96 CFPCR array was natural convection. The heat dissipation capability of the device depended on the natural convection coefficient and the surface area available for convection. The convection coefficient was environmentally-dependent, so that the surface area had to be maximized to ensure adequate cooling. Thermofluidic simulations were used to identify configurations that increased the convection area and were achievable using reasonable microfabrication processes. One of the challenges in realizing a nanoliter CFPCR device was the cooling of the PCR cocktail in the microchannel as it passed from the denaturation zone temperature (95°C), to the 55°C of the renaturation zone over a short distance. The total length of the renaturation zone on the innermost microchannel was only 6 mm, which included sections for both the renaturation reaction and for the temperature transition from the denaturation to the renaturation zones.

Thermal convection and conduction are equally important in a microchannel [8] so a model including polycarbonate, copper plates, and microchannels, was created using ANSYS. The geometry and the boundary conditions for the model to assess the temperature distribution along the center of the microchannel from denaturation to renaturation are shown in Figure 2a. The lateral heat transfer orthogonal to the 20 parallel microchannels was neglected in

this simulation, with $\frac{dT}{dZ}=0$, due to the assumption that the controller would produce uniform temperatures over each temperature zone. The inlet and outlet were also considered

adiabatic, $\frac{dT}{dX}=0$ [8]. The lengths of the denaturation and renaturation sections in the model were 6 mm, which corresponded to the lengths of the denaturation and renaturation zones along the innermost microchannel. Heat fluxes, representing the two Kapton-mounted electrical resistance heaters, were applied to the two individual copper plates to maintain the denaturation and renaturation temperatures. The groove used to decrease the lateral heat transfer between zones in prior work was not adequate for the smaller footprint devices as shown in Figure 2b. A fin with a width of 400 μm , constrained by the diameter of the micromilling tools available for machining a trench in the mold insert, was added to the groove. Fin heights from 1.2 mm to 1.5 mm were examined parametrically to understand the temperature distribution along the center of microchannel as a function of the different exposed convection areas. Thermal properties, including thermal conductivity, specific heat and density, from the literature were used for polycarbonate and copper plate [9]. The temperature dependent thermal properties of water, were used to represent the PCR cocktail [37].

Figure 2b shows the steady-state temperature distribution of the PCR cocktail along the center of the microchannel at zero flow velocity with different height fins. Temperature tolerance bands, $95^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $55^{\circ}\text{C} \pm 2^{\circ}\text{C}$, were used to quantify the simulation data. The two inserts in Figure 2b show the enlarged areas at the start and end of the transition from denaturation to renaturation. The simulation results are summarized in Figure 3. With a 1.2 mm tall fin the temperature dropped from 93°C at 4.56 mm from the inlet to 57°C at 6.84 mm from the inlet, giving a temperature gradient of $15.8^{\circ}\text{C}/\text{mm}$. When the height of the fin was increased, the temperature gradient increased. All of the fins lead to the desired temperature profile, but with different temperature gradients due to the different convection areas. The 1.2 mm high fin was selected and added between the denaturation zone and the renaturation zone in each CFPCR device to keep the device footprint small and achieve the desired set point temperatures. Although the 1.5 mm high fin had the steepest temperature gradient, it would be more difficult to fabricate a trench on the mold insert with an aspect ratio of 3.75 using micromilling and harder to demold the chips hot embossed using the mold insert.

2.4 Thermal simulations of the CFPCR device

Each thermal reactor, with either 20 cycles or 25 cycles, was confined to an area of $8\text{ mm} \times 8\text{ mm}$ to adhere to the footprint of a titer plate and to allow sufficient space for reservoirs for input and output. To further understand the effects of thermal management techniques on the temperature distribution of each reactor, thermal simulations were carried out. Figure 4a shows the geometry and the boundary conditions for a single CFPCR device, which included three individual copper plates supporting the heaters and the polycarbonate CFPCR device. Grooves 1 mm wide by 1.2 mm deep separated the extension zone from the denaturation and renaturation zones. The 0.4 mm wide and 1.2 mm tall fin was placed in the groove between the denaturation to renaturation zones, creating parallel grooves.

A model using ANSYS was used to estimate the temperature distribution across the single CFPCR device. Three different heat fluxes were applied to the copper plates to achieve the desired temperatures for denaturation (95°C), extension (72°C), and renaturation (63°C). Natural convection was assumed on all exposed areas of the polycarbonate substrate and copper plates with an initial temperature of 25°C . The surface temperature distribution across the center of the microchannels was extracted and the data plotted using Matlab (7.0,

The MathWorks, Inc., Natick, MA). Figure 4b shows the thermal contours across the midpoints of the microchannels as a function of location. The temperature contours were within $\pm 2^\circ\text{C}$ of the target temperatures. Four straight line paths, indicated by the arrows in Figure 4b, were used to illustrate temperature variation and uniformity along the innermost microchannel. Path 1 estimated the temperature uniformity in the extension zone. The other paths showed the temperature variation and gradients across zone boundaries including: Path 2 from the extension to the renaturation zone, Path 3 from the renaturation to the denaturation zone, and Path 4 from the denaturation to the extension zone (as shown in Figure 4b). The temperature along each path is shown in Figure 5 with $\pm 2^\circ\text{C}$ temperature tolerance bands. The transition distances between zones for Path 2, Path 3, and Path 4 were 1.15 mm, 1.78 mm, and 1.37 mm corresponding to temperature gradients of $11.3^\circ\text{C}/\text{mm}$, $20.3^\circ\text{C}/\text{mm}$, and $13.9^\circ\text{C}/\text{mm}$, respectively. Along Path 1, the temperature varied by only 0.3°C . The estimated temperature distributions along all four paths were used to determine the percentage of the microchannels within $\pm 2^\circ\text{C}$ of the target temperatures. Table 1 shows the designed length of the innermost microchannel within each temperature zone compared to the length of channel within the $\pm 2^\circ\text{C}$ temperature bands estimated using the results shown in Figure 5. The designed lengths for the denaturation, renaturation, and extension zones of the innermost channel were 6.058 mm, 6.058 mm, and 12.116 mm, respectively. From the thermal simulations shown in Figure 5, the estimated length in each temperature zone that was within the temperature tolerance envelopes were 4.398 mm, 4.728 mm, and 10.796 mm, respectively; 72.5%, 78% and 89.1% of the length of the microchannel was within the tolerance band for the desired temperature zones.

2.5 Thermofluidic simulations of flow in a single microchannel

A thermofluidic model was used to study the temperature distribution along the microchannel as a function of the flow velocity. Thermal conduction in the copper plates and polycarbonate, and thermal conduction and convection in the PCR cocktail in the microchannel, were accounted for in the model. The heat loss to the ambient environment from the exposed surfaces and heat transfer in the microchannel caused by flow of the PCR cocktail were addressed. The associated geometry and boundary conditions are shown in Figure 6a. The dimensions of the innermost microchannel from the actual spiral configuration were used for this model, with a length of 6 mm for the denaturation section, 6 mm for renaturation, and 12 mm for extension. The total thickness was 3.55 mm including 1 mm thick copper plates for heating, a 0.25 mm thick PC cover sheet, and a 2.3 mm thick PC substrate. The working fluid was assumed to be water with temperature dependent thermophysical and thermodynamic thermal properties [37].

The uniform temperature distribution demonstrated in each temperature zone for the CFPCR device simulation (Figure 4b), validated the neglect of lateral heat transfer across the 20

microchannels, $\frac{dT}{dZ}=0$, assumed in Figures 2a and 5a. The inlet and outlet were also

considered adiabatic, with $\frac{dT}{dX}=0$. A constant temperature of 72°C was applied at the inlet because it was the outlet of the extension zone of the previous loop. Different heat fluxes were applied to the three individual copper plates to yield the required steady-state target temperatures in the microchannels of 95°C , 55°C , and 72°C while accounting for the temperature gradient from the heaters to the microchannels.

Figure 6b shows the temperature distribution in the PCR cocktail along the microchannel at flow velocities from 0 mm/s to 4 mm/s. The $\pm 2^\circ\text{C}$ temperature tolerance bands with respect to the target temperatures were used to quantify the thermal performance. Figure 7 shows the residence times in each temperature band and the transition times between zones. As the

flow velocity of the PCR cocktail increased, the temperature profile shifted toward the extension zone and the duration of the plateau at each residence temperature decreased, leading to less time at the desired temperatures for the chemical reactions. The temperature gradient between the denaturation and renaturation zones at a zero flow velocity was $16.2^{\circ}\text{C}/\text{mm}$, which was 2.5% more than the estimate of $15.8^{\circ}\text{C}/\text{mm}$ in Figure 2 which was for a 1.2 mm high fin. The $0.4^{\circ}\text{C}/\text{mm}$ difference is reasonable since the model used for sizing the fin only accounted for two temperature zones and this model incorporated all three temperature zones.

To clarify the temperature shift at higher flow velocities, the temperatures at the top and bottom of the channel at the midpoints of the denaturation and renaturation zones were calculated. The point in the denaturation zone was located 2.5 mm from the inlet and the point in the renaturation zone was 7.5 mm from the inlet. In the denaturation zone, the bottom surface temperature was 95.06°C at a flow velocity of 0 mm/s and dropped to 94.49°C at 4 mm/s, the difference between the top and bottom surface temperatures increased from 0.16°C at 0 mm/s to 0.19°C at 4 mm/s. In the renaturation zone, the temperature at the bottom surface was 55.26°C at a flow velocity of 0 mm/s and increased to 55.86°C at 4 mm/s, with the difference between the top and bottom surfaces 0.04°C at 0 mm/s and 0.01°C at 4 mm/s. The temperature on the bottom surface was always slightly higher than the top surface because the heating units were below the microchannel and the major cooling mechanism was convection from the surface above the microchannel. If the temperature of the bottom surface at zero velocity was taken as a reference like 95.06°C for denaturation and 55.26°C for renaturation, the temperature of the PCR cocktail was lower than the baseline in the denaturation zone and higher in the renaturation zone at higher flow velocities due to convective effects. Overall, the microchannel was within the targeted tolerance limits for each temperature zone.

Experimental Methods

3.1. A nanoliter polycarbonate CFPCR device

Two metallic mold inserts were designed and fabricated using UV-LIGA [38] and micromilling [39]. Double-sided hot embossing with the two mold inserts was used to pattern both sides of a 2.3 mm thick polycarbonate substrate to fabricate the 96 CFPCR array. Laser ablation with an excimer laser (RapidX 1000 Series, Resonetics, Inc., Nashua, NH) was used to drill through holes in the polymer substrate to serve as inlet/outlet reservoirs and interconnects. Thermal fusion bonding with a customized fixture was used to seal the microchannels with a 0.25 mm thick polycarbonate sheet (Figure 1a).

To evaluate the performance of the CFPCR devices and validate the simulation results, both 20-cycle CFPCR (Figure 1b) and 25-cycle CFPCR were carried out on various length DNA fragments.

3.2 Experimental apparatus

Three separate copper plates were used as the heating stages for the nanoliter CFPCR devices. Three commercial Kapton heaters (HK5209R31.7L24.0627, Minco, Minneapolis, MN) were attached to the backsides of the three copper plates to supply uniform heat fluxes. Three Type K thermocouples (5TC-TT-K-30-36, Omega, Stamford, Connecticut) were inserted into grooves micromilled on the back side of each copper plate and sandwiched between the copper plate and the heater. Uniform temperature distributions were maintained over the three copper plates using PID controllers (96 Series, Watlow, St. Louis, MO). Thermally conductive double-sided tape (509-1000-ND, Digi-Key, Thief River Falls, MN) was used to hold the microfluidic device on the three copper plates during the experiments.

Two polyaryletheretherketone (PEEK) capillaries (1577-12X, Oak Harbor, WA) were inserted in the inlet and outlet reservoirs to connect to a syringe on a syringe pump (PicoPlus, Harvard Apparatus, Holliston, MA) and a sample collection tube.

3.3 PCR cocktail preparation

Six different length DNA fragments, 99 bp, 125 bp, 150 bp, 200 bp, 500 bp, and 997 bp, were used in the experiments. A common forward primer and six different reverse primers were designed to replicate the six different DNA fragments from a 48 kbp λ -DNA c1857Sam7 template (USB, Cleveland, OH). The PCR cocktail contained 10 mM Tris-HCl (PH 8.3), 1.5 mM MgCl₂, 50 mM KCl (USB, Cleveland, OH); the concentration of the nucleotides was 200 μ M (USB, Cleveland, OH), the template was 4.46 ng/ μ L, each forward and reverse primer was 0.2 μ M (IDT, Coralville, IA), bovine serum albumin (BSA) was 0.5 μ g/ μ L, and *Taq* DNA Polymerase was 0.1 units/ μ L (USB, Cleveland, OH). The renaturation temperatures for the six different DNA fragments were determined on a commercial thermal cycler (Mastercycler ep, Eppendorf, Inc., Westbury, NY) and the optimized temperature for amplification of each DNA fragment was used in the experiments. The total time required for 20 cycles on the commercial system was 1.5–2 h including 2 min for preheating, 7 min for a final extension, and 20 cycles consisting of denaturation for 1 min, renaturation for 1 min, and extension for 1 min.

3.4 Amplification and detection methods

The PCR cocktail was injected into the CFPCR device using a syringe pump (PicoPlus, Harvard Apparatus, Holliston, MA) at four different linear velocities, 1 mm/s (30 s/cycle), 2 mm/s (15 s/cycle), 3 mm/s (10 s/cycle), and 4 mm/s (7.5 s/cycle). For the 99 bp amplicon, the set point temperatures were 96.7°C in the denaturation zone, 73.6°C in the extension zone, and 64.5°C in the renaturation zone. The required renaturation zone temperature varied based on the length of the DNA fragment; the corresponding set point temperatures were 66.3°C for the 125 bp amplicon, 70.3°C for the 150 bp amplicon, 63.5°C for the 200 bp amplicon, 56.5°C for the 500 bp amplicon, and 69.3°C for the 997 bp amplicon.

The amplicons were collected from the outlet reservoir, mixed with 1X Blue/Orange Dye (Promega, Madison, WI), and injected into an agarose gel (Bio-Rad, Hercules, CA) for electrophoretic size analysis. The products were imaged using a Gel Logic 200 Imaging System (Kodak, New Haven, CT) with UV light. The resultant bands were evaluated with ImageQuant (V. 5.2, Amersham Biosciences, Piscataway, NJ).

4. Results and Discussion

4.1. DNA amplification at different flow velocities

The objective of these experiments was to examine the effect of flow velocity, or residence time, ranging from 1 mm/s to 4 mm/s, on the CFPCR amplification efficiency. The estimated residence and transition times in each temperature zone at the flow rates investigated are shown in Figure 7. Theoretically, the time required for the denaturation and renaturation reactions was less than 1 s while the extension rate was approximately 100 bp/s [40]. Figure 7 shows that the estimated residence time in the denaturation and renaturation zones was greater than 1 s for flow rates less than or equal to 2 mm/s. The dwell time in the extension zone was sufficient at all flow velocities for DNA fragments less than 200 bp in length, but only enough for the 500 bp fragment at 1 mm/s and inadequate for the 997 bp fragment at all velocities. Figure 8(a) shows a gel image which includes a marker, a control representing the amplicon from a commercial thermal cycler, and 3 amplicons from a 20-cycle nanoliter CFPCR device at flow velocities of 1 mm/s (24.48 s/cycle), 2 mm/s (16.32 s/cycle), and 3 mm/s (8.16 s/cycle); no amplicon was observed at a flow velocity of 4 mm/s.

Figure 8(b) shows the relative intensity of the amplicons produced at the different flow velocities compared to the control amplicon; the yield was 73% of that obtained from the commercial thermal cycler at 1 mm/s, 59% at 2 mm/s, and 13% at 3 mm/s. Successful amplification of the 99 bp DNA fragment was achieved at flow velocities up to 3 mm/s, requiring a processing time of 2.72 min for 20 cycles at 8.16 s/cycle. The lower amplification efficiency at higher flow velocities are most likely caused by shorter residence times in the desired temperature zones as indicated in Figure 7, with loss of enzyme or template due to nonspecific adsorption to the microchannel walls contributing as well [41].

4.2. Amplification of different size DNA fragments

The second experiment demonstrated the versatility of the system for amplification of different sized DNA fragments. By simply adjusting the set point temperature in the renaturation zone and changing the primer, the same device can be used for any length DNA fragment. Six primer sets were designed to amplify six different length DNA fragments starting at the same sequence region as the DNA template (i.e., the same forward primer). The PCR cocktail was injected into the microchannel at an average flow velocity of 1 mm/s. Figure 8(c) shows a gel image of the amplification results. The CFPCR successfully amplified DNA fragments ranging in length from 99 bp to 997 bp.

4.3. Determination of minimal template initial concentrations

To determine the lowest number of template copies needed for successful amplification in the nanoliter CFPCR device, different concentrations of template were used for experiments. The target was a 99 bp DNA fragment and the concentrations ranged from 4.46×10^{-5} ng/ μ L (8×10^2 copies) to 4.46 ng/ μ L (8×10^7 copies). The PCR cocktail was injected into the microchannel at an average flow velocity of 1 mm/s. The 20-cycle CFPCR device was used to successfully generate 99 bp amplicons for the four higher template concentrations, while 25 cycles were needed for the three lower concentrations (Figure 8d). The smallest concentration for which a successful amplicon could be observed was at 4.46×10^{-4} ng/ μ L, corresponding to 8×10^3 copies. These results may be improved by using a more sensitive detection method, such as laser-induced fluorescence detection.

5. Conclusions

The performance of a 96 CFPCR array configured in a standard 96-well titer plate format was evaluated. Finite element analysis was used to select the appropriate dimensions for the nanoliter CFPCR based on a series of thermofluidic models. As with larger devices, three distinct temperature zones were obtained in an 8 mm $\times$ 8 mm footprint, by incorporating grooves to increase the thermal resistance on the back side of the substrate. Between the denaturation and renaturation zones, a 0.4 mm wide and 1.2 mm deep fin was also needed to provide sufficient thermal isolation and increase the cooling capability. Thermal performance was quantified in the simulation results using $\pm 2^\circ\text{C}$ temperature tolerance bands. For the innermost microchannel in the spiral, 72.5% of the denaturation section was within the temperature band, 75% of the renaturation zone was within the limits, and 89.1% of the extension sector was in the proper temperature range. The thermal analysis also estimated the temperature distribution along the microchannel at different flow velocities. When the flow velocity was increased, the PCR cocktail had shorter residence times in the target temperature zones and longer transition times between temperature zones. The temperatures of the top and bottom surfaces inside the microchannel showed that the PCR cocktail did not have enough time to reach the desired temperatures at higher flow velocities.

Experiments were used to assess the performance of the nanoliter CFPCR device: (1) determining the effect of flow velocity on amplification; (2) demonstrating amplification of different length DNA fragments, which required different renaturation temperatures; and (3) identifying the minimum number of template copies required for successful amplification. Successful amplification of a 99 bp DNA fragment at average flow velocities up to 3 mm/s, leading to a 20 cycles of amplification in 2.72 minutes (8.16 s/cycle) with a yield of 73% to the amplicons from control. Six different DNA fragments were amplified successfully using the CFPCR device and gel electrophoresis was used to validate the amplicons. The minimum number of copies for amplification of a 99 bp DNA template at an average flow velocity of 1 mm/s was 8000.

The construction of this high throughput, 96- CFPCR device will provide the ability to rapidly amplify DNA templates to provide timely results. In addition, standard sample handling hardware can be used to load and extract samples from the plate to allow simple integration into existing genetic analysis pipelines. Fabrication and performance of a 96-well solid-phase extraction microfluidic device configured into a standard titer plate format was also reported [38]. Integration of these two devices, and other modules for additional thermally-driven reactions, using the proper interconnect technology will provide the ability to generate fully integrated systems that can provide high throughput analyses.

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Figure 1.

(a) The physical dimensions of a 96 CFPCR array and a standard micro-titer plate; (b) A single CFPCR, 8 mm × 8 mm, including three distinct temperature zones compared to a penny.

Figure 2.

(a) Boundary conditions for the model used to determine the physical dimensions of the fin needed to obtain sufficient cooling between the denaturation and renaturation zones, including the thermal and fluidic boundary conditions; (b) The temperature distribution in the PCR cocktail at the center of a microchannel for different fin heights, with a fin width of 400 μm .

Figure 3.
The transition distance and the temperature gradient for different groove depths between the denaturation and renaturation zones.

Figure 4.

(a) Boundary conditions for the thermal simulation for a single CFPCR device; (b) The simulated temperature distribution at the midplane of 20 microchannels with the $\pm 2^\circ\text{C}$ thermal contour lines, which also could be used to elucidate the temperatures of the 20 microchannels as a function of location on the chip.

Figure 5.

Temperature distribution along the paths defined in Figure 4(b), which are located in the innermost microchannel.

Figure 6.

(a) The boundary conditions used for the thermofluid model to estimate the temperature distribution along the center of the microchannel at different flow velocities; (b) The temperature distribution along a single microchannel at flow velocities from 0 mm/s to 4 mm/s.

Figure 7.

The total time of each thermal cycle and the residence and transition times (s) for the PCR cocktail in each temperature zone as a function of flow velocity (mm/s). Isothermal heating zones are represented as solid segments and transitions between isothermal zones are shown filled with lines sloping up or down, depending on the direction of the temperature transition.

Figure 8.

(a) Agarose gel image of amplicons from a commercial thermal cycler (Control), and the nanoliter CFPCR at linear flow velocities of 1 mm/s, 2 mm/s, and 3 mm/s. (b) The relative intensity of the amplification efficiency at each flow rate compared to the amplicon from a commercial thermal cycler (b) Amplification results for different DNA fragments from 99 bp, 125 bp, 150 bp, 200 bp, 500 bp, and 997 bp at a flow velocity of 1 mm/s (c) Amplification results for a 99 bp amplicon at different initial concentrations of template from 4.46×10^{-5} ng/ μ L to 4.46 ng/ μ L.

Table 1

The percentage of the innermost microchannel length located within a $\pm 2^{\circ}\text{C}$ temperature tolerance band of each target temperature.

	Designed Length (mm)	Estimated Length (mm)	Percentage (%)
Denaturation (95°C)	6.058	4.398	72.5
Renaturation (55°C)	6.058	4.728	78
Extension (72°C)	12.116	10.796	89.1