VIP Microreactors

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A Pocket-Sized Convective PCR Thermocycler**

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The ability to make technologies for rapid diagnosis of infectious disease broadly available in a portable, low-cost format would mark a revolutionary step forward in global public health.[1,2] A critical challenge to these efforts is that a large segment of the population that is most in need of these advances resides in locations that offer limited or nonexistent laboratory infrastructure.[3,4] At the same time, many diagnostic assays rely on the polymerase chain reaction (PCR), which requires thermocycling instruments that are relatively slow and consume considerable electrical power to perform repeated heating and cooling steps.[5] Herein, we introduce an innovative thermocycling system that harnesses natural convection phenomena to amplify DNA rapidly by the PCR in a greatly simplified format. A key element of this design is an architecture that allows the entire thermocycling process to be actuated pseudo-isothermally by simply maintaining a single heater at a constant temperature, thereby enabling a pocket-sized battery-powered device to be constructed at a cost of about US$10. These devices are straightforward to design and build, easy to operate, and uniquely address a critical need for expanded availability of PCR-based diagnostics.

The PCR continues to be an indispensable tool in a diverse array of genomic analysis applications including medical diagnostics, pathogen and infectious disease detection, forensics, population-scale polymorphism, and mutation studies.[6,7] The development of capabilities to amplify long targets (>1 kb) and to coamplify multiple targets simultaneously (multiplex PCR) has further extended the inherently robust capacity of the PCR for efficient DNA replication. Despite these advances, the timescales required to perform a typical reaction generally remain on the order of hours—a rate much slower than would be expected on the basis of kinetics alone. This is because conventional thermocycling instruments typically employ a hot-plate design consisting of a metal block whose high heat capacity, combined with the relatively low thermal conductivity of the plastic tubes or multiwell plates used to contain the reagents, severely limits achievable heating and cooling rates. Consequently, the majority of time and electrical power consumed is expended regulating the temperature of the instrument's structural components rather than driving the reaction.

Buoyancy-driven natural convection phenomena offer an attractive way to overcome these limitations. By applying a static temperature gradient across an appropriately designed reactor geometry (e.g., a cylindrical cavity or closed loop), a continuous circulatory flow can be initiated that will repeatedly transport PCR reagents through temperature zones associated with each stage of the reaction.[8–11] This arrangement is highly advantageous because the need for active heating and cooling is eliminated, which greatly simplifies instrument design. Furthermore, rapid thermocycling is achievable since reagents quickly attain local thermal equilibrium as they travel through the temperature field. Despite this promise, the development of convective thermocycling technology has not yet advanced beyond the proof-of-concept level, with results obtained over a very limited range of template and target sizes (without multiplex capability) and employing instrument designs that are cumbersome for routine use.[12–15] Herein, we show that these shortcomings can be addressed through the design of simplified closed-loop convective thermocyclers capable of rapidly amplifying a wide range of targets in both single and multiplex formats.

First, we illustrate the basic concept in a thermocycling device constructed by positioning two thermoelectric heaters along the perimeter of a fluoropolymer tubing loop that follows a triangular path (Figure 1a and the Supporting Information). PCR reagents are pipetted directly into 9-cm-long tubing segments and then the free ends are joined together by a small sleeve of larger diameter tygon tubing to yield a loop that is affixed around the heater assembly. The heaters are independently adjusted to maintain denaturation (95 °C) and extension (72 °C) temperatures across the two inclined legs, while the horizontal leg passively attains annealing conditions (ca. 58 °C). In this way, thermocycling is passively actuated by the unidirectional convective flow generated in response to the imposed thermal gradient (the heater temperatures are held constant during the reaction). The fluoropolymer tubing used to construct the flow loops is stable at high temperatures (up to 205 °C) and is chemically inert, which eliminates the need for surface pretreatment processes that are often required to minimize nonspecific

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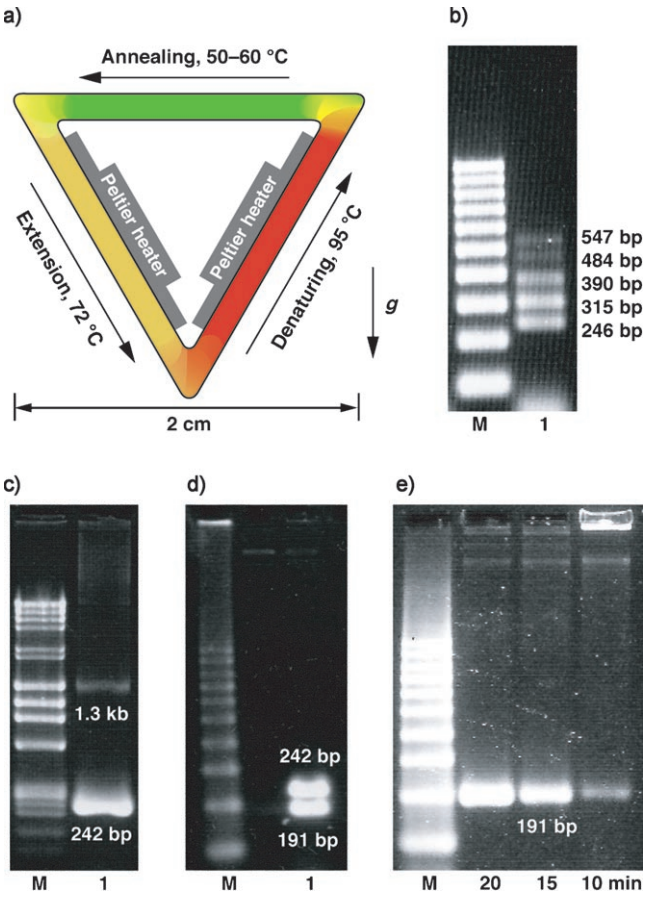

Figure 1. Design and performance of a simplified convective thermocycler. a) Thermocycler assembly constructed with a fluoropolymer tubing reactor and scaffold incorporating two thermoelectric heaters (see the Supporting Information for a photograph and more detailed description). g = direction of gravity. b–e) Agarose gel electrophoresis analysis of PCR products from different template/target combinations under various thermocycling conditions. M = marker. b) Multiplex amplification of five different human respiratory infection associated virus targets in 50 minutes with a 16-μL flow loop. c) Coamplification of 1.3-kb λ-DNA and 242-bp L32 gene targets in 50 minuntes with a 16-μL loop. d) Coamplification of 191-bp influenza virus and 242-bp L32 gene targets in 50 minutes with a 16-μL loop. e) Amplification as a function of reaction time for a 191-bp target associated with membrane channel proteins M1 and M2 of the influenza-A virus with a 25-μL flow loop.

binding interactions in many miniaturized PCR systems.[16–17] No modifications to standard reaction protocols are necessary, and the tubing is optically transparent, which makes it amenable for use in real-time PCR applications.

The versatility of this convective-flow thermocycling system is demonstrated by its ability to amplify a multiplex mixture of targets associated with five different respiratory viruses in 50 minutes with a 16-μL flow-loop reactor (Figure 1b). Simultaneous coamplifications were also performed in mixtures containing primers and template for a 1.3-kb target from a λ-DNA template and a 242-bp human L32 gene target (Figure 1c), as well as 191-bp influenza-A virus and 242-bp human L32 gene targets (Figure 1d) in 50 minutes with a 16-μL loop. Finally, we characterized amplification speeds in 10-, 16-, and 25-μL closed-loop reactors (same length but different diameters of tubing) with corresponding cycling times of 102, 69, and 42 s, respectively (flow velocities in the loops scale with tubing diameter). Amplification of a 191-bp target with a 25-μL loop was detectable by agarose gel analysis after as little as 10 minutes of reaction time (Figure 1e). Smaller-volume flow loops (i.e., constructed from smaller-diameter tubing) deliver longer cycling times and produce detectable products in 20–30 minutes (see the Supporting Information). This level of speed and versatility is comparable to that achievable in many advanced thermocyclers, but in a format that offers an exceptional degree of electronic and mechanical simplicity.

Next, we demonstrate how the convective thermocycling concept can be adapted for use in portable diagnostic applications by introducing a pocket-sized device incorporating three aluminum blocks, each corresponding to one of the three PCR temperature zones. Thermal interconnections (e.g., screws made from materials of different thermal conductivity) are incorporated to regulate the heat flow between blocks so that when the denaturing block is heated to 95 °C, the remaining blocks passively attain the desired annealing and extension conditions (Figure 2a and the Supporting Information). This arrangement makes it possible to actuate the entire thermocycling process pseudo-isothermally by simply maintaining a single heater at constant temperature (i.e., 95 °C). Furthermore, the temperatures at each stage of the reaction can be independently adjusted by purely mechanical means (by appropriate selection of screw size, material, and number of screws), which significantly reduces the cost and complexity. Another feature of this design is that the 72 °C extension zone is structured to occupy the largest fraction of the loop perimeter to promote amplification of longer targets (>1 kb). We incorporated this architecture into a prototype by employing a simple on–off temperature control circuit that allows the entire device to be powered by two AA-size batteries (Figure 2b). Despite its simplicity and low cost (hardware costs approximately US$10; disposable tubing costs only a few cents per reaction), a 1.3-kb target can be amplified from a λ-DNA template in 50 minutes with a 7-μL reaction volume (Figure 2c).

Finally, we present a simplified analytical model that highlights the underlying physics and establishes general design criteria for convective-loop thermocyclers. The key physical parameters that influence convective-loop flows become evident by first noting that the magnitude of the destabilizing buoyant forces relative to the thermal and viscous restoring forces can be expressed in terms of a dimensionless Rayleigh number $Ra = (g\cos\theta)\beta(\Delta T)D^3/\nu\kappa$. Here, $g\cos\theta$ is the component of gravitational acceleration acting along the flow direction (i.e., θ is the angle that a segment of the flow loop makes with respect to the vertical), ΔT is the temperature difference imposed between the two heaters, D is the tube diameter, and β, κ, and ν are fluid properties (thermal-expansion coefficient, thermal diffusivity, and kinematic viscosity, respectively). Consequently, the magnitude of Ra (and hence the flow velocity) increases with increasing ΔT and with increasing tube diameter, which is consistent with data obtained by monitoring fluorescent

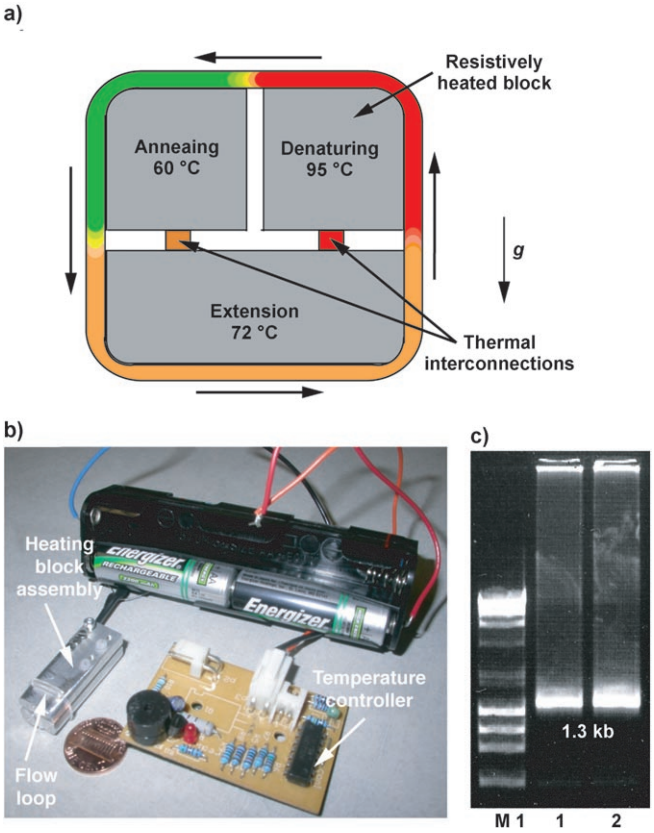

Figure 2. A pocket-sized battery-powered convective thermocycler. a) Design schematic depicting three blocks joined by thermal interconnections (i.e., screws of different thermal conductivity) that regulate heat flow such that when the denaturing block is heated to 95 °C, the remaining blocks passively attain the desired reaction temperatures. The 72 °C block is larger to allow extension to occur during a greater fraction of time in each cycle. See the Supporting Information for a photograph and more detailed description. b) The assembled PCR thermocycler shown with accompanying temperature-control circuit. As the design in (a) makes it possible to perform the reaction by maintaining a single heater at constant temperature, the entire device can be powered by two AA-size batteries. c) Products from two independent amplifications of a 1.3-kb target from a λ-DNA template in 50 minutes with a 7-μL loop in the device shown in (b).

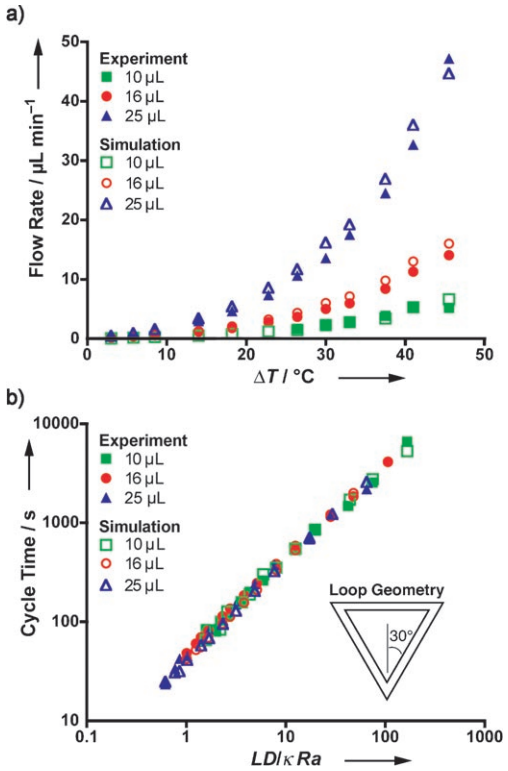

Figure 3. Experimental and computational analysis of the convective flow in the triangular loop configuration depicted in Figure 1 a. Average flow rates were measured by observing the motion of fluorescent microsphere tracers, and 2D flow simulations were performed by using FLUENT software. a) Volumetric flow rate as a function of the temperature difference applied between heaters (ΔT) and the reactor volume (volumes of 10, 16, and 25 μL correspond to tubing diameters of 320, 400, and 510 μm, respectively). b) Flow data plotted as a function of cycle time follow a scaling that allows the points to collapse onto a master design curve. The temperature dependence of fluid properties β, κ, and ν is accounted for in these calculations; reactor volumes are determined from the tubing length and inside diameter; $\theta = 30°$.

microsphere tracers inside the flow loops (Figure 3 a). Under PCR conditions, average flow velocities of approximately 0.9, 1.3, and 2.1 mm s^{-1} were obtained in 10-, 16-, and 25-μL loops, respectively.

Over the range of flow conditions of interest for PCR, analysis of the momentum and energy-balance relationships for the case of a loop geometry symmetric about the vertical centerline (e.g., the triangular configuration in Figure 1 a) suggests that Ra should scale with uD/κ (u is the average velocity; see the Supporting Information). This scaling is evident when cycling times determined both from flow visualization experiments and from computational simulations are plotted versus the parameter $LD/\kappa Ra$ (L is the loop length): data for various reactor volumes collapse onto a single curve (Figure 3 b). This relationship permits strategic design of reactors that satisfy virtually any combination of volume and cycling time.

Experimental Section

Thermocycler construction (see the Supporting Information for further details): Convective-flow loop reactors were constructed by using 9-cm lengths of thin-wall fluorinated ethylene propylene (FEP) tubing (Zeus Industrial Products, Inc.). Loops were constructed from three different tubing diameters: inside diameter 320, 400, and 510 μm (28-, 26-, and 24-gauge sizes), corresponding to reactor volumes of 10, 16, and 25 μL, respectively. Lengths of tubing were filled with PCR reagents and then the two open ends were joined together by using a short sleeve of tygon tubing.

After being loaded, the loops were mounted on a fixture incorporating two independently controlled thermoelectric Peltier heaters (Melcor Corporation) positioned on an aluminum scaffold designed to create a triangular flow path. The flow loops were affixed directly to the heater surfaces by using aluminum adhesive tape (Axygen Scientific) to ensure thermal contact and a uniform temperature distribution. Flat thermocouple probes (Model #SA1-(K), Omega Engineering, Inc.) were affixed to the heater surfaces to monitor their temperature. The heaters were independently maintained at 95 °C (denaturing) and 72 °C (extension), while the third (annealing) arm of the loop passively attained a temperature of 58 °C. The two Peltier heaters were powered by independent external DC power supplies, and temperatures were maintained by manually

adjusting the input power to each heater. At an ambient temperature of 22 °C, the input power requirement was 3.1 V at 660 mA and 1 V at 460 mA for heaters at 95 °C and 72 °C, respectively. A ceramic fiber insulation strip was wrapped around the triangular assembly to reduce heat loss and to insulate the loop from ambient temperature fluctuations.

A battery-powered three-block thermocycler was constructed and operated in a similar fashion, except that one of the blocks was fitted with a resistance cartridge heater (Omega Engineering) and thermocouple probes were inserted into each block to monitor the temperature in each reaction zone. The blocks were interconnected by threaded screws of materials with different thermal conductivities (e.g., nylon and stainless steel) such that the temperature in each zone could be set by simply inserting the appropriate number of screws between blocks. A homebuilt on–off temperature control circuit was constructed that allowed the heater temperature to be powered and regulated so that a 50-min amplification reaction could be performed by using two AA-size batteries (Energizer NiMH rechargeable, 2200 mAh). See the Supporting Information for a circuit diagram. The temperature controller shares the same power source as the heater and consumes minimal power so that the majority of battery power can be directed to operate the heating element. No computer or electronic interface is required for the user to select cycling parameters. The flow-loop geometry incorporated a 5.5-cm length of 400-μm diameter tubing (volume 7 μL).

PCR protocols: Amplification performance was characterized by using several reaction systems (see the Supporting Information). Reagents for reactions involving amplification of the following targets were supplied in kits from Maxim Biotech, Inc.: a) a 191-bp target associated with the membrane channel proteins M1 and M2 of the influenza-A virus from a 3.9-kb template (Catalog #SP-10377), and b) a multiplex system incorporating a primer mix for amplification of five different respiratory infection associated virus targets (264-bp respiratory syncytial virus (RSV), 315-bp corona virus, 390-bp influenza virus, 484-bp adenovirus, and 547-bp rhino virus) (Catalog #MP-70178). Standard 50-μL reaction mixes contained optimized buffer/dNTP mix (30 μL; dNTP = deoxynucleotide triphosphate), primer mix (10 μL), doubly distilled H_2O (8.75 μL), template DNA (1 μL), and AmpliTaq polymerase (0.25 μL, 5 units μL^{-1}; Applied Biosystems). After the reactions were complete, the products were aspirated from the flow loops, run on a 2% agarose gel at 60 V for 1 h, and stained with SYBR-Green I.

A kit for amplification of a 1.3-kb target from a λ-DNA template was also used (Phusion High-Fidelity PCR Kit; New England Biolabs, Inc.). Standard 50-μL reaction mixes contained H_2O (34 μL), 5× Phusion HF buffer (10 μL), 10 mM dNTP (1 μL), primers (2.5 μL), λ-DNA control template (2 μL, 0.5 ng μL^{-1}), and Phusion DNA polymerase (0.5 μL, 2 units μL^{-1}). The remainder of the reaction protocol was identical to that described above.

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