Exploring the limits of ultrafast polymerase chain reaction using liquid for thermal heat exchange: A proof of principle

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Thermal ramp rate is a major limiting factor in using real-time polymerase chain reaction (PCR) for routine diagnostics. We explored the limits of speed by using liquid for thermal exchange rather than metal as in traditional devices, and by testing different polymerases. In a clinical setting, our system equaled or surpassed state-of-the-art devices for accuracy in amplifying DNA/RNA of avian influenza, cytomegalovirus, and human immunodeficiency virus. Using Thermococcus kodakaraensis polymerase and optimizing both electrical and chemical systems, we obtained an accurate, 35 cycle amplification of an 85-base pair fragment of *E. coli* O157:H7 Shiga toxin gene in as little as 94.1 s, a significant improvement over a typical 1 h PCR amplification. © *2010 American Institute of Physics*. [doi:10.1063/1.3530452]

Real-time polymerase chain reaction has revolutionized biochemistry.[1–4] These reactions utilize selective primers to simultaneously identify and amplify specific nucleic acid molecules. By allowing users to monitor the progress of polymerase chain reaction (PCR) as it occurs, real-time PCR (Refs. 5 and 6) provides information on the DNA in a sample,[7,8] removing many of the limitations of earlier end-point PCR. More recent advances include techniques for precise, rapid thermal control of nanoliter and microliter analyte volumes.[9–14] Traditionally, however, diagnostic tests are performed only in large, expensive thermal cyclers with slow thermal ramp rates that take about 1 h to complete a PCR amplification. This paper presents a method for ultrafast real-time thermal cycling through liquid thermal transfer.

Our thermal cycler (Fig. 1) used a liquid interface sandwiched between two high-powered Peltier devices (Marlow XLT2393, Dallas, TX) that worked in tandem to cycle the temperature rapidly. They rejected waste heat to a pair of fan-cooled heat sinks. These high efficiency heat sinks were sized to allow for increased cooling rates due to their ability to bias the heat exchange toward lower temperatures, optimizing power usage at less than 400 W. A phenolic gasket held 2.3 ml of thermal transfer medium (gallium eutectic) between the two face surfaces of the Peltiers, making direct thermal contact between them and the sample capillary tube. The liquid allowed for excellent thermal contact to the glass capillary tube. The small volume needed to make thermal contact required less than 200 J to raise the medium's temperature from 60 to 95 °C. In a typical thermal cycler, the thermal resistance between the junction of the solid metal block and the sample tube is a dominant factor in reducing the ability to transfer heat in and out of the sample, resulting in slow thermal cycling. A series of 6 μl PCR reaction mixtures was contained by thin-walled glass capillary tubes (Idaho Technology, Salt Lake City, UT). We controlled thermal cycling with a temperature control board (Oven Industries ENG1566, Mechanicsburg, PA) calibrated to in-sample temperature using a bare-wire type K thermocouple.

We supplied different voltages during heating and cooling via a separately designed circuit that worked in conjunc-

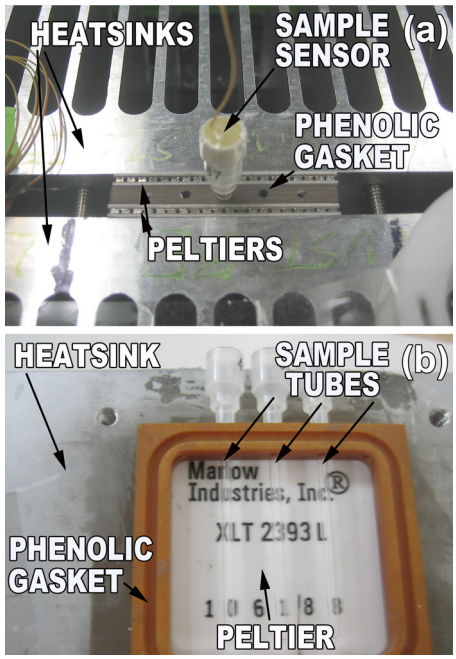

FIG. 1. (Color online) (a) Top view of the ultrafast polymerase chain reaction device. Visible is the heatsink-Peltier-phenolic-Peltier-heatsink sandwich that contained the gallium eutectic used for thermal transfer to the sample tube placed where the dummy sample sensor is visible in this photo. (b) Cross section of the heatsink-Peltier-phenolic-Peltier-heatsink sandwich where the sample tube and 2.3 ml gallium eutectic reservoir are visible.

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TABLE I. Characteristics of primers used for real-time PCR amplification of samples.

Primer sequence	Final concentration (mmol)	Amplicon size (bp)	Specificity
Forward: ATGCCCCAAATATGTGAAATCA Reverse: TGTCTGCAGCGTACCCACTC	0.5 0.5	201	H5N1 avian influenza
Forward: TCAGCCCAGAAGTAATACC Reverse: GCAGCTTCCTCATTGAT	0.5 0.5	139	Human immunodeficiency virus
Forward: GGCCTCTGATAACCAAGCC Reverse: GCACCATCCTCCTCTTCCT	0.5 0.5	337	Cytomegalovirus
Forward: CTGAGGCAAGAGCGATGTTACGGTTTG Reverse: TTGTACGAAATCCCCTCTGTATTTGCCG	0.5 0.5	85	Shiga toxin

tion with the control computer—thus taking advantage of increased Joule heating for faster heating ramp rate, while still maintaining optimum efficiency during cooling as described by the Peltier equation

$$\text{Peltier constant} = [Q + I^2R(t)/2]/I,$$

where Q represents the transferred heat, I represents the electrical current through the device, and R represents the Peltier's temperature-dependent electrical resistance. The Joule heating (I^2R) term of this equation always contributes waste heat and is determined by the temperature-dependent resistance of the Peltier junctions. We empirically determined the optimum voltages for our system and supplied them through the control system and achieved approximate maximum measured ramp rates of 44 deg/s heating and 17 deg/s cooling.

Accuracy cannot be sacrificed for speed. At the Ramathibodi Hospital in Bangkok, Thailand, we compared the performance of our liquid thermal device to standard diagnostic assays already run at the hospital. We completed assays for avian influenza (H5N1), and human immunodeficiency virus (HIV) under clinical conditions, running over 150 clinical samples of both RNA and DNA viruses under both typical thermal cycling regimes and increased thermal ramp rates. Real-time fluorescence data were collected and melt-curve end point detection was carried out on the LIGHTCYCLER 2.0 system for direct comparison.

H5N1. We tested our ultrafast real-time PCR instrument on samples from H5N1-infected chickens. Using the hospital's clinical real-time PCR primers (Table I) and thermal cycling profile, we amplified the *in vitro* transcribed H5 gene RNA to serve as a positive control, then analyzed H5N1 virus RNA from infected chicken samples.

H5N1 virus was analyzed using a LIGHTCYCLER RNA Master HybProbe kit (Roche No. 03-01895400) with 4 mmol $Mn(Oac)_2$ and 0.4 μmol probes (probe 1, Infa_TH5_fl; probe 2, Infa_TH5_lc). 5 μl of template RNA (10 pg −1 μg) were added and amplified with denaturation at 95 °C, annealing at 53 °C, and extension at 72 °C.

Melting curves of split-samples produced by the Roche LIGHTCYCLER and our portable real-time PCR system did not differ greatly. Both indicated a hybridization probe melting temperature of approximately 82.9 °C (±2.5 °C) [Figs. 2(a) and 2(b)], confirming that our ultrafast system is capable of amplifying H5N1 samples accurately.

HIV. Both PCR instruments analyzed RNA from patient blood samples, using a primer set (Table I) corresponding to the HIV-1 *gag* gene (GenBank Accession No. EF680874). This method used a two-probe hybridization mix for detection of the complete subtype of HIV. The HIV DNA was analyzed using a SuperScript™ one-step RT-PCR kit with Platinum® Taq (Invitrogen, No. 10928-042, Carlsbad, California), 0.4 mmol of each dNTP, 2.4 mmol $MgSO_4$, and 0.4 μmol probes (probe 1, gag-5LC* set 1; probe 2, gag-3FL* set 1; probe 3, gag-5LC* set 2; probe 4 gag-3FL* set 2). Template RNA (10 pg−1 μg) was added and amplified with denaturation at 95 °C, annealing at 50 °C, and extension at 72 °C. To enhance amplification, 10% bovine serum albumen was used to coat the capillary.

The DNA melting temperature of approximately 82.2 °C (±2.5 °C) [Figs. 2(c) and 2(d)] was similar for split-samples amplified using both PCR systems, confirming that our ultrafast system can amplify HIV samples accurately.

In the HIV tests, we began increasing the ramp rate and decreasing annealing and extension times, thereby limiting the total time of the reaction. Assays were completed in as little as 675 s, including a shortened 2 min reverse transcription and a total thermal cycling time of 555 s for amplifying real clinical samples, showing promise for a dramatic increase in sample-to-answer time for real-time PCR testing.

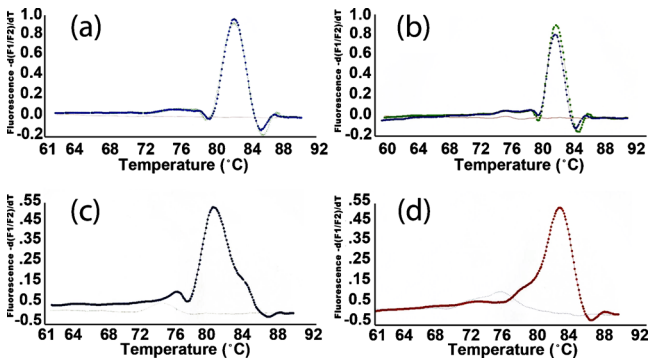

FIG. 2. (Color online) (a) End point melt curve of the clinical H5N1 split-sample amplified on the Roche LIGHTCYCLER 2.0. (b) End point melt curve of the clinical H5N1 split-sample amplified on the ultrafast PCR system. The melting curves of samples produced by both the Roche LIGHTCYCLER 2.0 and the ultrafast PCR system do not differ greatly and indicated a hybridization probe melting temperature of approximately 82.9 °C (±2.5 °C). (c) End point melt curve of the clinical HIV split-sample amplified on the Roche LIGHTCYCLER 2.0. (d) End point melt curve of the clinical HIV split-sample amplified on the ultrafast PCR system. The melting curves of samples produced by both the Roche LIGHTCYCLER 2.0 and the ultrafast PCR system do not differ greatly and indicated a hybridization probe melting temperature of approximately 82.2 °C (±2.5 °C).

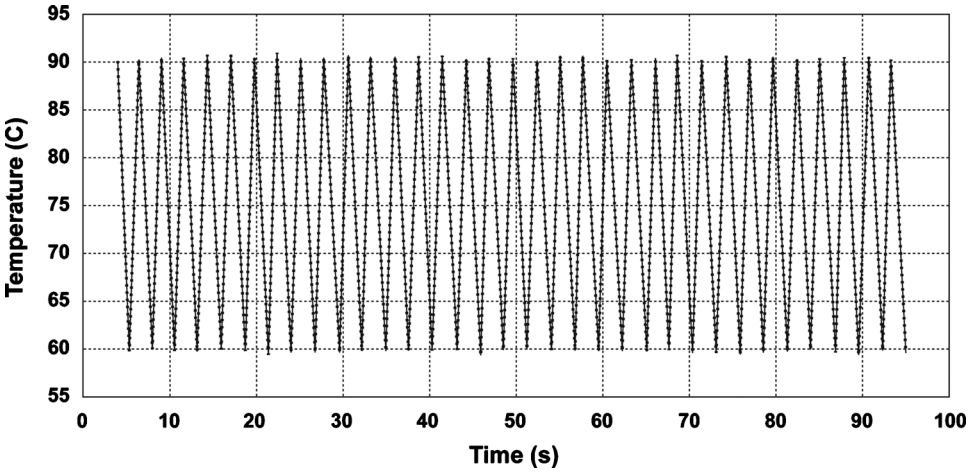

FIG. 3. Thermal profile used to achieve 94.1 s amplification of an 85-base pair fragment of *E. coli* O157:H7 Shiga toxin gene (*Stx*). These data were taken with a thermocouple placed directly in the sample.

To explore the extreme limits of rapid thermal cycling in our system, we amplified an 85-base pair fragment of *E. coli* O157:H7 Shiga toxin gene (*Stx*).[15] The DNA denaturation temperature was set to 86 °C and annealing temperature was set at 62 °C, with no wait time at each set point. Figure 3 shows thermal cycling data recorded by an in-sample thermocouple used in calibrating the instrument. We performed another set of experiments to determine the fastest possible amplification, varying thermal cycling ramp rates, polymerase type, and concentration in order to optimize the reaction for fastest amplification time. Shorter ramp times decreased the amplification efficiency while speeding the amplification, as the gel in Fig. 4 illustrates for *Thermococcus kodakaraensis* (KOD) polymerase. PCR products were analyzed on 12% nondenaturing polyacrylamide minigel [1× tris-borate-ethylenediaminetetraacetic acid (EDTA)] and stained with ethidium bromide. The gel shows increasingly stronger bands with slower PCR cycling conditions. The quality of the PCR reaction at this speed can be seen from the gel; only one band appears at an identical level in all samples, showing good specificity and lack of primer-dimer formation. *Thermus aquaticus* (Taq) polymerase required slower ramp rates to generate a product that is detectable on a gel. The reaction had to be slowed to over 2 min for 35 cycles in order to produce even a faint gel signal with Taq. KOD polymerase, however, worked well even with faster ramp rates, producing a gel signal with a 94.1 s, 35 cycle amplification (Figs. 3 and 4).

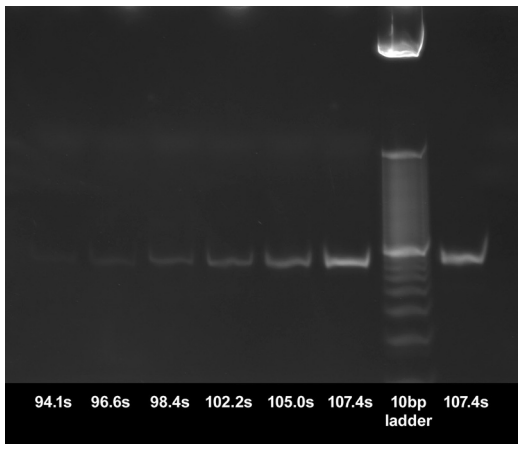

FIG. 4. Amplified fragment of *E. coli* O157:H7 Shiga toxin gene (*Stx*) analyzed on 12% nondenaturing polyacrylamide minigel (1× tris-borate-EDTA) and stained with ethidium bromide. Decreasing ramp times (faster ramp rates) were used to speed the amplification using *Thermococcus kodakaraensis* (KOD) polymerase. The gel shows increasingly stronger bands with slower PCR cycling conditions. The quality of the PCR reaction at this speed can be seen from the gel; only one band appears at an identical level in all samples, showing good specificity and lack of primer-dimer formation.

The technique of using liquid for thermal transfer in PCR needs further development and testing. We have demonstrated herein the principle, however, that accurate amplification can be obtained in a little over 1.5 min. The remaining task is to further refine the method. In a clinical environment, increased ramp rates and uniformity of liquid thermal transfer should allow faster, lower-cost testing for a wide variety of applications.

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