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Taking It to the Extreme: PCR at Wittwerspeed

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If the start times of PCR thermal cycling were plotted relative to frequency, we could expect to easily see sharp peaks of start times immediately before morning/afternoon coffee breaks, lunch times, and end of day, by operators safe in the knowledge that the amplification would take 1–2 hours to complete. Perhaps it is a little faster in many, but not most, diagnostic laboratories. Indeed, careful experimental planning when I was a student allowed leisure activities at opportune times “while the PCR was running.” At the time, these PCRs were typically 50–100 μ L in volume and used relatively thick 0.5-mL tubes.

In the late 1990s, the Guinness Book of World Records held an entry for “fastest DNA fingerprint” of approximately 11 min (essentially rapid real-time PCR detection). Further demonstrating that faster amplification concepts are not new, the term “rapid-cycle PCR” was coined in the early 1990s to define a thermal cycling program performed in 10–30 min (1). The Guinness entry and rapid-cycle PCR have a common denominator: the world record was held by the original capillary-based LightCycler, and similar capillaries were used for the rapid-cycle PCR—both in the hands of Carl Wittwer from the University of Utah.

The work of Professor Wittwer and his team is no stranger to these pages, as they previously published real-time PCR applications on the LightCycler (2) as well as early papers on the now well-adopted genotyping and variant scanning methodology of high-resolution melting analysis (HRMA) developed in his laboratory (3, 4)—work that more than justified Wittwer as the subject of an “Inquiring Minds” article, also in *Clinical Chemistry* (5). Since these developments, he has returned to his first molecular interest: increasing the speed of PCR.

Total PCR analysis time has decreased largely because of the advent of real-time PCR and now digital droplet PCR, in which gel electrophoresis bands have been replaced with amplification curves and scatter plots. Yet the major time component in the PCR itself is the thermal cycling

time. Recent efforts to decrease these times have used chip-based reactions using microvolume reactions in <6 min (6) or a rapid-cycling instrument with high convective heat transfer in <3 min (7). New companies are heating the reactions on particles (rather than heating reaction volumes) as a means to increasing ramp rates (the speed at which reactions heat and cool among the various temperature cycles) (8). Despite these newer methods, most have yet to reach the commercial market, and none have come close to penetrating the dominance of the slower Peltier-based technology.

Recent work in Wittwer’s laboratory has characterized the effect of PCR buffer and additive components on DNA polymerase activity using a novel nonradioactive assay (9), thus paving the way for further improvements in PCR. In this issue of *Clinical Chemistry*, Farrar and Wittwer return to these original reaction capillaries (thinner than the current LightCycler capillaries and thus allowing faster heat transfer to the reaction volumes) and describe their concept of Extreme PCR, pushing down the time of amplification to <15 s—as little as 14.7 s for 35 cycles, in fact (10). Such short times demand better accuracy.

Having frequently challenged the dogmas that existed in PCR and DNA melting kinetics, Wittwer’s laboratory has overturned the belief that higher primer and enzyme concentrations always lead to nonspecific amplifications. That belief is true if typical thermal cycling rates are used; but under extreme PCR conditions, the amplifications result in sharp specific bands (and melting peaks), presumably because of a lack of time for off-target products to be generated. This increased specificity was also observed for certain targets in the original rapid-cycle PCR of 10–30 min compared with thermal cycling of 2–3 h (1). Extreme PCR has also used existing enzymes successfully, as well as newer-generation highly processive systems. These current enzyme systems have been successfully used previously in reactions of <3 min (7).

Thus the extreme PCR technique requires 3 main parameters to ensure the robust, efficient, and sensitive amplifications we typically enjoy: rapid temperature cycling (very rapid!), higher primer concentrations (typically 20-fold higher than what would be considered usual), and higher enzyme concentrations (approximately 15-fold higher). Standard enzymes are less expensive now due to expiration of the *Taq* patents, and thus the chemistry modifications of extreme PCR look to have little

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effect on reagent costs. Interestingly, the reactions do not use any hot-start chemistry, noting the limitations of antibody amounts required and chemical modification activation times. Reaction setup on ice would be of limited practical use in the field, and instantaneous technologies such as aptamers would appear to be an attractive means of obtaining hot-start activation (11).

Amplification (and concurrent real-time fluorescent detection) in less than a minute highlights the considerable future potential of PCR. Although it is a mature technology, the wider prospect of field-based PCR is only starting to emerge in small field-based instruments (some field instruments having been made available previously to military or specialized agencies). However, the throughput vs time-to-result considerations of current instruments may limit their utility with relatively low sample size and run times of 30–60 min. The ability to perform extreme PCR on a field-based instrument will obviate the need for high-sample-number capacity. Such an instrument could be rapidly deployed in biosecurity cases for dangerous diseases or organisms where suspect samples may preferably be quarantined and prevented from travel to a testing laboratory. Extreme PCR run times further demonstrate the potential for point-of-care diagnostics in areas ranging from rapid viral diagnostics (such as in the 2014 Ebola outbreak) to testing and immediate treatment for sexual health patients with *C. trachomatis* infections—patients who often miss treatment due to time delays between samples taken and test results being made available. The rapid implementation of HRMA for genotyping could allow a patient to be tested for a susceptibility variant immediately before receiving a medication, to ensure appropriate efficacy.

The demonstration in this issue's report shows the speed at which amplification can occur, traditional speeds once again being limited by instrumentation. It uses rapid switching between 2 water baths (once more, harking back to the early days of PCR). It will be of great interest to observe the instrumentation developed that exploits this new technique: whether samples will indeed switch between steady temperatures or whether the sample can remain in a static position, as with most current instrumentation, and yet be rapidly heated and cooled in a reliable fashion using, for example, lasers (8), infrared light (12), or induction.

Although the time for testing of nucleic acid samples has decreased because of real-time PCR, and now vastly shortened cycling conditions have been shown with extreme PCR, the effort and time in nucleic acid extraction becomes further highlighted. A number of rapid extraction methods exist, using novel enzymes now commercially available (13) or high-efficacy 1-tube extraction

buffers (14). However, many of these systems have not been characterized for applications such the high-sensitivity requirements of pathogen detection. Other options—again, for applications in which targets are in abundance—may be the “direct PCR” enzyme and buffer systems that work directly with the sample material, removing the need for nucleic acid extraction altogether. However, whether these system enzymes and buffers have the processivity for extreme PCR remains to be seen.

So we now look forward to the meeting of extreme PCR with superlative sample extraction. The days of the coffee break (or paragliding stint) while the PCR thermal cycling runs may soon be over. But in the time it took to read these words, your nucleic acids results are in and a patient is already being treated.

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References

1. Wittwer CT, Garling DJ. Rapid cycle DNA amplification: time and temperature optimization. *Biotechniques* 1991;10:76–83.
2. Lay MJ, Wittwer CT. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin Chem* 1997;43:2262–7.
3. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003;49:853–60.
4. Zhou L, Errigo RJ, Lu H, Poritz MA, Seipp MT, Wittwer CT. Snapback primer genotyping with saturating DNA dye and melting analysis. *Clin Chem* 2008;54:1648–56.
5. Landau M. Inspiring minds. *Clin Chem* 2009;55:1744–6.
6. Neuzil P, Zhang C, Pipper J, Oh S, Zhuo L. Ultra fast miniaturized real-time PCR: 40 cycles in less than six minutes. *Nucl Acids Res* 2006;34:e77.
7. Wheeler EK, Hara CA, Frank J, Deotte J, Hall SB, Benett W, Spadaccini C, Beer NR. Under-three minute PCR: probing the limits of fast amplification. *Analyst* 2011;136:3707–12.
8. Stehr J, Hrelescu C, Sperling RA, Raschke G, Wunderlich M, Nichtl A, et al. Gold nanostoves for microsecond DNA melting analysis. *Nano Lett* 2008;8:619–23.
9. Montgomery JL, Wittwer CT. Influence of PCR reagents on DNA polymerase extension rates measured on real-time PCR instruments. *Clin Chem* 2014;60:334–40.
10. Farrar JS, Wittwer CT. Extreme PCR: efficient and specific DNA amplification in 15–60 seconds. *Clin Chem* 2015;61:145–53.
11. Dang C, Jayasena SD. Oligonucleotide inhibitors of Taq DNA polymerase facilitate detection of low copy number targets by PCR. *J Mol Biol* 1996;264:268–78.
12. Huhmer AFR, Landers JP. Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. *Anal Chem* 2000;72:5507–12.
13. Moss D, Harbison SA, Saul DJ. An easily automated, closed-tube forensic DNA extraction procedure using a thermostable proteinase. *Int J Legal Med* 2003;117:340–9.
14. Chomczynski P, Rymaszewski M. Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. *BioTechniques* 2006;40:454–8.



Extreme PCR: Efficient and Specific DNA Amplification in 15–60 Seconds

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BACKGROUND: PCR is a key technology in molecular biology and diagnostics that typically amplifies and quantifies specific DNA fragments in about an hour. However, the kinetic limits of PCR are unknown.

METHODS: We developed prototype instruments to temperature cycle 1- to 5- μ L samples in 0.4–2.0 s at annealing/extension temperatures of 62 °C–76 °C and denaturation temperatures of 85 °C–92 °C. Primer and polymerase concentrations were increased 10- to 20-fold above typical concentrations to match the kinetics of primer annealing and polymerase extension to the faster temperature cycling. We assessed analytical specificity and yield on agarose gels and by high-resolution melting analysis. Amplification efficiency and analytical sensitivity were demonstrated by real-time optical monitoring.

RESULTS: Using single-copy genes from human genomic DNA, we amplified 45- to 102-bp targets in 15–60 s. Agarose gels showed bright single bands at the expected size, and high-resolution melting curves revealed single products without using any “hot start” technique. Amplification efficiencies were 91.7%–95.8% by use of 0.8- to 1.9-s cycles with single-molecule sensitivity. A 60-bp genomic target was amplified in 14.7 s by use of 35 cycles.

CONCLUSIONS: The time required for PCR is inversely related to the concentration of critical reactants. By increasing primer and polymerase concentrations 10- to 20-fold with temperature cycles of 0.4–2.0 s, efficient (>90%), specific, high-yield PCR from human DNA is possible in <15 s. Extreme PCR demonstrates the feasibility of while-you-wait testing for infectious disease, forensics, and any application where immediate results may be critical.

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PCR is now fundamental to molecular biology and diagnostics (1, 2). Temperature cycling triggers 3 distinct but overlapping *in vitro* events—DNA denaturation, primer annealing, and polymerase extension—that result in exponential DNA amplification (3). Despite its origin 30 years ago, details of the kinetics of PCR are not well understood. Initially PCR took hours to perform, but research in the early 1990s reduced times to 10–30 min (20–60 s/cycle) and sought to define its kinetic requirements (4, 5). These early rapid-cycle PCR investigations suggested that the time required for PCR was limited by instrumentation and not biochemistry.

In the last 20 years, many groups have tried to reduce PCR times to <5 min (10 s/cycle) (6, 7). Recently, systems using hot and cool water for heat transfer through metal blocks have achieved 5.25- and 4.6-s cycles (8, 9). Infrared-mediated temperature cycling demonstrated 3-s cycles (10); vapor pressure-augmented, continuous-flow PCR, 3-s cycles (11); and PCR within glass capillaries submerged in heated and cooled liquid gallium, 2.7-s cycles (12). However, all of these attempts to increase the speed of PCR led to compromises in efficiency and yield. As PCR cycle times were reduced from 20 to 2 s, efficiency and yield were compromised and then PCR typically failed entirely (12–14). Previous investigators also focused on simple targets (previously amplified PCR products, plasmids, viruses, bacteria) at high initial template concentrations (7). Practical PCR amplification in <5 min from complex human genomic DNA has been elusive.

We reasoned that shorter cycling times might require increased concentrations of critical reagents to maintain PCR efficiency. To test this proposition, we first prototyped instruments to cycle at extreme speeds from 0.4 to 2.0 s/cycle (hence the name extreme PCR), and then increased the rates of primer annealing and polymerase extension by increasing primer and polymerase concentrations to match the cycling speed.

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Methods

EXTREME PCR PROTOTYPES

We used a hot water bath of 95.5 °C (the temperature of boiling water in Salt Lake City, UT) and cool water baths of 25 °C–74 °C to change the temperature of reaction containers (see Supplemental Fig. 1, which accompanies the online version of this article at <http://www.clinchem.org/content/vol61/issue1>). The water baths (4.5-quart stainless steel dressing jars, Laboratory Safety Supply) were heated on electric hotplates with magnetic stirring and programmable temperature control (Digital Hotplates, Fisher Scientific). A stepper motor (Applied Motion Products) rotated samples between each water bath in <0.1 s. The stepper motor was powered and controlled with commercial hardware driven by custom LabView software (based on NI-Motion, National Instruments).

We used a thermocouple (Omega type T precision fine wire thermocouple, 0.003-inch diameter with Teflon insulation) centered in a dedicated control tube to measure temperature. Thermocouple amplification and linearization was performed with an Analog Devices AD595 chip. The thermocouple voltage was first calculated from the AD595 output as type T voltage = (AD595 output/247.3) – 11 μ V. The thermocouple voltage was then converted to temperature by use of National Institute of Standards and Technology coefficients for the voltage/temperature correlation of type T thermocouples. The analog signal was digitized and processed by LabView software (National Instruments). Stepper motion was triggered at 85 °C–92 °C for denaturation and 62 °C–70 °C for annealing/extension to obtain desired temperature cycling profiles. Alternatively, stepper motion was triggered after a programmed hold time after reaching position in each water bath.

Sample containers for PCR were composite glass/plastic LC24 capillary reaction tubes (0.8-mm inner diameter and 1.0-mm outer diameter, BioFire Diagnostics) or 19-gauge hypodermic needles (0.042-inch inner diameter, 0.075-inch outer diameter, Becton Dickinson). We sealed the open end of the metal tubing by heating to a red-white color with a gas flame and compressing in a vise. Samples were spun down to the bottom of each tube by brief centrifugation and sealed with clay capillary tube sealant.

For real-time PCR, epifluorescent illumination of the glass capillary was accomplished with a fiber optic cable (600- μ m fiber core diameter, Ocean Optics) placed 1–2 mm distant from and inline with the sample capillary, when positioned in the cooler water bath (see online Supplemental Fig. 2). The fiber optic cable entered the water bath through a port in the side of the

container. Light from a light-emitting diode (LED)³ light source (Ocean Optics) was guided by another fiber optic cable into an optics block with a 440 $\pm$ 20-nm excitation interference filter, a beam-splitting 458-nm dichroic, and a 490 $\pm$ 5-nm emission filter (Semrock). Emission detection was with a photomultiplier tube (PMT) module (Hamamatsu). The analog signal from the PMT module was digitized and processed by LabView software. A custom LabView program recorded fluorescence and temperature measurements and controlled stepper motion to obtain desired temperature cycling profiles. Fluorescence was averaged during cooling each cycle and plotted against cycle number to generate real-time PCR amplification plots.

POLYMERASE QUANTIFICATION

We obtained KlenTaq1™ DNA polymerase from either AB Peptides or Wayne Barnes (Washington University, St. Louis, MO). KlenTaq has a molecular weight of 62.1 kDa and an absorptivity at 280 nm of 69 130 mol/L⁻¹cm⁻¹, as calculated from the sequence (US Patent No. 5436149). Mass spectrometry confirmed a predominant molecular weight of 62 kDa, and denaturing polyacrylamide gels showed that the major band was >80% pure by integration. Using the absorbance and purity to calculate the concentration indicated an 80- μ mol/L stock in 10% glycerol (Wayne Barnes) or 40- μ mol/L stock in 50% glycerol (AB Peptides). One micromole per liter KlenTaq is equivalent to 3.8 U/ μ L, with a unit defined by the manufacturer as the amount of enzyme required to catalyze the incorporation of 10 nmol dNTP into acid-insoluble material in 30 min at 72 °C (pH 9.1) by use of activated salmon sperm DNA as the template. We used KlenTaq1 in all experiments except when KAPA2G™ Fast DNA polymerase was used, a gift from Kapa Biosystems as a 44- μ mol/L stock in 37.5% glycerol. One micromole per liter KAPA2G Fast is equivalent to 4.5 U/ μ L.

PCR PROTOCOLS

Unless otherwise indicated, we performed PCR in 5- μ L reaction volumes containing 50 mmol/L Tris (pH, 8.3), 3 mmol/L MgCl₂, 200 μ mol/L of each dNTP (dATP, dCTP, dGTP, dTTP), 500 μ g/mL nonacetylated BSA (Sigma), 2% (vol/vol) glycerol (Sigma), 50 ng purified human genomic DNA, and 1 $\times$ LCGreen® Plus (BioFire Diagnostics). Because of the high concentrations of polymerase and primers, reactions were prepared on ice, quickly transferred to the instrument, and immediately amplified without delay. The concentration of the

³ Nonstandard abbreviations: LED, light emitting diode; PMT, photomultiplier tube; T_m , melting temperature; Cq, quantification cycle.

primers and the polymerase varied according to the specific experimental protocols. Final polymerase concentrations were between 0.064 and 16 $\mu\text{mol/L}$. Primers were synthesized by the University of Utah Core Facility and desalted, and concentrations were determined by absorbance at 260 nm. The final concentrations of each primer varied from 0.5 to 20 $\mu\text{mol/L}$. Standard real-time PCR on the LightCycler (Roche Applied Science) or LS32 (BioFire Diagnostics) used final concentrations of 0.064 $\mu\text{mol/L}$ KlenTaq1 polymerase, 0.5 $\mu\text{mol/L}$ of each primer, and 0.1% (vol/vol) glycerol. Reactions using KAPA2G Fast DNA polymerase substituted 1 $\times$ KAPA2G Buffer A instead of 50 mmol/L Tris (pH 8.3 at 25 $^{\circ}\text{C}$) and had a final glycerol concentration of 1.3% (vol/vol).

We obtained synthetic DNA templates from Integrated DNA Technologies as either standard desalted oligonucleotides (100-bp template) or duplex gBlocks™ (200- to 500-bp templates). Synthetic template sizes were verified by agarose gel electrophoresis. The synthetic templates were serially diluted in 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0 (at 25 $^{\circ}\text{C}$), and quantified by absorbance at 260 nm and standard real-time PCR on an LS32 thermal cycler (Biofire). For primer and product sequences, see Methods in the online Supplemental Data.

EXTREME PCR EFFICIENCY

Human genomic DNA samples at a stock concentration of 50 ng/ μL were serially diluted in 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0 (at 25 $^{\circ}\text{C}$), to obtain template concentrations across 4 orders of magnitude. For each dilution, samples were run in quadruplicate (*NQO1*) or quintuplicate (*KCNE1*),⁴ and quantification cycles (*C_q*) were determined by use of a custom LabView program. The program determined *C_q* for each curve by (a) arithmetically normalizing fluorescence by subtracting the minimum fluorescence from each point on the curve; (b) adding back a residual fluorescence to all points so that the logarithm could be taken (typically 0.1%–1% of the fluorescence range); (c) displaying the data on a plot of log fluorescence vs cycle number; (d) providing cursors so that a range of points could be dynamically selected by the user that are log-linear by observing the best-fit log-linear line between the cursors while (e) maximizing the slope of the best fit log-linear line; (f) calculating the fluorescence mean of the log-linear upper and lower bound points included in the selected range; and (g) determining the fractional cycle number from the fluorescence mean by

interpolation along the log-linear line. From multiple curves with varied initial template copies, *C_q* was plotted against $\log_{10}$ (initial template copies), and the amplification efficiency was calculated as a percentage, $E = (10^{(-1/\text{slope})} - 1) \times 100$, with the slope determined by linear regression. Because of higher variation at very low template concentrations, the 1.5 copy/reaction tubes were not included in the efficiency calculations.

PRODUCT VERIFICATION

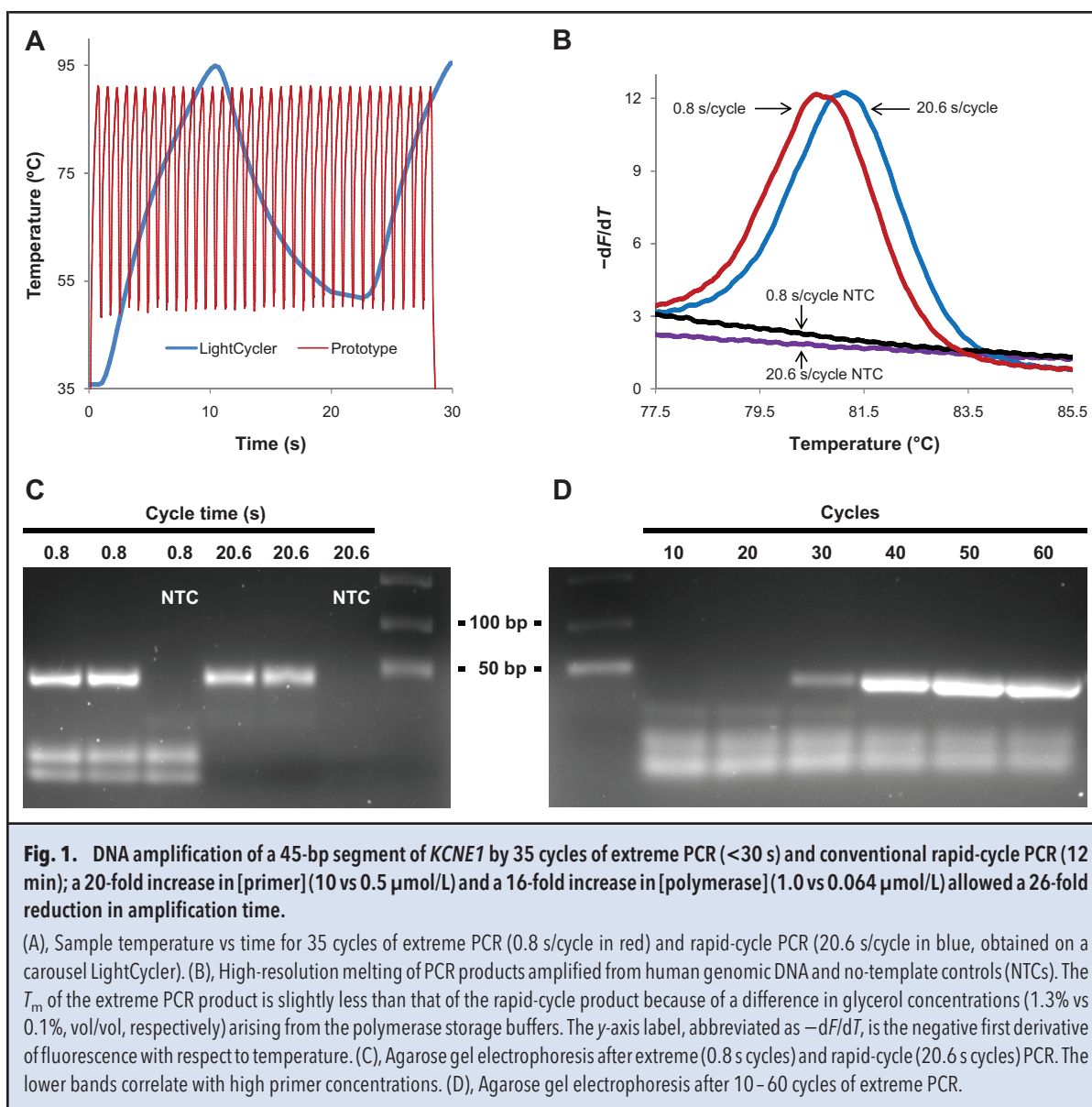
High-resolution melting curves were generated immediately after PCR by use of an HR-1 instrument (BioFire Diagnostics) modified to accept LC24 capillaries. High-resolution melting data were analyzed with custom software written in LabView and viewed as derivative melting curves (15). Fluorescence was corrected for excitation power before analysis. PCR products and no-template controls were also separated on 4% NuSieve 3:1 or 2% SeaKem LE agarose gels and imaged after ethidium bromide staining.

Results

We constructed a prototype instrument to temperature cycle 1- to 5- μL samples in 0.4–2.0 s by heat transfer from hot or cold water (see online Supplemental Fig. 1). Samples in thin capillary tubes or steel needles were mechanically flipped between water baths to obtain the desired temperature cycling profile. The PCR solution temperature was measured by a fine wire thermocouple in a parallel control tube.

A 45-bp fragment of the single-copy gene *KCNE1* (potassium voltage-gated channel, Isk-related family, member 1) was amplified from human genomic DNA by either extreme PCR or conventional rapid-cycle PCR on a carousel LightCycler® instrument (Fig. 1). Thirty-five cycles of extreme PCR required 28 s (0.8 s/cycle), whereas rapid-cycle PCR was completed in 12 min (20.6 s/cycle). Extreme PCR was finished before 2 cycles could be completed on the rapid-cycle instrument (Fig. 1A). Both methods produced amplicons with similar melting curves and strong, specific bands on gel electrophoresis, although extreme PCR showed greater yield than rapid-cycle PCR (Fig. 1, B and C). The reaction components for extreme and rapid-cycle PCR were identical except for the amount of polymerase and primers. Robust PCR using 0.8-s cycles required primer concentrations ≥ 10 $\mu\text{mol/L}$ and a polymerase concentration ≥ 1 $\mu\text{mol/L}$. High polymerase and primer concentrations have been reported to increase yield in rapid-cycle and real-time PCR (4, 16). Despite high polymerase and primer concentrations, extreme PCR generated only the specific product. Low molecular weight primer bands were visible in both reactions with and without template. When evaluated every 10 cycles, product was first vis-

⁴ Human genes: *KCNE1*, potassium voltage-gated channel, Isk-related family, member 1; *IL10RB*, interleukin-10 receptor, β ; *NQO1*, NAD(P)H dehydrogenase, quinone 1; *AKAP10*, A kinase (PRKA) anchor protein 10.



ible at 30 cycles, plateauing at 40–60 cycles (Fig. 1D). At the genomic template concentrations used, C_q values at high efficiency are usually in the low 20s, so we would expect that gel bands might be absent at 20 cycles, especially with dye detection of a small 45-bp product.

The effect of polymerase and primer concentrations in PCR with <1-s cycles was studied by use of a 49-bp fragment of *IL10RB* (interleukin-10 receptor, β), shown as both 2-dimensional (Fig. 2A) and 1-dimensional (Fig. 2, B and C) data. Product yield was determined after 35 cycles, with a mean cycle time of 0.73 s between 92 °C and 63 °C. Only with primer concentrations $\geq 5 \mu\text{mol/L}$ and polymerase concentrations $\geq 1 \mu\text{mol/L}$ were PCR

products detected. Using 19-gauge hypodermic needles as reaction containers, 20 $\mu\text{mol/L}$ primers, and 8 $\mu\text{mol/L}$ polymerase, the time to amplify the 49-bp fragment was reduced further to 16 s using 35 cycles between 92 °C and 65 °C (Fig. 2D). Furthermore, genotyping by high-resolution melting was successful on a 58-bp segment of *IL10RB* flanking an A>G variant after a 38-s PCR with 10 $\mu\text{mol/L}$ primers and 2 $\mu\text{mol/L}$ polymerase (see online Supplemental Fig. 3).

Optimal polymerase and primer concentrations were also studied with a 102-bp fragment of *NQO1* using 1.93-s cycles between 92 °C and 72 °C (see online Supplemental Fig. 4). Similar to the 49-bp *IL10RB* target, amplification required both increased primers (≥ 2

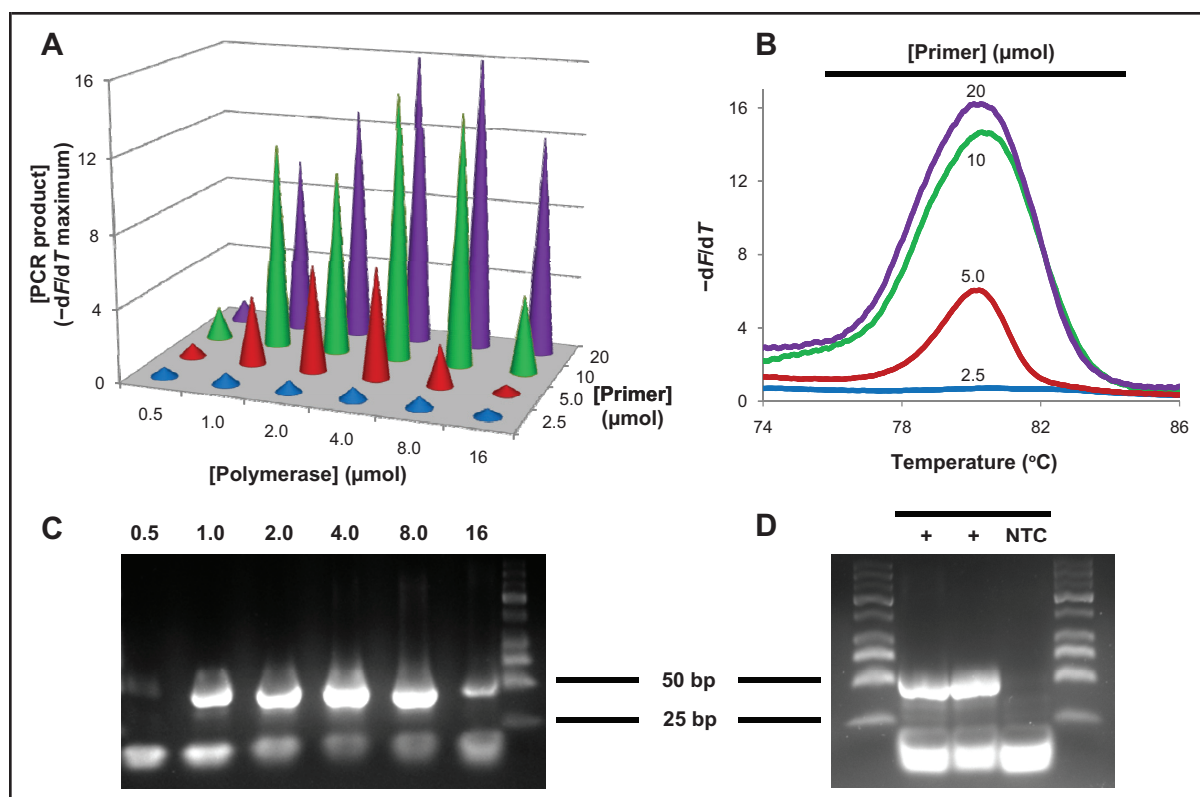


Fig. 2. Extreme PCR amplification of a 49-bp segment of *IL10RB*.

(A), Polymerase and primer optimization for product yield after 35 cycles of 0.73 s each. PCR product yield was measured as the peak of derivative melting curve plots. (B), Derivative melting curves demonstrate the effect of primer concentration on product yield when using 4 $\mu\text{mol/L}$ polymerase. (C), Agarose gel showing the effect of polymerase concentration on amplification yield using 10 $\mu\text{mol/L}$ of each primer. (D), Sixteen-second amplification of the 49-bp segment of *IL10RB* (35 cycles, 0.45 s/cycle) using 19-gauge hypodermic needles as reaction containers with 20 $\mu\text{mol/L}$ of each primer and 8 $\mu\text{mol/L}$ polymerase. NTC, no-template control. $-dF/dT$, negative first derivative of fluorescence with respect to temperature.

$\mu\text{mol/L}$) and polymerase (≥ 0.5 $\mu\text{mol/L}$). However, as the fragment size increases, longer annealing/extension times are needed for complete extension, and lower reagent concentrations can be used.

The extreme PCR prototype was further modified to include optics for real-time monitoring (see online Supplemental Fig. 2 and Supplemental Video). The quantitative performance of extreme PCR for the 102-bp fragment of *NQO1* [NAD(P)H dehydrogenase, quinone 1] (Fig. 3) and the 45-bp fragment of *KCNE1* (see online Supplemental Fig. 5) was assessed by use of a dilution series of human genomic DNA. With a dynamic range of at least 4 logs, the amplification efficiencies calculated from the calibration curves were 95.8% for *NQO1* and 91.7% for *KCNE1*, similar to conventional PCR. Control reactions without template did not amplify after 50 cycles. Single-copy replicates (mean copy number of 1.5 copies per reaction) were similar in amplification curve shape and intensity to higher concentrations (Fig. 3 and

online Supplemental Fig. 5). At a mean copy number of 0.15 copies/reaction, 2 reactions were positive out of 17 (combining both *NQO1* and *KCNE1* trials), with a calculated expectation of 0.13 copies/reaction by binomial expansion (uCount for digital PCR, <https://dna.utah.edu/ucount/uc.php>; accessed May 7, 2014).

We also examined the extension time required for different product lengths by use of real-time PCR (Fig. 4). To avoid possible confounding effects of different primers, synthetic templates of 100–500 bp with common high melting temperature (T_m , 77 $^{\circ}\text{C}$) primers were used. Optimal concentrations of primers and polymerase were first determined for the 300-bp product by use of a 4-s combined annealing/extension segment with 4.9-s cycles (Fig. 4A). Identical primer (4 $\mu\text{mol/L}$) and polymerase (2 $\mu\text{mol/L}$) concentrations were then used for all product lengths, and minimum extension times were determined (see online Supplemental Fig. 6). For each product length, increased annealing/extension times re-

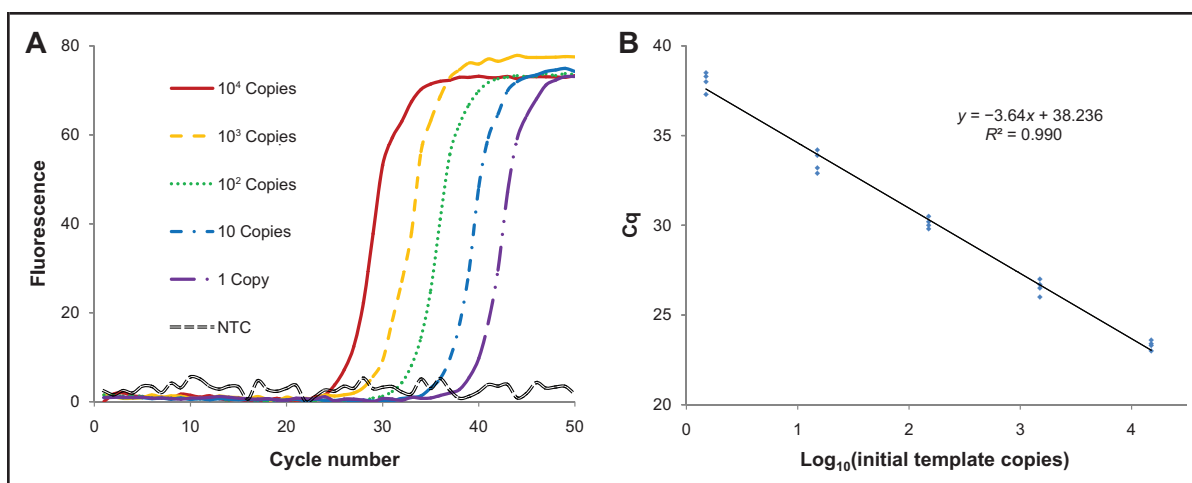


Fig. 3. Efficiency and sensitivity are not compromised by extreme PCR.

(A), Fluorescence versus cycle number. (B), Cq versus log₁₀ (initial template copies) plots for amplification of a 102-bp fragment of *NQO1* (50 cycles, 1.93 s/cycle). Reactions were performed in quadruplicate with 2 μmol/L polymerase and 8 μmol/L of each primer. The calculated efficiency was 95.8%.

sulted in decreased fractional Cq values until no further change was observed, reflecting the minimum extension time required for efficient PCR. For example, amplification curves by use of the KAPA2G FAST polymerase for the 500-bp product are shown in Fig. 4B. The minimum extension time by use of KAPA2G FAST polymerase was 3 s, compared to 7 s by use of KlenTaq1 (a deletion mutant of *Taq* polymerase). Longer products required longer extension times (Fig. 4C). For KlenTaq1 polymerase, about 1 s is required for each 60 bps, whereas for KAPA2G FAST, 1 s is required for each 158 bps.

High Mg²⁺ concentrations facilitate extreme PCR. When a 60-bp fragment of *AKAP10* [A kinase (PRKA) anchor protein 10] was amplified for 35 cycles with 20 μmol/L primers and 8 μmol/L polymerase, no product was observed on gels at 2–3 mmol/L, minimal product at 4 mmol/L, and a large amount of specific product at 5–7 mmol/L MgCl₂ (see online Supplemental Fig. 7). At 5 mmol/L MgCl₂, the minimum cycle time for efficient amplification was 0.42 s (see online Supplemental Fig. 7), demonstrating that specific, high-yield 60-bp product can be obtained in <15 s from human genomic DNA (35 cycles in 14.7 s).

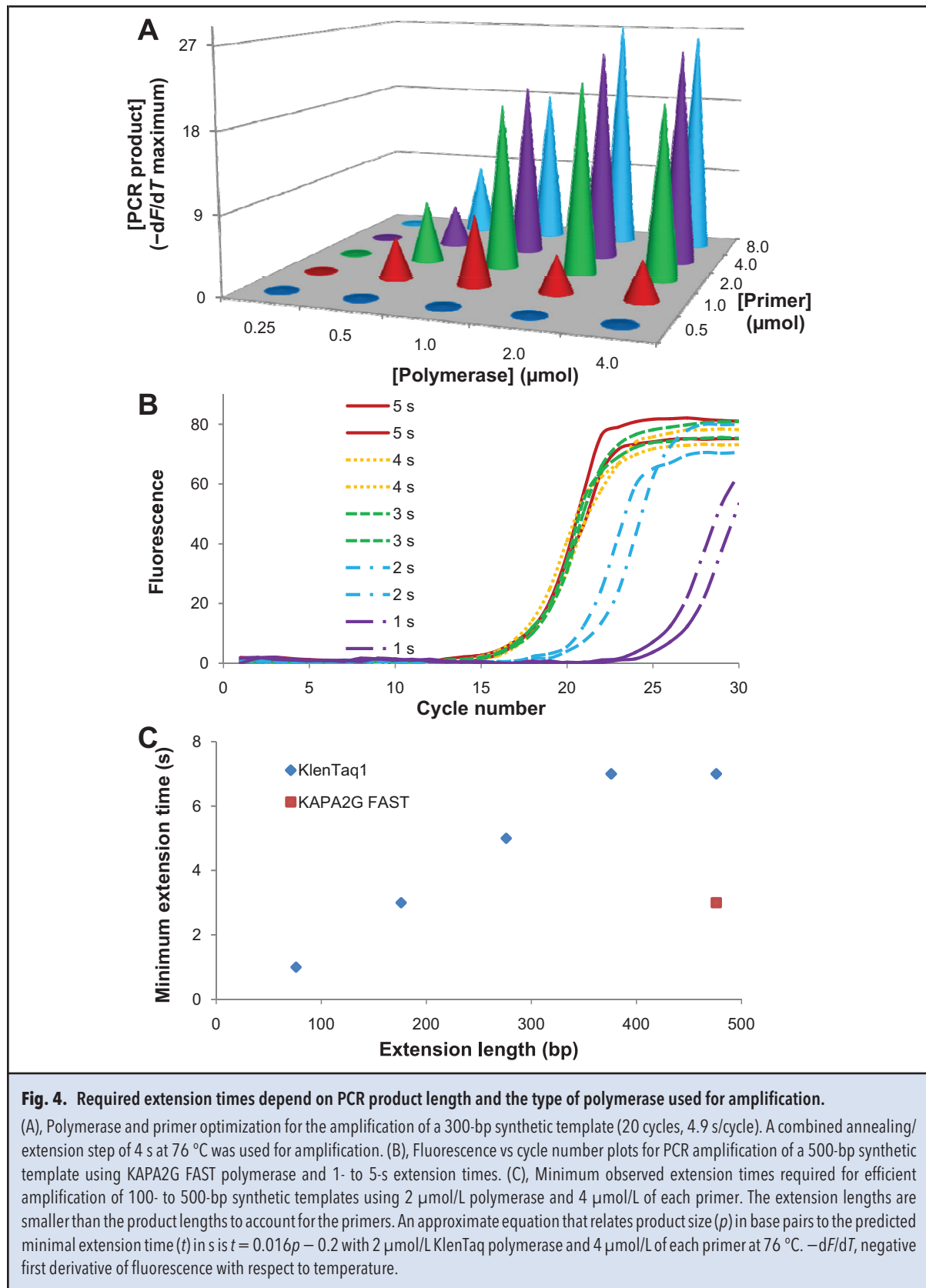
Discussion

Great progress in the throughput of molecular testing has occurred over the last several years. Throughput can be increased by larger batch size or faster turnaround time. Batch size improves as we move from handling individual tubes to arrays of 96, 384, or even 1536. Processing is easier when either the sample number or assay number

can be limited or standardized; for example, microfluidic handling of 96 samples with 96 assays can produce 9216 results. Furthermore, microchips produce millions of results on single samples, and massively parallel sequencing with sequence barcoding on tens of samples can produce billions of results. Research studies and clinical laboratories thrive on high numbers.

The importance of turnaround time often gets lost in the race for higher and higher numbers of results. Individual patients care only about their own test results and how quickly they can be returned. Although point-of-care testing is recognized as an important trend, progress in molecular testing has been slow because of its complexity. Recently, rapid sample-to-answer systems have appeared that can return results in about an hour, ranging from single assays to multiplexed assays focused on patient syndromes (17, 18). Further improvements in turnaround time on these systems primarily depend on reducing PCR times. By increasing the concentration of critical reagents, we have been able to reduce PCR times to less than a minute. The critical reagents that limit PCR speed are the polymerase and the primers. Successful completion of both primer annealing and polymerase extension must occur during the combined annealing/extension segment used in extreme PCR.

When annealing times are reduced, primer hybridization can become rate-limiting. Primer annealing to template DNA is a second-order process, with a rate determined by both primer and template DNA concentration (19). However, during most of PCR, the primer concentration is much greater than the template DNA, and the extent of annealing completion each step is di-



rectly proportional to the primer concentration in a pseudo-first-order reaction. For every doubling of the primer concentration, the time required for an equivalent percentage of product to anneal to primer is reduced by a factor of 2. The specificity advantage of short annealing times has been known for many years (5, 20, 21).

When extension times are reduced, the amount and/or speed of the polymerase can become rate limiting. The time required for polymerase extension depends on many factors, including the length of the amplified product, temperature, and the polymerase used for extension. Recent estimates for KlenTaq1 and KAPA2G at 75 °C are 101 and 155 nucleotides/s, respectively (22), although rates are strongly dependent on pH and the Mg^{2+} , monovalent cation, dye, and T_m depressor concentrations (23). During typical PCR, the amount of polymerase starts in great excess to template but ends up limiting (24), requiring each polymerase molecule to recycle and extend many templates each cycle. In extreme PCR, increasing the polymerase concentration allows the simultaneous extension of more primed templates later into the PCR, resulting in lengthening the exponential phase and increasing the yield of product. However, in some cases (Fig. 2), high polymerase concentrations appear to inhibit product yield, an effect we have yet to explain.

Hot start methods are difficult to apply to extreme PCR and were not used here. High polymerase concentrations make the use of anti-*Taq* antibodies impractical, and chemical hot starts require too much time. Perhaps the best solution is an integrated microfluidic system where automatic mixing of reagents occurs at high temperature for a true hot start, and small sample volumes can be used to reduce cycling times, similar to some integrated systems (18). Accurate measurement of sample temperature is perhaps the most difficult problem, one that might be solved by monitoring temperature with fluorescence (25).

In summary, efficient PCR from human genomic DNA can be completed in 15–60 s. When paired with high-resolution melting analysis (26), DNA variants can be amplified and genotyped in less than a minute. Extremely fast amplification and analysis has obvious advantages for any application where time to result is critical. We hope that the demonstrated feasibility of extreme PCR will encourage further work on practical implementation that may lead to commercial devices that improve the turnaround time for molecular testing, leading to better patient satisfaction and care.

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References

- Mullis K, Ferre F, Gibbs R, eds. The polymerase chain reaction. Boston: Birkhauser; 1994.
- Bustin SA, ed. The PCR revolution: basic technologies and applications. New York: Cambridge University Press; 2010.
- Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487–91.
- Wittwer CT, Fillmore GC, Garling DJ. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal Biochem* 1990;186:328–31.
- Wittwer CT, Garling DJ. Rapid cycle DNA amplification: time and temperature optimization. *Biotechniques* 1991;10:76–83.
- Zhang C, Xing D. Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. *Nucl Acid Res* 2007;35:4223–37.
- Roper MG, Easley CJ, Landers JP. Advances in polymerase chain reaction on microfluidic chips. *Anal Chem* 2005;77:3887–93.
- Wheeler EK, Hara CA, Frank J, Deotte J, Hall SB, Benett W, et al. Under-three minute PCR: probing the limits of fast amplification. *Analyst* 2011;136:3707–12.
- Terazono H, Takei H, Hattori A, Yasuda K. Development of a high-speed real-time polymerase chain reaction system using a circulating water-based rapid heat-exchange. *Jpn J Appl Phys* 2010;49:06GM05.
- Huhmer AFR, Landers JP. Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. *Anal Chem* 2000;72:5507–12.
- Fuchiaki Y, Nagai H, Saito M, Tamiya E. Ultra-rapid flow-through polymerase chain reaction microfluidics using vapor pressure. *Biosens Bioelectron* 2011;27:88–94.
- Maltezos G, Johnston M, Taganov K, Srichantaratamee C, Gorman J, Baltimore D, et al. Exploring the limits of ultrafast polymerase chain reaction using liquid for thermal heat exchange: a proof of principle. *Appl Phys Lett* 2010;97:264101.
- Kopp MU, Mello AJ, Manz A. Chemical amplification: continuous-flow PCR on a chip. *Science* 1998;280:1046–8.
- Hashimoto M, Chen PC, Mitchell MW, Nikitopoulos DE, Soper SA, Murphy MC. Rapid PCR in a continuous flow device. *Lab Chip* 2004;4:638–45.
- Palais R, Wittwer CT. Mathematical algorithms for high-resolution DNA melting analysis. *Meth Enzymol* 2009;454:323–43.
- Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnol* 1993;11:1026–30.

17. Pancholi R, Kelly C, Raczkowski M, Balada-Llasat JM. Detection of toxigenic *Clostridium difficile*: comparison of the cell culture neutralization, Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays. *J Clin Microbiol* 2012;50:1331–5.
18. Poritz MA, Blaschke AJ, Byington CL, Meyers L, Nilsson K, Jones DE, et al. FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. *PLoS ONE* 2011;6:e26047.
19. Wetmur JG, Davidson N. Kinetics of renaturation of DNA. *J Mol Biol* 1968;31:349–70.
20. Wittwer CT, Herrmann MG. Rapid thermal cycling and PCR kinetics. In: Innis M, Gelfand D, Sninsky J, eds. *PCR methods manual*. San Diego: Academic Press; 1999: 211–29.
21. Wittwer CT, Marshall BC, Reed GH, Cherry JL. Rapid cycle allele-specific amplification: studies with the cystic fibrosis delta F508 locus. *Clin Chem* 1993;39:804–9.
22. Montgomery JL, Rejali N, Wittwer CT. Stopped-flow DNA polymerase assay by continuous monitoring of dNTP incorporation by fluorescence. *Anal Biochem* 2013;441:133–9.
23. Montgomery JL, Wittwer CT. Influence of PCR reagents on DNA polymerase extension rates measured on real-time PCR instruments. *Clin Chem* 2014;60:334–40.
24. Wittwer CT, Kuskawa N. Real-time PCR and melting analysis. In: Persing DH, Tenover FC, Hayden R, Nolte F, Tang YW, Belkum AV, eds. *Molecular microbiology: diagnostic principles and practice*. Washington (DC): ASM Press; 2011. p 63–82.
25. Sanford LN, Wittwer CT. Monitoring temperature with fluorescence during real-time PCR and melting analysis. *Anal Biochem* 2013;434:26–33.
26. Vossen RH, Aten E, Roos A, den Dunnen JT. High-resolution melting analysis (HRMA): more than just sequence variant screening. *Hum Mutat* 2009;30: 860–6.