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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-μL 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.

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 μV. 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-μm 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 ± 20–nm excitation interference filter, a beam-splitting 458-nm dichroic, and a 490 ± 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/$L^{-1}cm^{-1}$, 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-μmol/L stock in 10% glycerol (Wayne Barnes) or 40-μmol/L stock in 50% glycerol (AB Peptides). One micromole per liter KlenTaq is equivalent to 3.8 U/μL, 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-μmol/L stock in 37.5% glycerol. One micromole per liter KAPA2G Fast is equivalent to 4.5 U/μL.

PCR PROTOCOLS

Unless otherwise indicated, we performed PCR in 5-μL reaction volumes containing 50 mmol/L Tris (pH, 8.3), 3 mmol/L $MgCl_2$, 200 μmol/L of each dNTP (dATP, dCTP, dGTP, dTTP), 500 μg/mL nonacetylated BSA (Sigma), 2% (vol/vol) glycerol (Sigma), 50 ng purified human genomic DNA, and 1× 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 μmol/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 μmol/L. Standard real-time PCR on the LightCycler (Roche Applied Science) or LS32 (BioFire Diagnostics) used final concentrations of 0.064 μmol/L KlenTaq1 polymerase, 0.5 μmol/L of each primer, and 0.1% (vol/vol) glycerol. Reactions using KAPA2G Fast DNA polymerase substituted 1× KAPA2G Buffer A instead of 50 mmol/L Tris (pH 8.3 at 25 °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 °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 °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 (Cq) were determined by use of a custom LabView program. The program determined Cq 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, Cq 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 μmol/L and a polymerase concentration ≥1 μmol/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.

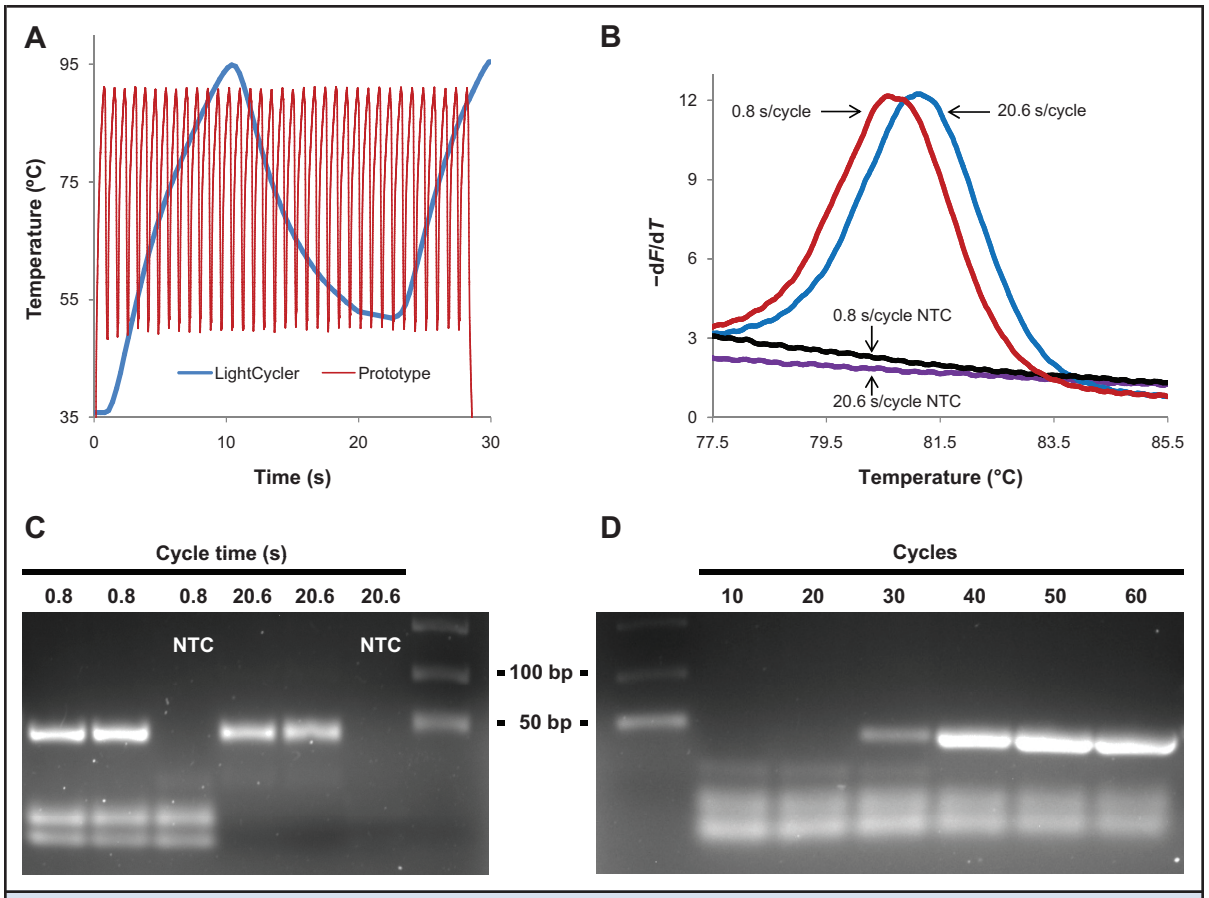

Fig. 1. DNA amplification of a 45-bp segment of *KCNE1* by 35 cycles of extreme PCR (<30 s) and conventional rapid-cycle PCR (12 min); a 20-fold increase in [primer] (10 vs 0.5 μmol/L) and a 16-fold increase in [polymerase] (1.0 vs 0.064 μmol/L) allowed a 26-fold reduction in amplification time.

(A), Sample temperature vs time for 35 cycles of extreme PCR (0.8 s/cycle in red) and rapid-cycle PCR (20.6 s/cycle in blue, obtained on a carousel LightCycler). (B), High-resolution melting of PCR products amplified from human genomic DNA and no-template controls (NTCs). The T_m of the extreme PCR product is slightly less than that of the rapid-cycle product because of a difference in glycerol concentrations (1.3% vs 0.1%, vol/vol, respectively) arising from the polymerase storage buffers. The *y*-axis label, abbreviated as −d*F*/d*T*, is the negative first derivative of fluorescence with respect to temperature. (C), Agarose gel electrophoresis after extreme (0.8 s cycles) and rapid-cycle (20.6 s cycles) PCR. The lower bands correlate with high primer concentrations. (D), Agarose gel electrophoresis after 10–60 cycles of extreme PCR.

ible at 30 cycles, plateauing at 40–60 cycles (Fig. 1D). At the genomic template concentrations used, Cq 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 ≥5 μmol/L and polymerase concentrations ≥1 μmol/L were PCR products detected. Using 19-gauge hypodermic needles as reaction containers, 20 μmol/L primers, and 8 μmol/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 μmol/L primers and 2 μmol/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

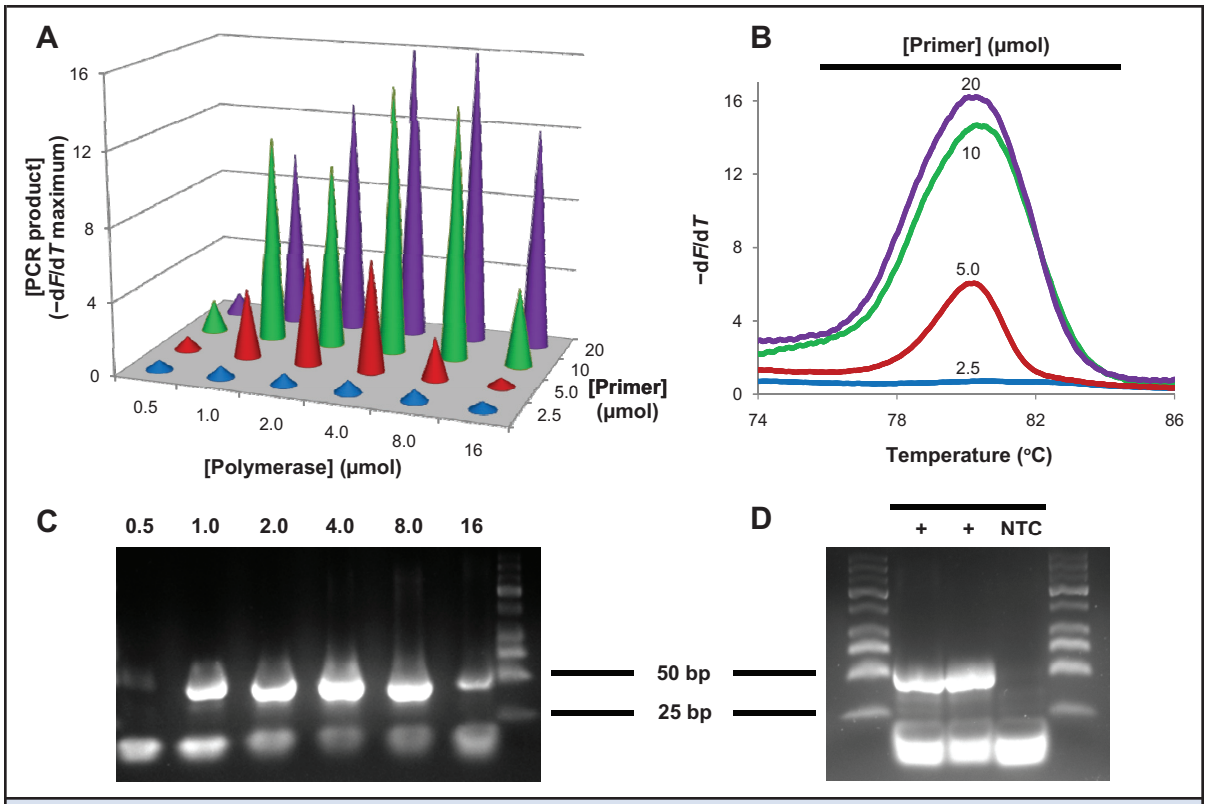

Fig. 2. Extreme PCR amplification of a 49-bp segment of *IL10RB*.

(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 μmol/L polymerase. (C), Agarose gel showing the effect of polymerase concentration on amplification yield using 10 μmol/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 μmol/L of each primer and 8 μmol/L polymerase. NTC, no-template control. −d*F*/d*T*, negative first derivative of fluorescence with respect to temperature.

μmol/L) and polymerase (≥0.5 μmol/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 °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 μmol/L) and polymerase (2 μmol/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-

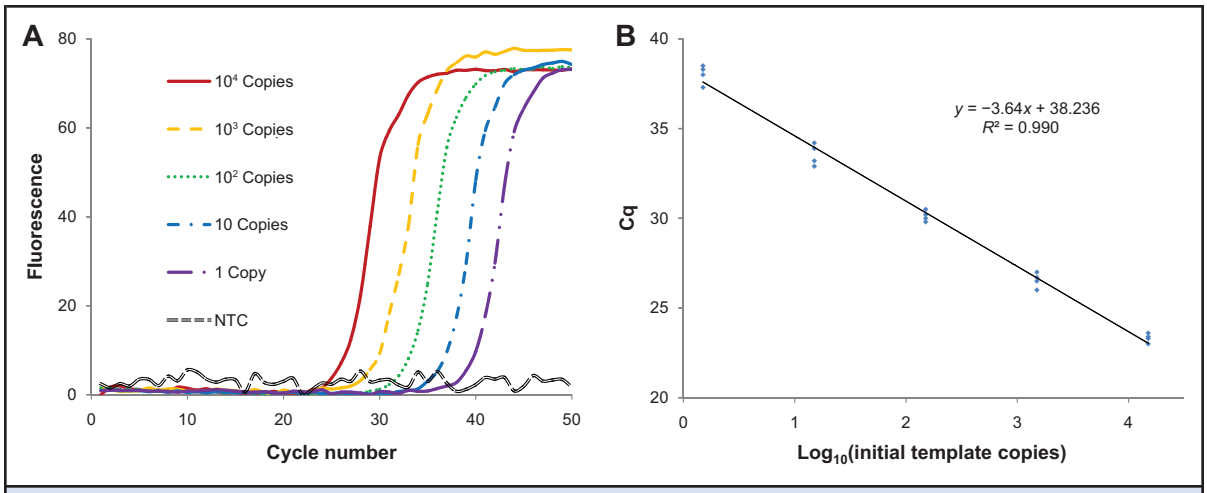

Fig. 3. Efficiency and sensitivity are not compromised by extreme PCR.

(A), Fluorescence versus cycle number. (B), Cq versus $\log_{10}$ (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 Mg^{2+} 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 $MgCl_2$ (see online Supplemental Fig. 7). At 5 mmol/L $MgCl_2$, 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-

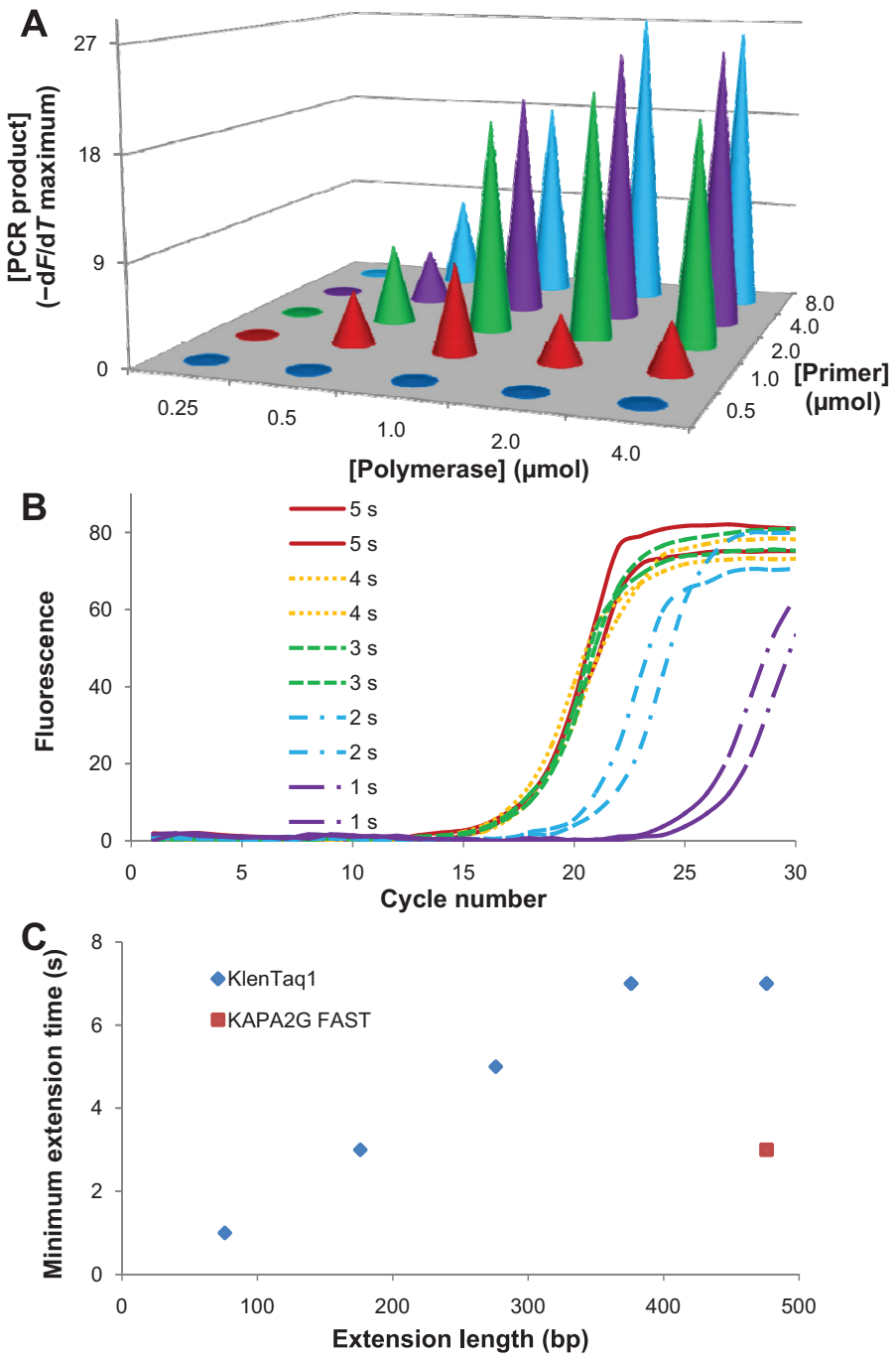

Fig. 4. Required extension times depend on PCR product length and the type of polymerase used for amplification.

(A), Polymerase and primer optimization for the amplification of a 300-bp synthetic template (20 cycles, 4.9 s/cycle). A combined annealing/extension step of 4 s at 76 °C was used for amplification. (B), Fluorescence vs cycle number plots for PCR amplification of a 500-bp synthetic template using KAPA2G FAST polymerase and 1- to 5-s extension times. (C), Minimum observed extension times required for efficient amplification of 100- to 500-bp synthetic templates using 2 μmol/L polymerase and 4 μmol/L of each primer. The extension lengths are smaller than the product lengths to account for the primers. An approximate equation that relates product size (p) in base pairs to the predicted minimal extension time (t) in s is $t = 0.016p - 0.2$ with 2 μmol/L KlenTaq polymerase and 4 μmol/L of each primer at 76 °C. $-dF/dT$, negative first derivative of fluorescence with respect to temperature.

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.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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