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Adaptive PCR Based on Hybridization Sensing of Mirror-Image L-DNA

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Abstract

Polymerase chain reaction (PCR) is dependent on two key hybridization events during each cycle of amplification, primer annealing and product melting. To ensure that these hybridization events occur, current PCR approaches rely on temperature set points and reaction contents that are optimized and maintained using rigid thermal cycling programs and stringent sample preparation procedures. This report describes a fundamentally simpler and more robust PCR design that dynamically controls thermal cycling by more directly monitoring the two key hybridization events during the reaction. This is achieved by optically sensing the annealing and melting of mirror-image L-DNA analogs of the reaction's primers and targets. Because the properties of L-DNA enantiomers parallel those of natural D-DNAs, the L-DNA reagents indicate the cycling conditions required for effective primer annealing and product melting during each cycle without interfering with the reaction. This hybridization-sensing approach adapts in real time to variations in reaction contents and conditions that impact primer annealing and product melting and eliminates the requirement for thermal calibrations and cycling programs. Adaptive PCR is demonstrated to amplify DNA targets with high efficiency and specificity under both controlled conditions and conditions that are known to cause traditional PCR to fail. The advantages of this approach promise to make PCR-based nucleic acid analysis simpler, more robust, and more accessible outside of well-controlled laboratory settings.

Graphical Abstract

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Author Contributions

N.M.A. and F.R.H. conceived the basic idea and supervised the work. N.M.A. designed the experiments. W.E.G. constructed and programmed the Adaptive PCR device. N.M.A., W.E.G., and A.N.H. performed experiments and analyzed data. N.M.A. and F.R.H. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supplementary Table 1, Supplementary Figure 1, and Supplementary Figure 2.

Notes

The authors declare no competing financial interest.

Keywords

Polymerase chain reaction; PCR; mirror-image DNA; L-DNA; Adaptive PCR; thermal cycler; thermal cycling

INTRODUCTION

Polymerase chain reaction (PCR) is the gold standard for amplification and detection of a specific DNA fragment. Since its conception in the early 1980's,^{1,2} the method has been widely used in the diverse fields of molecular biology, genetics, forensic science, ecology, food quality assurance, and diagnostics. Despite its undisputed success, PCR remains constrained by a fundamental dependence on indirect temperature measurements of the reaction to achieve target melting and primer annealing. Because efficient and specific amplification only occurs within a narrow range of reaction conditions,³⁻⁵ discrepancies between the estimated and actual hybridization states of the targets and primers cause PCR failure. Consequently, temperature set points, cycle timing, and reaction contents must be precisely maintained to ensure complete target melting, stringent primer annealing, and efficient polymerase activity during each cycle.⁶⁻⁸ Achieving these conditions at the programmed temperature set points, however, is often compromised by variations in prepared DNA samples,^{3,9,10} error in the volumes of pipetted reagents,^{3,6,11} or inaccuracies in thermal cycler temperature calibrations.^{6,12,13} These challenges are particularly acute in applications seeking to make PCR accessible outside of well-controlled laboratory settings, such as in point-of-care settings where the contents of prepared samples and reaction temperatures are more difficult to control.^{10,13-16} Methods that more directly monitor the reaction using fluorescence probes have shown promise for overcoming some of the challenges with indirect thermal sensing,¹⁷⁻²² yet these methods still rely on temperature calibrations to estimate primer annealing and target melting and do not compensate for variation in reaction contents that impact primer and target hybridization. To enable PCR-based nucleic acid amplification in settings that have insufficient resources to precisely maintain sample contents and reaction temperatures, new PCR designs are needed that more directly monitor the key hybridization events during the reaction, rather than relying on rigid temperature cycling programs and consistent sample preparation. The challenge with this approach is that, to date, it has not been possible to directly detect these hybridization events in real-time during thermal cycling without interfering with the reaction.

Mirror-image L-DNA oligonucleotides were first synthesized in the early 1980's as unnatural left-helical enantiomers of naturally occurring right-helical D-DNA.²³ The original promise of L-DNA oligonucleotides was that they could be used as nuclease-resistant "anti-genes" to anneal to complementary D-DNA sequences and block the expression of abnormal or harmful genes. Although the authors determined that the L-DNAs did indeed resist enzymatic degradation, they found it "particularly disappointing" that L-DNAs do not interact with complementary D-DNAs.²³ Other investigators confirmed that the mirror-image L-DNAs do not participate in traditional interactions with natural enzymes and nucleic acids.^{24–28} It was also determined that L-DNAs possess many of the same physical properties as their D-DNA counterparts, including identical conformation transitions in the presence of salts,²⁹ hybridization responses during heating and cooling,^{29,30} and interactions with non-chiral DNA intercalators.²⁶ More recently, the properties of L-DNAs have been exploited to develop nuclease-resistant aptamers,^{28,31,32} non-cross-reactive microarray tags,^{30,33} and biologically inert molecular probes for monitoring cellular processes.^{34,35} Building on this repertoire of novel applications, we take advantage of the unique properties of L-DNA to dynamically control thermal cycling during PCR by optically sensing the hybridization state of fluorophore-labeled L-DNAs identical in sequence to the PCR target and primer sequences (Figure 1). By adding the L-DNAs directly to the PCR sample, the L-DNA primer and target analogs indicate when the reaction conditions have been achieved for optimal PCR primer annealing and target melting without interfering with the reaction. This thermal cycling approach adapts to variations in the reaction contents, compensates for errors in the sample preparation, and mitigates the need for accurate thermal calibrations. In this report, we demonstrate that this adaptive PCR approach enables successful two-step PCR cycling similar to traditional PCR and under conditions and in samples that are known to cause traditional PCR to fail. To differentiate this design from standard PCR, we refer to this approach as Adaptive PCR.

MATERIALS AND METHODS

Design and synthesis of oligonucleotides

The primers and hydrolysis probe sequences for the PCR assay were designed using Roche's Universal ProbeLibrary assay design tools using the *Mycobacterium tuberculosis* IS6110 gene sequence (access. no. X17348) as the input. The compatible Probe #26 (prod. no. 04687574001) was purchased from Roche Diagnostics, and the primers and 77-mer double-stranded target sequences were purchased from Integrated DNA Technologies. The Roche hydrolysis probe was labeled with a 5'-FAM dye and a 3'-quencher dye. L-DNA probes were designed with sequences identical to the 77-mer sense and antisense PCR target sequences and the 19-mer forward primer sequence. The sense L-DNA target analog was labeled with a 5'-HEX dye, the antisense L-DNA target analog was labeled with a 3'-Black Hole Quencher 2 (BHQ2) dye, and the forward primer L-DNA analog was labeled with a 5'-Texas Red dye. The labeled L-DNAs were synthesized at medium scale and purified by HPLC by Bio-Synthesis, Inc. (Lewisville, TX) and Biomers.net GmbH (Ulm, Germany). Control D-DNA versions of the labeled sequences were also synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The 137-mer mismatch sequence was designed to simulate a non-target template for non-specific product formation by appending a 100-mer *Plasmodium*

falciparum gene fragment with forward and reverse primer binding regions of the target sequence including three nucleotide substitutions near each end. The non-target mismatch sequence was synthesized by Integrated DNA Technologies. PCR assay sequences, L-DNAs, and the mismatch sequence are provided in Supplementary Table 1.

Hybridization characteristics of L-DNA and D-DNA

The hybridization response of complementary L-DNA strands or complementary D-DNA strands to chemical additives was analyzed by combining 1 μ M of the L-DNA or D-DNA primer with 1 μ M of the respective L-DNA or D-DNA anti-sense target sequence in 10 mM Tris-HCl buffer at pH 8.0, 50 mM KCl, and 1.5 mM MgCl₂ (unless otherwise noted). Hybridization was analyzed in the presence of a range of concentrations of MgCl₂ (0, 0.25, 1, 4, 8 mM total concentration), ethanol (0, 8, 12, 16, 24%), NaCl (0, 50, 100, 400 mM), or trehalose (0, 250, 500, 750 μ M) in 25 μ L reaction volumes. Samples were placed in a Qiagen Rotor-Gene Q 5-plex HRM instrument and heated to 95 °C for 30 seconds, then cooled in 1 °C increments from 95 to 50 °C. The temperature was held for 60 seconds at each increment and Texas Red fluorescence was measured using the orange channel (585 nm excitation, 610 nm detection). The derivatives of the fluorescence values were plotted in the Rotor-Gene software. To facilitate comparisons between the hybridization responses of L-DNAs and D-DNAs, the derivatives were normalized to a 0 – 100 unit scale. For the cross-hybridization studies, 1 μ M of L-DNA or D-DNA primers were combined with 1 μ M of L-DNA, D-DNA, or no anti-sense target sequence in 10 mM Tris-HCl buffer at pH 8.0, 50 mM KCl, and 2.5 mM MgCl₂ in 25 μ L reaction volumes, and hybridization analysis was performed using the Rotor-Gene instrument as described above.

PCR performance with and without L-DNA additives

Samples were prepared for PCR with and without L-DNA using a KAPA2G Fast PCR kit (Kapa Biosystems, prod. no. KK5009) in 25 μ L reaction volumes. For reactions without L-DNA, samples were prepared with final concentrations of 1 $\times$ KAPA2G buffer with 1.5 mM MgCl₂, 200 μ M of dNTPs, 400 nM of each primer, 400 nM of the probe, and 0.05 U/ μ L of KAPA2G Fast DNA polymerase. For reactions with L-DNA, samples were prepared identically, but with the addition of 100 nM of the forward L-DNA primer, 100 nM of the antisense L-DNA target, and 20 nM of the sense L-DNA target (cost per assay of L-DNAs was ~\$0.28 from Biomers.net). Each standard dilution series was prepared with DNA template concentrations of 5×10^8 , 5×10^7 , 5×10^6 copies per reaction. A standard Rotor-Gene PCR instrument was programmed to repeat 40 cycles of a 95 °C melt temperature step for 1 second and a 60 °C annealing temperature step for 1 second. Fluorescence was measured using the green channel (470 nm excitation, 510 nm detection). PCR efficiencies with and without L-DNA and cycle threshold values for each amplification curve were calculated using the Rotor-Gene software based on a best-fit linear regression.

Design, construction, and operation of the Adaptive PCR instrument

The Adaptive PCR instrument was constructed using a Wagner HT400 forced-air heater (prod. no. 0503038) and two dual-channel Qiagen ESElog USB confocal fluorescence detectors. A green channel (470 nm excitation, 520 nm detection) was used to detect FAM fluorescence, an orange channel (565 nm excitation, 625 nm detection) was used to detect

Texas Red fluorescence, and a yellow channel (520 nm excitation, 570 nm detection) was used to detect HEX fluorescence. The heater and optics were oriented to focus the heat flow and the optical paths on the sample chamber of a 25 μ L Cepheid SmartCycler[®] reaction tube (prod. no. 900–0003). The structural components were made from optical stages, stands, and 3D printed parts. The heater was attenuated to ~10% power using a voltage reducer. The relay for controlling the heater and monitoring the thermocouples interfaced with LabVIEW software using a Multifunction USB-DAQ Controller (National Instruments, prod. no. USB-6009). A LabVIEW master program was developed to actuate the heater relay based on the optical inputs from the two ESElog fluorimeters, which were controlled and monitored using in-house libraries written in LabVIEW software. An algorithm was developed to monitor the hybridization state of the L-DNA probes and to switch between phases of heating and air-cooling, in a two-step PCR cycle format (i.e., cycling between target melting and primer annealing steps with the majority of the reaction time spent in thermal transition). During heating, the software monitored HEX fluorescence on the yellow channel as the L-DNA target strands melted. During cooling, the software monitored Texas Red fluorescence quenching on the orange channel as the L-DNA primer annealed to the antisense L-DNA target. The derivatives of the absolute fluorescence values were plotted in real-time during heating and cooling, and Gaussian fits of the derivatives were used to indicate the ratio of targets melted or primers annealed. The number of standard deviations (σ) from the center or mean ($\bar{x}$) of the Gaussian distribution was the metric used to trigger the switch points between heating or cooling. To monitor target amplification, a single measurement of FAM fluorescence was taken on the green channel and plotted immediately after the annealing switch point of each cycle. The software was programmed to end the experiment after completing 40 cycles.

Adaptive PCR performance studies

Samples were prepared for Adaptive PCR using a KAPA2G Fast PCR kit (Kapa Biosystems, prod. no. KK5009) in 25 μ L reaction volumes with final concentrations of 1 $\times$ KAPA2G buffer with 1.5 mM $MgCl_2$, 200 μ M of dNTPs, 400 nM of each primer, 400 nM of the probe, 100 nM of the forward L-DNA primer, 100 nM of the antisense L-DNA target, 20 nM of the sense L-DNA target, and 0.05 U/ μ L of KAPA2G Fast DNA polymerase. Additional $MgCl_2$ (4 mM total) or ethanol (4 – 20%) was included in the samples prepared for the experiments that evaluated their effects on Adaptive PCR performance. A DNA template concentration of 5×10^8 copies per reaction was added to each reaction. For the experiments that evaluated the effects of $MgCl_2$ on PCR specificity, the non-target mismatch template was added to the reaction at a concentration of 5×10^9 copies per reaction. Each time Adaptive PCR was performed, samples were split for comparison on the standard Rotor-Gene PCR instrument. For the experiments evaluating the effects of increased ambient temperature, the Rotor-Gene and Adaptive PCR instruments were placed in the same room and the room was heated to 35 $^{\circ}$ C using convection heaters distributed throughout the room. During development, reaction temperatures during thermal cycling were collected by placing a thermocouple probe directly into the reaction. This was done by punching a small hole in the reaction tube lid, feeding a fine-gauge thermocouple (Omega Engineering, Inc., prod. no. 5TC-TT-K-36–36) through the hole in the lid the distance required to reach the reaction chamber, and gluing it in place using thermal epoxy. The thermocouple-modified lids were

reused up to eight times using a series of bleach washes and water rinses after each run. To facilitate comparisons between the Adaptive PCR and Rotor-Gene instruments, the amplification curves from each instrument were normalized by fitting the fluorescence values to a 0 – 100 unit scale. Cycle threshold values were determined using LinRegPCR analysis software (available at <http://linregpcr.nl>), by setting the fluorescence threshold above the fluorescence values of the negatives.^{36,37}

Melt analysis of PCR products

Melt analysis was performed using the PCR products from the experiments that evaluated the effects of MgCl₂ on PCR specificity. Samples were prepared at a final concentration of 2 μM SYTO[®] 9 green fluorescent nucleic acid stain (Molecular Probes, prod. no. S34854), 10 mM Tris buffer at pH 8.0, 1.5 mM MgCl₂, and 5 μL of the PCR products. Melt analysis was run using the High Resolution Melt program on the Rotor-Gene instrument. Samples were heated in 0.1 °C increments from 75 °C to 95 °C. The temperature was held for 4 seconds at each increment and SYTO 9[®] fluorescence was measured using the HRM channel (460 nm excitation, 530 nm detection). The derivatives of the fluorescence values were plotted in the Rotor-Gene software. The expected melt peaks for the DNA target and the non-target sequence were identified based on the results from control samples containing the DNA target sequence or a non-target primer-matched sequence alone.

RESULTS AND DISCUSSION

L-DNAs have key properties for reporting reaction hybridization conditions

Three important features make L-DNA particularly fitting for use as a surrogate for indicating D-DNA hybridization conditions. First, L-DNA has the same physical properties as natural D-DNA³⁰ and therefore responds similarly to sample conditions. To confirm this, we demonstrated that L-DNAs and D-DNAs have identical melt responses to several known effectors of DNA hybridization during PCR, including magnesium, ethanol, sodium, and trehalose (Figure 2A - D). Second, L-DNAs do not cross-hybridize with complementary D-DNA strands,^{28,35} and therefore do not interfere with primer annealing or target melting. We confirmed this by performing a melt analysis of complementary L-DNA:D-DNA strands, which produced no hybridization signal, while complementary L-DNA:L-DNA or D-DNA:D-DNA strands produced a distinct hybridization signal (Figure 2E). And third, L-DNAs do not interfere with the amplification of PCR targets.³³ We demonstrated this by performing PCR using a standard Qiagen Rotor-Gene Q instrument and observing equal amplification efficiencies in reactions with and without L-DNAs added (Figure 2F). These unique features make L-DNA an ideal PCR “informant,” reporting when the reaction conditions for optimal PCR primer annealing and target melting have been achieved, without interfering with or participating in the amplification reaction.

L-DNA hybridization sensing successfully controls thermal cycling

To demonstrate the feasibility of controlling thermal cycling based on the hybridization state of the L-DNAs, a prototype instrument was developed and evaluated using a standard PCR reaction containing L-DNA analogs of the primers and targets. The thermal cycler was constructed using a forced-air heater, two Qiagen ESElog dual-channel fluorimeters, and

importantly, no thermal sensors (Figure 3A). The fluorimeters were used to monitor i) target melting using labeled L-DNA target analogs, ii) primer annealing using labeled L-DNA primer analogs, and iii) DNA amplification using a hydrolysis probe (sequences provided in Supplementary Table 1). The derivatives of the absolute fluorescence values of L-DNA target melting or L-DNA primer annealing were plotted and fit to a Gaussian curve in real-time, and the Gaussian fit was used to monitor target melting and primer annealing (Figure 3B). The ideal switch-point between heating and cooling was determined to be two standard deviations from the mean ($\bar{x} + 2\sigma$) of the Gaussian-fitted derivative (Supplementary Figure 1), which assuming a normal distribution, is equivalent to 97.8% of L-DNA targets melted or L-DNA primers annealed. Importantly, because the control algorithm is based on changes in hybridization state rather than absolute fluorescence values or temperature measurements, no optical or thermal calibrations are required.

The Adaptive PCR approach was first demonstrated to perform independent of thermal measurements and without the use of programmed temperature set points using a PCR assay developed for a 77-mer *Mycobacterium tuberculosis* DNA biomarker sequence using a standard Qiagen Rotor-Gene Q PCR instrument. Under baseline reaction conditions, Adaptive PCR successfully amplified the DNA template (Figure 4, left y-axis), producing an amplification curve similar to the Qiagen Rotor-Gene instrument at the same template concentration (compare to Figure 2C). Passive temperature measurements collected during the Adaptive PCR reaction by temporarily inserting a thermocouple directly into the reaction revealed that the reaction was switched from heating to cooling at a 94.8 ± 0.4 °C melt temperature and from cooling to heating at a 60.7 ± 0.3 °C annealing temperature (Figure 4, right y-axis), which align with the 95 °C melting and 60 °C annealing temperatures that were determined to be optimal on the Rotor-Gene instrument.

Adaptive PCR overcomes challenges with indirect temperature sensing designs

Precise temperature regulation is a fundamental challenge with the design of traditional PCR instruments. To control thermal cycling, the reaction temperature is typically estimated using thermocouple probes positioned outside of the reaction tube (i.e., usually in an aluminum block or in the air), and the PCR sample and thermocouple are insulated from the effects of ambient temperature. Factory calibration algorithms are programmed to estimate the temperature inside of PCR sample tube based on the thermal properties of the system. Because heat transfer does not scale linearly with the thermal gradient outside of the tube and because measurement errors are disproportionately propagated at higher and lower temperatures, the thermal calibrations of PCR instruments are only valid within a relatively narrow range of ambient temperatures. In contrast to the temperature-focused design of traditional thermal cyclers, Adaptive PCR focuses on the hybridization state of L-DNA probes, which eliminates the requirement for thermal monitoring, calibrations, or insulation and overcomes the challenges associated with indirect temperature measurements.

The advantages of the calibration-free design of Adaptive PCR were demonstrated by running it at an extreme ambient temperature that was expected to cause standard PCR to fail. Adaptive PCR was compared to a standard PCR instrument at 35 °C, which exceeds the specified 18 – 30 °C operational range for which many PCR instruments are calibrated. The

Adaptive PCR and Rotor-Gene instruments produced similar amplification curves at room temperature (22 °C) (Figure 5A, solid blue and red curves, respectively), with cycle threshold values of 13.1 and 14.2, respectively. At the elevated ambient temperature (35 °C), the Adaptive PCR cycle threshold was 14.1, which was similar to those produced at room temperature (dotted blue curve), while the Rotor-Gene shifted to a much later cycle threshold of 17.9 (dotted red curve). Notably, each cycle of Adaptive PCR increased by 8.1 ± 0.3 seconds in response to the longer air-cooling time required at elevated ambient temperatures (Figure 5B). These results demonstrate that Adaptive PCR dynamically adapts cycling conditions to compensate for ambient temperatures that fall outside the calibration range of standard PCR without the need for the thermal monitoring, calibrations, or insulation that are required for standard PCR instruments.

Adaptive PCR adapts to changes in the hybridization properties of DNA

Another challenge with traditional PCR is that sample-to-sample variation and sample preparation error can introduce reagents that can negatively impact PCR. Two of the most common PCR inhibitors are i) direct inhibitors of polymerase (e.g., heme, urea, heparin, guanidine) and ii) molecules that impact DNA hybridization properties (e.g., salts, sugars, alcohols). While the development of inhibitor-tolerant polymerases is an area of active research with many products coming to market, not much attention has been given to addressing the effects of hybridization stabilizers and destabilizers. Adaptive PCR is designed to overcome the effects of this second class of PCR inhibitors. The unexpected presence of molecules in the reaction that change hybridization properties of nucleic acids leads to non-optimal primer annealing or target melting at the programmed set points. This results in poor amplification specificity or efficiency. Because Adaptive PCR monitors the hybridization state of L-DNA probes, the approach is anticipated to compensate for the effects of these molecules and adapt thermal cycling conditions to achieve efficient primer annealing and target melting.

The potential for Adaptive PCR to adapt to changes in DNA hybridization properties was demonstrated by adding to the reaction examples of reagents known to stabilize or destabilize DNA. The unexpected presence of chemicals that stabilize DNA hybridization increases the likelihood of promiscuous primer binding to non-target sequences and decreases amplification specificity by increasing the annealing temperature of primers above the programmed annealing temperature. To determine if Adaptive PCR overcomes the effects of a DNA hybridization stabilizer, nonspecific amplification was evaluated in response to variation in the concentration of magnesium, an essential polymerase cofactor that is present in each reaction for efficient polymerase activity. For these studies, we added to the reaction a non-target 137-mer DNA sequence containing primer-binding regions similar to the target DNA template but with three nucleotide mismatches in each (sequence provided in Supplementary Table 1). Non-specific amplification of the non-target sequence was evaluated using melt analysis of the PCR products obtained using baseline magnesium concentrations (1.5 mM) and with additional magnesium added (4.0 mM total). Rotor-Gene reactions containing baseline 1.5 mM MgCl_2 (Figure 5C, solid red curve) produced a distinct target product peak near 90 °C, as expected. Using 4.0 mM total MgCl_2 , however, the Rotor-Gene produced a distinct non-target product peak near 84 °C (dotted red curve),

indicating nonspecific product formation. Adaptive PCR reactions containing either 1.5 or 4.0 mM MgCl₂ (solid or dotted blue curves, respectively) each produced a distinct target product peak near 90 °C. These results demonstrate that the hybridization-focused approach adapts to the increased magnesium levels and amplifies with greater specificity than standard PCR, which does not adapt to the increased stability of primer annealing.

The potential for Adaptive PCR to overcome the effect of reagents that destabilize DNA hybridization was also demonstrated. The presence of DNA destabilizers decreases the annealing temperature of primers, which results in an insufficient number of primers binding to the target sequences at the programmed annealing temperature and decreases amplification efficiency. Adaptive PCR performance was compared to standard PCR in the presence of ethanol, a known DNA destabilizer that is introduced into reactions as carry over from DNA sample preparation, where it is often used in high concentrations. The level of amplification was nearly identical for both instruments with no ethanol in the reactions, but began to diverge when ethanol was present (Figure 5D). On the Rotor-Gene instrument, amplification plateau levels decreased by ~30% with 4% ethanol added to the reaction, decreased by ~90% with 5% ethanol, and failed completely with 8% ethanol (red curves). Using Adaptive PCR, amplification plateau levels decreased by ~16% with 8% ethanol added to the reaction, decreased by ~35% with 12% ethanol added, decreased by ~65% with 16% ethanol added, and failed completely with 24% ethanol added (blue curves). While these ethanol concentrations are much greater than anticipated in standard reactions, these results indicate that Adaptive PCR adapts to the decreased stability of primer annealing in the presence of a common DNA destabilizer, whereas standard PCR does not.

Because standard PCR relies on rigid temperature cycling programs, it cannot adjust cycling conditions to compensate for the presence of unexpected DNA stabilizers and destabilizers in the reaction. Adaptive PCR, on the other hand, dynamically adapts to the reaction contents by adjusting cycling conditions in real time based on the hybridization state of L-DNA analogs of the primers and targets. We measured the adaptive thermal response of Adaptive PCR to the presence of an example of a common DNA stabilizer, magnesium, and a common DNA destabilizer, ethanol by inserting a thermocouple into the reactions during thermal cycling. It was confirmed that Adaptive PCR switched to heating at lower or higher annealing temperatures in response to the presence of ethanol or magnesium, respectively (Figure 6). These differences in the temperatures at the annealing switch points are consistent with the relative shifts in annealing temperatures in the presence of these reagents reported in Figure 2A - B. Taken together, the results of these experiments indicate that Adaptive PCR enables efficient and specific amplification in the presence of DNA destabilizers and DNA stabilizers by dynamically selecting lower or higher annealing switch points, respectively.

CONCLUSION

In summary, Adaptive PCR dynamically controls cycling conditions by optically monitoring primer annealing and target melting of L-DNA probes added to the reaction. This approach offers a solution for issues with standard PCR that arise from the mismatch between indirect temperature monitoring and actual primer annealing or target melting. While Adaptive PCR

does not overcome the effects of all molecules that negatively impact PCR, it adapts to the presence of chemicals that alter DNA hybridization, and enables efficient and specific PCR amplification under conditions that cause PCR to fail. Current efforts are focused on incorporating robust polymerase enzymes that are resistant to common inhibitors into Adaptive PCR for a more complete solution to the wide range of inhibitors that confound PCR. Adaptive PCR has the potential to make PCR-based diagnostics more accessible outside of well-controlled laboratories, such as in point-of-care or field settings that lack the resources to accurately control the reaction temperature or perform high quality sample preparation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Basic concept for controlling thermal cycling based on L-DNA hybridization. During heating, melting of the L-DNA target analog strands is monitored using a HEX dye (orange curve). The melt switch point is triggered when all L-DNA targets are melted (orange arrow). During cooling, annealing of the L-DNA primer and target analogs is monitored using a Texas Red dye (red curve). The anneal switch point is triggered when all L-DNA primers are annealed (red arrow). The quencher-labeled target is added in excess to avoid competition between the HEX-labeled target and the Texas Red-labeled primer.

Figure 2.

Validation of L-DNA probes as hybridization reporters for PCR. (**A - D**) Double stranded L-DNAs (solid blue curves) and D-DNAs (dotted red curves) have identical annealing responses to increasing concentrations of MgCl_2 (**A**), ethanol (**B**), NaCl (**C**), and trehalose (**D**). (**E**) L-DNA primers hybridize to L-DNA targets (blue curves) and D-DNA primers hybridize to D-DNA targets (red curves), while L-DNA primers or targets do not cross-hybridize with D-DNA targets or primers (green and purple curves). L-DNA primers (dark blue curves) and D-DNA primers (orange curves) alone were used as no-hybridization controls. (**F**) PCR reactions prepared with a standard dilution series of template DNA with or without L-DNA added to the reactions amplified on a standard Rotor-Gene PCR instrument with similar efficiencies (0.81 and 0.80, respectively). Each curve is the average of three independent amplification reactions. The cycle threshold (C_t) values for each curve are listed in the table inset.

Figure 3.

Design and development of the prototype Adaptive PCR instrument and control program. (A) Photographs of the prototype Adaptive PCR thermal cycler. The device was constructed using a forced-air heater, two spot fluorimeters focused on a 25 μ L reaction tube, and a desktop computer running LabVIEW software to monitor the fluorescence and control heating and cooling (not shown). (B) Example fluorescence response curves from the L-DNA probes during a single cycle of heating (left panel) and cooling (right panel). As the reaction is heated, the HEX-labeled L-DNA target sequence melts from the BHQ-labeled complement, increasing the HEX fluorescence (left panel, orange points). As the reaction is cooled, the Texas Red-labeled L-DNA primer sequence anneals to the BHQ-labeled complement, decreasing the Texas Red fluorescence (right panel, red points). The derivatives of these fluorescence curves (grey points) are plotted in real-time, and a Gaussian fit of the derivatives (black curves) is used to determine the switch point between heating and cooling (orange and red arrows) as a function of the number of standard deviations (σ) from the mean ($\bar{x}$).

Figure 4. Raw fluorescence data from Adaptive PCR amplification using a positive sample containing DNA template and a negative sample containing no template (left y-axis, black curves). Thermocouple measurements in the sample indicate the reaction temperatures at each of the melt and anneal switch points during the 40 cycles (right y-axis, red triangles).

Figure 5.

Performance of the Adaptive PCR approach. **(A)** Normalized PCR amplification curves from the Adaptive PCR (blue curves) and Rotor-Gene (red curves) instruments at room temperature (22 °C) (solid curves), and at the elevated ambient temperature (35 °C) (dotted curves). Each curve is the average of three independent amplification reactions. Raw data from Adaptive PCR is included in Supplementary Figure 2. **(B)** Passively collected thermocouple measurements for four representative cycles of Adaptive PCR. An increase in cycle timing was observed in reactions performed at 35 °C (dotted curve) compared to 22 °C (solid curve). **(C)** Melt analysis results from Adaptive PCR and Rotor-Gene (blue and red curves, respectively) reactions after 40 cycles of amplification with 1.5 or 4.0 mM total MgCl₂ added (solid and dotted curves respectively). The melt temperature was 84 °C for the non-target mismatch product and 90 °C for the target product. Each melt curve is the average of four independent reactions. **(D)** Normalized PCR amplification curves from the Adaptive PCR (blue curves) and Rotor-Gene (red curves) instruments with increasing concentrations of ethanol added to the reactions. Each curve is the average of three independent amplification reactions. Raw data from Adaptive PCR is included in Supplementary Figure 2.

Figure 6.

Thermal cycling temperatures for ~5 cycles focused on the range of temperatures before and after the annealing switch points for reactions containing baseline conditions (1.5 mM MgCl_2 and 0% ethanol, blue curves), high magnesium conditions (4 mM MgCl_2 and 0% ethanol, dark grey curves), or high ethanol conditions (8% ethanol and 1.5 mM MgCl_2 , crimson curves). Temperature measurements were collected by inserting a thermocouple probe directly into the reaction. Average annealing switch point temperatures are listed for each condition to the right.