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Isothermal amplification of long DNA segments by quadruplex priming amplification

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Abstract

Amplification of long DNA segments with the highest possible specificity and lowest bias is one of the main goals of modern genomics. Quadruplex priming amplification (QPA) is a single-primer isothermal method, which employs the free energy of quadruplex structures as the driving force for DNA amplification without any extra reaction components. As a result, QPA represents one of the simplest isothermal assays and was previously shown to be suitable for amplification of relatively short DNA sequences. The current study reveals that single-primer QPA can be used for both exponential and linear amplification of relatively long DNA segments (>100 nt), and switching between these modes can be accomplished by simple re-design of the primer used. While exponential amplification resulted in production of some undesired higher molecular weight species, linear QPA demonstrated highly specific amplification of the target molecules without any side products.

Keywords

DNA amplification; isothermal; quadruplex

INTRODUCTION

One of the main challenges of next-generation sequencing (NGS) is to accomplish DNA amplification of target molecules with high specificity and low bias [1; 2; 3]. Polymerase chain reaction (PCR), which allows amplification of DNA from specimens containing minute quantities of a nucleic acid target, is the most common method for DNA amplification. High sensitivity is achieved by using excess amounts of PCR components (i.e., primers, polymerase), which ensures that final complex formation (target/primer/polymerase) is thermodynamically and kinetically favorable. However, excess reaction components can also result in undesired non-specific hybridization events (i.e., non-specific priming, primer dimerization) and undesired polymerase activities. Constant temperature cycling, which completely unfolds DNA duplexes also facilitates non-specific priming,

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CONFLICTS OF INTEREST

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including self-priming of the amplicons. To eliminate thermal cycles, several isothermal assays were developed [4; 5; 6]. Most of these isothermal methods introduce additional components, which can result in generation of non-specific products, or are suitable only for amplification of short DNA segments. For instance, loop-mediating priming amplification and similar assays employ several “bumper” primers (4–6) to displace the initial primers and achieve isothermal amplification [7]. However, these methods require very careful design, are unable to amplify long targets, and suffer from strong background activity. Isothermal assays based on nicking enzymes are robust; however, due to simultaneous non-specific activities of the enzymes (i.e., nicking outside of the specific site or unprimed polymerization), they are characterized by very strong background amplification [8; 9]. Helicase-dependent amplification employs extra protein components including a helicase to unfold the DNA duplex and single-strand binding proteins to keep the primer binding site (PBS) duplex unfolded [10]. Since any additional component in the PCR mixture is an extra source for background activity, the isothermal nature of existing assays is achieved at the expense of specificity and sensitivity of amplification.

Since polymerases with strand-displacement ability exist, the main purpose of temperature cycling is to free a PBS for the next cycles of primer annealing. Therefore, the simplest way to transform PCR into an isothermal reaction would be to design a specific primer capable of self-dissociation and folding into a stable secondary or tertiary structure. This approach is employed in quadruplex priming amplification (QPA), which uses the free energy of DNA quadruplexes to convert PCR into an isothermal assay [11]. A key aspect of QPA is that the 5′-GGGTGGGTGGGTGGG-3′ (G3T) sequence is capable of forming a quadruplex with significantly more favorable thermodynamics than the corresponding DNA duplex. In contrast, a truncated version of G3T (missing G or GG at the 3′ end) is not able to form a quadruplex and readily binds to the PBS. Upon polymerase elongation, the truncated primer is extended to form a full-length G3T, which self-dissociates from the PBS and folds into a stable monomolecular quadruplex. As a result, the PBS is accessible for annealing to an incoming primer without temperature cycling or any additional components. Moreover, QPA requires only one primer, which further simplifies the reaction mixture and allows both exponential and linear amplification. The latter represents the most unbiased amplification since only the original targets are replicated. The only QPA requirement is to incorporate a QPA-specific PBS (i.e., CCCACCCACCCACCC) in the target molecules, which can easily be accomplished via adapter attachment to the target DNA. Thus, QPA represents the simplest isothermal assay developed to date and has the potential to amplify DNA with low bias and high specificity.

Earlier studies demonstrated that QPA can be successfully adopted for molecular diagnostics, which requires amplification of short (< 30 nucleotides (nt)) DNA segments [12; 13; 14]. For instance, QPA can achieve 10¹⁰-fold amplification of short AT-rich sequences in less than 40 min [9]. However, it was also shown that increasing the target length or GC-content significantly impedes QPA [14]. In the present study, the potential of QPA to amplify relatively long DNA molecules is investigated, demonstrating that single-primer QPA can be used for both exponential and linear amplification. Switching between exponential and linear amplification can be achieved by changing the design of the primers used. While exponential amplification resulted in production of some undesired higher

molecular weight species, linear QPA demonstrated highly specific amplification of only the target molecules.

MATERIALS AND METHODS

Enzymes and DNA substrates

DNA polymerases (*Vent*(exo-) and *Bst* 2.0), dNTPs and isothermal buffer (10 mM (NH₄)₂SO₄, 50 mM KCl, 2 mM MgSO₄, 0.1% Tween® 20, 20 mM Tris-HCl, pH 8.8) were purchased from New England BioLabs. All oligonucleotides were obtained from Integrated DNA Technologies with the exception of 3-methylisoxanthopterin (3MI)-containing primer purchased from Fidelity Systems. The 3'-ends of the templates were protected by a 3C spacer to inhibit polymerase extension. The concentration of DNA oligonucleotides was determined by measuring UV absorption at 260 nm as described earlier [15]. All experiments were performed in the buffer conditions suitable for DNA polymerases: 50 mM monovalent cations (K⁺ and Cs⁺), 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.7. The concentration of Cs⁺ ions, which was included in some experiments to partially destabilize quadruplexes, is indicated in the figure legends. Primers with a stem-loop (SL) attachment at the 5'-end contain the 14-nt segment, 3'-*CGCGCTGGTGC*CG-5', in addition to a quadruplex-derived segment. The SL sequence forms a 5-bp stem (shown in italics) and 4-nt loop, to avoid secondary structure formation with the quadruplex primer.

Fluorescence measurements

Fluorescence measurements (3MI, Ex 348 nm, Em 431 nm) were performed using a Varian spectrophotometer (Cary Eclipse, USA) and ESEQuant Tube Scanner (Qiagen, Germany). QPA reactions were carried out directly in the quartz cuvettes inserted in the spectrophotometer or in 0.2 mL PCR tubes using the tube scanner. Fluorescence was expressed in relative fluorescence units (RFU).

3MI-based monitoring of QPA

Isothermal QPA with a fluorescent primer was used to estimate the rate of linear amplification. The 3MI-containing QPA primer, 5'-GGG(3MI)GGGCGGGTGG-3', demonstrates a strong increase in fluorescence upon polymerase extension and subsequent quadruplex formation, which permits simple and effective quantification of the amplicons [8; 16]. These QPA reactions were typically carried out in a reaction mixture containing 2 μM primer, 800 μM mix of all four dNTPs or 100 μM dGTP using *Bst* 2.0 or *Vent*(exo-) DNA polymerases. The reactions were carried out in quartz cuvettes or PCR tubes. The reaction mixtures were vortexed for 2–3 sec and inserted into a cell or tube holder equilibrated at the indicated reaction temperature for 30–60 min. In some cases, the temperature was varied, as indicated in the figure legends. In the case of real-time monitoring of QPA through 3MI fluorescence, the fluorescence measurements were initiated immediately upon insertion into the instrument. QPA rates were determined from the initial slopes of the kinetic curves conducted at different temperatures. Each value corresponds to the average of three independent determinations. After each experiment, the cuvettes were washed with DNAzap solution (Invitrogen) for complete elimination of DNA products.

Quantitative polymerase chain reaction (qPCR)

The primer pair (5'-CTGGAAGAGGGCTAATAAGG-3' and 5'-AGAGACCACACTTCATCAACC-3') was designed to quantify QPA products by qPCR. Each reaction (20 µL) contained qPCR mix (Roche Diagnostics, Germany), 500 nM of each primer and 4 µL of QPA product. Before quantification, the QPA product was purified using a MinElute PCR Purification Kit (Qiagen, Germany). For qPCR, an initial 5 min denaturation step at 95 °C was followed by 35 cycles of 10 sec hybridization at 60 °C, replication at 72 °C and denaturation at 95 °C. Each data point corresponds to the average of three separate measurements collected at different time points.

Gel electrophoresis

Agarose gels (1.5%) were prepared in TBE buffer with ethidium bromide. A 100-bp ladder (New England BioLabs) and a 92-bp stem loop were used as markers. The latter was obtained by *Bst* 2.0 extension (duplexing) of the template shown in Fig. 3A.

RESULTS AND DISCUSSION

Role of AT-rich segment between QPA-PBS and target sequence

Linear QPA reactions conducted with short targets were studied earlier [8; 17]. When a 14-nt 5'-GGGTGGGTGGTGG-3' primer binds to a 15-nt 3'-CCCACCCACCCACCC-5' target, the extended primer, or full-length quadruplex, self-dissociates from the target, allowing the next priming cycle. This reaction is very fast and, as expected, strongly depends on the primer concentration [8; 17]. We also showed that elongation of the target molecule by a 10-nt AT-rich segment does not affect the QPA rate significantly [14]. However, inserting a GC-rich segment at the 3'-end of the target significantly slowed down the rate of QPA amplification and shifted the optimal activity to higher temperatures [14]. We hypothesize that the slower rate of QPA with longer targets is attributed to the increased thermostability of the resulting longer duplex, which inhibits quadruplex self-dissociation. Here, to overcome this shortcoming we systematically studied the effect of target elongation and nt composition on the rate of linear QPA. As a positive control, we used dGTP-QPA (QPA conducted with only dGTP). In this reaction, after adding the single G to the primer, the polymerase is unable to continue elongation and dissociates from the target (see Fig. 1A). Thus, dGTP-QPA on longer targets mimics dNTP-QPA (QPA conducted with all four dNTPs) on short (i.e., 15-nt) targets and reflects the highest achievable rate for dNTP-QPA with long targets. In contrast to dGTP-QPA, in dNTP-QPA, the polymerase produces full-length amplicons and performs strand-displacement amplification (Fig. 1B). In addition, since dNTP-QPA produces quadruplexes appended to longer segments of DNA, liberating the PBS is expected to be significantly slower than for dGTP-QPA.

The dNTP-QPA cycle (Fig. 1B) includes: (i) primer binding to the PBS; (ii) polymerase binding to the primer/template complex; (iii) extension, which includes dNTP binding, conformational change and nucleotide incorporation; (iv) polymerase dissociation; (v) quadruplex formation and next primer binding; and (vi) strand-displacement synthesis with concomitant product dissociation. It is known that for nonprocessive DNA synthesis, enzyme dissociation is the rate-limiting step [18; 19; 20]. Systematic studies using 11- and

15-nt targets revealed that the rate-limiting step of QPA is amplicon dissociation and primer annealing [8; 17]. We hypothesized that placement of an AT-rich segment between the PBS and the target sequence would facilitate dNTP-QPA by facilitating self-dissociation of the quadruplex as a separate domain. To test this idea, we studied the effect of the length of AT-rich inserts on the QPA rate. All constructs contained a 14-nt GC-rich sequence at the 5'-end and a QPA-PBS at the 3'-end (Fig. 2A). These two segments were separated by 34-nt, 20-nt or 5-nt AT-rich segments. The QPA rate was measured over the temperature range of 58 °C to 70 °C using *Bst* 2.0 polymerase. dGTP-QPA using a 45-nt long DNA target containing the 20-nt AT-rich segment (target 2, Fig. 2B, black curve) revealed a rate of ~30 amplicons/min, in good agreement with previous linear QPA measurements using 11-nt or 15-nt targets [14]. As expected, dNTP-QPA proceeds with significantly lower rates (Fig. 2B, colored curves). Between 58 °C and 60 °C, all targets demonstrate an increase in QPA rate (Fig. 2C). At 60–62 °C, the rates reach a maximum, and further increase in temperature is accompanied by a decline in QPA rate. A similar dependence of the QPA rate on temperature was observed earlier due to the thermodynamic nature of QPA [8; 17]. Specifically, QPA properties are determined by the thermal stabilities of the primer/template complexes before and after elongation. Isothermal QPA requires both priming and duplex dissociation at the same temperature. Below the optimal temperature, the primer binds more efficiently to the target; however, product dissociation slows down due to higher stability of the complex. At temperatures > 62 °C, product dissociation is facilitated; however, priming becomes less efficient, which is manifested in a decreased QPA rate (Fig. 2C). The QPA rate increases as the length of the AT-rich segment increases (Fig. 2C). For example, at 62 °C, the shortest target with a 5-nt AT-rich segment (target 3) has a QPA rate of 1.2 amplicons/min, while the rate of target 2 at this temperature is 1.7 amplicons/min. Elongation of the AT-rich segment to 34-nt is accompanied by a further increase in the rate to 2.5 amplicons/min. Thus, AT-rich segments facilitated dNTP-QPA and the reaction rate is proportional to the length of the AT-rich segment.

Characterization of QPA products using a 110-nt template

Next, a series of experiments was performed using the 110-nt template shown in Fig. 3A and a variant lacking the 5'-end hairpin structure. This construct consists of the following segments starting from the 3' end: (i) 15-nt long QPA-PBS; (ii) 15-nt long AT-rich segment to facilitate isothermal self-dissociation of the quadruplex; (iii) 50-nt segment with ~50% GC composition and two PBSs for qPCR quantification of the QPA product; and (iv) 30-nt stem-loop segment, which serves as a primer to initiate duplexing of the amplicons and minimize formation of non-specific products. Amplification of this 110-nt DNA construct was characterized by fluorescence, electrophoretic and qPCR methods.

All amplification reactions were tested using this construct with some variations within the QPA-PBS segment (Fig. 3A). The constant part of the construct is designated in the figures as “template”. Modifications in the QPA-PBS segment enabled optimization of the amplification temperature and switching between exponential and linear QPA. We hypothesized that more stable QPA-PBS segments would allow amplification at elevated temperatures and therefore facilitate strand-displacement. The 3'-ends of all constructs were protected by a C3 spacer to avoid polymerase elongation of original templates.

Rate of linear QPA determined by 3MI fluorescence—Fig. 3B illustrates the steps of the QPA reaction using the 110-nt template shown in Fig. 3A. Briefly, following primer annealing, the polymerase extends the primer resulting in a 110-bp duplex. The quadruplex sequence then dissociates and allows the next primer to anneal. Following strand-displacement synthesis, the product of the first round dissociates and the 3'-end hairpin serves as the primer for synthesis of the 92-bp stem loop. Quadruplex dissociation allows the incoming primer to anneal. Strand invasion synthesis generates the 2nd QPA-PBS at the 3'-end, allowing exponential amplification of the 190-bp duplex. Initially, the linear QPA rate was determined using a 110-nt template, similar to the sequence shown in Fig. 3A, but with an altered 5'-end (green segment) that was unable to form the stem-loop structure. This was done to avoid exponential amplification. The linear QPA rate was monitored in real-time through 3MI fluorescence using the primer shown in Fig. 2A employing *Bst* 2.0 and *Vent* (exo-) polymerases under different experimental conditions (Fig. 4). K⁺ ions are key elements of QPA [11] and the amplification was measured in two different buffers containing 10 mM K⁺ (panels A, C) and 50 mM K⁺ (panels B, D). As expected, signal amplification in the presence of only dGTP was significantly faster than dNTP-QPA under all conditions examined. At 10 mM K⁺, *Bst* 2.0 and *Vent* (exo-) demonstrated ~25-fold decreased (from 35 to 1.5 amplicons/min) and ~10-fold decreased (from 10 to 1 amplicons/min) rates in dNTP-QPA relative to dGTP-QPA, respectively. At 50 mM K⁺, all reactions were accelerated and the no-template controls (NTC) revealed some background activity. This is likely due to quadruplex formation of unextended 14-nt primers at increased K⁺. As previously observed, background activity can be inhibited by using 13-nt primers missing GG at the 3'-end [17]. Therefore, in the final amplification experiments we employed primers missing 3'-end GG (see Fig. 5–8). Thus, both K⁺ concentration and primer length can be optimized to inhibit the background activity of linear QPA, which is not associated with production of non-specific amplicons, but arises from spontaneous folding of the primers into quadruplexes.

We also performed QPA in isothermal buffer from NEB, which is specifically designed for isothermal amplification and results in the optimal activity of *Bst* polymerases. The buffer contains 50 mM KCl and 10 mM (NH₄)₂SO₄, which are suitable for quadruplex formation (NH₄⁺ is considered one of the best quadruplex-forming monovalent cations [21]). The NEB buffer also contains 0.1% Tween 20, which is a known destabilizer of DNA duplexes and facilitates strand-displacement replication. The results of the reactions performed in the NEB buffer are shown in Fig. 4E. Interestingly, in this buffer the *Bst* 2.0 dGTP-QPA rate decreased ~4-fold to 9 amplicons/min, while dNTP-QPA demonstrated increased activity (3 amplicons/min) relative to the reactions performed in 50 mM K⁺ in the absence of the detergent (Fig. 4A–D). Thus, the detergent has both positive and negative effects on QPA. As mentioned above, the rate-limiting step in QPA reactions is the combined process of amplicon dissociation and primer annealing. Since the detergent is a non-specific destabilizer of DNA duplexes, it can both facilitate product dissociation and inhibit the priming process. Thus, the observed decrease of the dGTP-QPA rate can be attributed to the detergent's more pronounced negative effect on the priming process than on the already fast product dissociation in this case. However, in dNTP-QPA, destabilization of the product molecules is more pronounced, which accelerates the overall reaction. Since the isothermal

buffer contains high amounts of the quadruplex-forming cations (K^+ and NH_4^+), the NTC reaction also shows some activity.

Primer attachment serves as a switch between exponential and linear QPA—

Since exponential amplification introduces bias [2], one of the goals of the current study was to design a linear QPA approach, which replicates only original templates. As shown in Fig. 3B, before step (iv), QPA is a linear process because it employs only original targets. The last step of linear QPA creates fully duplexed monomolecular duplexes. Linear amplification requires that the quadruplex (red segment) doesn't self-dissociate from the long duplex and stays base paired with its complement. In contrast, for exponential amplification, the quadruplex must self-dissociate and allow the next priming event followed by strand-displacement replication and quadruplex invasion (step v). This creates a 2nd QPA PBS in the new amplicon (step vi) and initiates exponential amplification. It was shown earlier, that DNA polymerases are capable of invading even very stable quadruplexes [9]. When we allow exponential QPA to proceed by using 13-nt QPA primers, DNA amplicons of various lengths (Fig. 5, lane 1) in addition to the expected linear (92-bp) and exponential (190-bp) products are formed, most likely due to non-specific activities (i.e., template switching) of the polymerases [22; 23]. To prevent exponential amplification or to stop QPA at step (v) (Fig. 3B), we appended a 14-nt 5'-CGCGCTGGTGC GCG-3' sequence to the primers. This sequence can fold into a SL structure to avoid intramolecular interaction with the primer. After duplexing the initial product, the resulting quadruplex/PBS domain is significantly stabilized and quadruplex self-dissociation is inhibited. In this case, only one band of the expected size corresponding to the linear amplification product is observed (Fig. 5, lane 2), demonstrating complete inhibition of exponential QPA. Based on the intensity of the band in lane 2 relative to a 92-bp control, we estimate ~50-fold amplification of 0.1 nM target in 60 min. This is in general agreement with amplification rates measured from direct fluorescence measurements (Fig. 3 and 4).

To confirm that inhibition of exponential QPA is the result of stabilization of the quadruplex domain, temperature oscillation reactions were performed. In these reactions, QPA reactions were periodically moved between two water baths, equilibrated at certain temperatures, and incubated for various times as indicated in the figure legends. We hypothesized that narrow temperature oscillation (i.e., between 65 °C and 77 °C), insufficient to unfold the duplexed amplicon, would produce only linear product. However, wider temperature oscillation (i.e., between 65 °C and 85 °C) would be sufficient to unfold the duplexed amplicon and exponential products would be detected. In agreement with this hypothesis, 12 °C temperature oscillation using the extended primer demonstrated only one product band (Fig. 6A, lane 3), while a 20 °C oscillation shows two products associated with exponential amplification (Fig. 6B, lane 3). As expected, a reaction using a short 13-nt primer yielded the exponential products under both conditions (Fig. 6A and 6B, lane 1). We hypothesized that a 13-nt primer with T to U substitution in position 11 (primer 2) might prevent formation of QPA-PBS in the amplicons and thus reduce exponential QPA. This modification decreased the amount of the exponential product (Fig. 6A and 6B, lane 2 vs lane 1), but didn't eliminate it completely. Thus, extension of the 5'-end of the primer represents a more efficient approach to inhibit exponential QPA. Interestingly, compared to

the completely isothermal approach where multiple product bands were observed (Fig. 5, lane 1), single product bands of the expected size were obtained using the temperature oscillation approach.

To estimate the linear amplification product yield, temperature oscillated QPA of 0.1 nM target and the SL-containing primer was performed between 75 °C and 81 °C with 10 sec incubation at each temperature. Lanes 1, 2 and 3 in Fig. 7 corresponding to 20, 50 and 100 cycles, respectively, demonstrated a linear increase of the final product with increasing cycles. The observed product yield agrees well with 2 nM, 5 nM and 10 nM of a loaded control 92-bp duplex (Fig. 7, lanes 4, 5 and 6), confirming the expected band size and yield.

Rate of linear QPA determined by qPCR—To more accurately quantify the product yield, isothermal amplification of 1 pM target was carried out at 74 °C and quantified by qPCR. The data demonstrate linear amplification, which plateaus after 30 min likely due to polymerase inactivation (Fig. 8). During the first 30 min, the rate of QPA is ~1 amplicon/min. This value is in general agreement with the QPA rate determined from real-time fluorescence measurements at 10 nM target concentration (Fig. 4). Thus, the QPA rate is independent of the target concentration.

CONCLUSIONS

The present study demonstrated that QPA can be adopted for isothermal amplification of long DNA segments. The biggest advantage of QPA over other isothermal assays is its simplicity. QPA is a single-primer assay and does not use any extra reaction components. It is capable of amplifying target DNA in a linear manner with high specificity and low background. In contrast, exponential isothermal amplification resulted in undesired higher molecular weight species, likely due to template switching activity of the polymerase. These undesired products can be eliminated by temperature oscillation. In addition, to ensure that only linear amplification is achieved, longer 5'-end extended SL primers can be used. The rate of linear QPA is ~2–3 amplicons/min, which can be accelerated by a narrow (± 3 °C) temperature oscillation. Linear QPA has the potential to decrease the cost of NGS by amplification of target molecules with high accuracy and specificity.

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Fig. 1. Scheme demonstrating dGTP-QPA (QPA in the presence of only dGTP) (A) and dNTP-QPA (QPA in the presence of all dNTPS) (B) using long targets.

Fig. 2. Rate dependence of linear QPA on the length of AT-rich segment. (A) Targets and primer sequences; targets 1, 2 and 3 contain 34-nt, 20-nt and 5-nt AT-rich segments, respectively. (B) Typical dGTP-QPA (black) and dNTP-QPA profiles (colored) obtained using 0.5 μ M primer, 10 nM target 2, 100 μ M dGTP or 800 μ M dNTP, 0.08 U/ μ L *Bst* 2.0 polymerase in 10 mM KCl, 40 mM CsCl, 2 mM MgCl₂ and 10 mM Tris-HCl, pH 8.7 at 62 °C. Dashed line corresponds to no-template control (NTC). (C) Temperature dependence of the rate of linear QPA using target 1 (blue), target 2 (red) and target 3 (black).

Fig. 3. QPA of a 110-nt template. (A) Sequence of the template containing a 3' 15-nt QPA-PBS (red), a 15-nt AT-rich segment, a 50-nt sequence with ~50% GC content and two qPCR PBSs (italic bold) and a 12-bp duplex segment (green) (see text for detailed explanation). (B) Scheme showing the steps of linear QPA (steps I–iv) and exponential QPA (steps i–vii).

Fig. 4.

Comparison of dNTP-QPA vs dGTP-QPA using 0.04 U/ μ L *Vent*(exo-) (A, B) and 0.08 U/ μ L *Bst* 2.0 (C, D, E) in 10 mM KCl buffer (10 mM KCl, 40 mM CsCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.7) (A, C), 50 mM KCl buffer (50 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.7) (B, D) and NEB isothermal buffer (see Materials and Methods) (E). These representative reactions are conducted with the template shown in Figure 1 using 1 μ M GGG(3MI)GGGCGGGCGG primer, 10 nM target, 800 μ M dNTP or 100 μ M dGTP. All experiments are conducted at 70 °C (A–D) or 72 °C (E). Arrows in panel E indicate times when the second batch of enzymes were added; at ~40 and ~45 min, the enzyme was added in dNTP-QPA and dGTP-QPA, respectively, which resulted in the fragmentation of the curves. Black lines correspond to no-template controls (NTC).

Fig. 5.

Agarose gel showing isothermal QPA products using primers and template shown at the top. SL is the 3'-CGCGCTGGTGCGCG-5' extension designed to block experimental amplification. Lanes labeled 1 and 2 correspond to amplicons produced with primers 1 and 2 at 76 °C for 60 min using 0.08 U/μL of *Vent* (exo-). Amplifications were conducted in NEB isothermal buffer at [primer] = 2 μM, [target] = 0.1 nM and [dNTP] = 800 μM. The 92-b control corresponds to the stem-loop construct described in Material and Methods.

Fig. 6.

Agarose gels showing amplicons produced by temperature oscillation QPA using primers and template shown at the top. Lanes 1, 2 and 3 correspond to amplicons produced with primers 1, 2 and 3 following 20 cycles of temperature oscillations between 65 °C and 77 °C (panel A) and between 65 °C and 85 °C (panel B). The reactions were incubated for 30 sec at each temperature. Amplifications were conducted in NEB isothermal buffer at [primer] = 2 µM, [target] = 1 nM, [dNTP] = 800 µM and 0.04 U/µL of *Vent* (exo-). SL is defined in the legend of Fig. 5.

Fig. 7.

Agarose gel showing amplicons produced by temperature oscillation QPA between 75 °C and 81 °C (10 sec at each temperature). Lanes 1, 2 and 3 correspond to 20, 50 and 100 temperature oscillations in NEB isothermal buffer at [primer] = 2 μM, [target] = 0.1 nM, [dNTP] = 800 μM and 0.04 U/μL of *Vent* (exo-). Lanes 4, 5 and 6 correspond to 2 nM, 5 nM and 10 nM control 92-bp stem-loop (see Materials and Methods). SL is defined in the legend of Fig. 5.

Fig. 8.

Quantification of linear isothermal QPA by qPCR. QPA was performed at 74 °C in NEB isothermal buffer using 2 μ M primer, 1 pM target, 800 μ M dNTP and 0.04 U/ μ L of *Vent* (exo-). Primers and experimental conditions are given in the Materials and Methods. SL is defined in the legend of Fig. 5.