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Real-Time Detection of Isothermal Amplification Reactions with Thermostable Catalytic Hairpin Assembly

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Supporting Information

ABSTRACT: Catalytic hairpin assembly (CHA) is an enzyme-free amplification method that has previously proven useful in amplifying and transducing signals at the terminus of nucleic acid amplification reactions. Here, for the first time, we engineered CHA to be thermostable from 37 to 60 °C and in consequence have generalized its application to the real-time detection of isothermal amplification reactions. CHA circuits were designed and optimized for both high- and low-temperature rolling circle amplification (RCA) and strand displacement amplification (SDA). The resulting circuits not only increased the specificity of detection but also improved the sensitivity by as much as 25- to 10000-fold over comparable real-time detection methods. These methods have been condensed into a set of general rules for the design of thermostable CHA circuits with high signals and low noise.

Isothermal nucleic acid amplification reactions such as nucleic acid sequence-based amplification (NASBA),[1] signal-mediated amplification of RNA technology (SMART),[2] rolling circle amplification (RCA),[3] strand displacement amplification (SDA),[4] and loop-mediated isothermal amplification (LAMP)[5] may be much more tractable than the polymerase chain reaction (PCR) for the amplification of nucleic acid analytes in point-of-care applications. However, the robust detection of amplicons remains analytically difficult and in many cases still relies on the detection of final products by methods such as electrophoresis followed by dye staining (using ethidium bromide or SYBR Green)[6−8] or through monitoring the increase in calcein fluorescence[9] or solution turbidity due to pyrophosphate release.[10,11] These methods can allow for real-time monitoring of signals but sacrifice specificity because any accumulation of nonspecific or parasitic amplicons yields a false-positive signal. While some sequence-specific detection methods have also been developed, such as fluorescence resonance energy transfer (FRET) probes,[12,13] molecular zippers,[14] molecular beacons,[15,16] and hybridization with DNA-coated gold nanoparticles,[17,18] these approaches merely mirror the development of amplicons and provide no opportunity for programming how amplicon signals might be amplified, integrated, and displayed in real time.

In contrast, nonenzymatic nucleic acid circuits have previously provided a useful means for both amplifying and transducing signals from nucleic acid analytes.[19−25] One of these circuits, catalytic hairpin assembly (CHA), has been engineered to yield hundreds-fold catalytic amplification with negligible background and can transduce analyte binding to a variety of detection modalities, such as fluorescent and electrochemical signals.[26−29] Recently, CHA has been used as a specific end-point transducer[30] for enzyme-based isothermal amplification reactions such as LAMP that sometimes produce parasitic amplicons and result in a high false-positive rate.[30−32]

The further adaptation of such circuits to real-time detection would likely advance their general utility as molecular transducers, just as the use of exonuclease-sensitive (Taqman) probes[33] has led to great improvements in PCR sensitivities. In particular, the use of CHA transducers in real-time detection schemes may lead to concomitant improvements in the convenience, sensitivity, and programmability of isothermal reactions. However, the development of real-time CHA detection has previously posed several challenges. Many isothermal amplification reactions are carried out at 60 °C or above,[34] a temperature that may increase the background due to hairpin breathing.[35] Therefore, we have for the first time engineered a thermostable CHA circuit that can operate at temperatures up to 60 °C without sacrificing performance. This achievement has enabled the use of CHA as a real-time sequence-specific detector for two isothermal amplification reactions (RCA and SDA). The development of real-time CHA transduction has also yielded insights into circuit design that could be immediately applied to the creation of circuits that operate under different reaction conditions.

As a first step, we investigated whether circuits could be designed to operate at high temperatures. A circuit designed to perform at 37 °C in the presence of 140 mM NaCl and 5 mM KCl at pH 7.5 [hereafter called the TNaK reaction; Table S4 in the Supporting Information (SI)] was initially chosen and monitored for performance at higher temperatures. In this circuit (Figure 1), domain 1 (8-base, Table S1) on strand C1 (here termed $C1_{LT}$, where "LT" denotes "low temperature") serves as a toehold to bind domain 1* on $H1_{LT}$ and initiates a branch migration reaction to open the $H1_{LT}$ stem (16-base). Domain 3 (8-base) on $H1_{LT}$ then serves as a second toehold to initiate another strand displacement reaction to open the $H2_{LT}$ stem (11-base), forming the $C1_{LT}$−$H1_{LT}$−$H2_{LT}$ intermediate. $C1_{LT}$ can dissociate from $H1_{LT}$ and catalyze additional hairpin assembly reactions. $H1_{LT}$−$H2_{LT}$ initiates strand displacement (via a 7-base toehold) of a Black(R) fluorescence quencher

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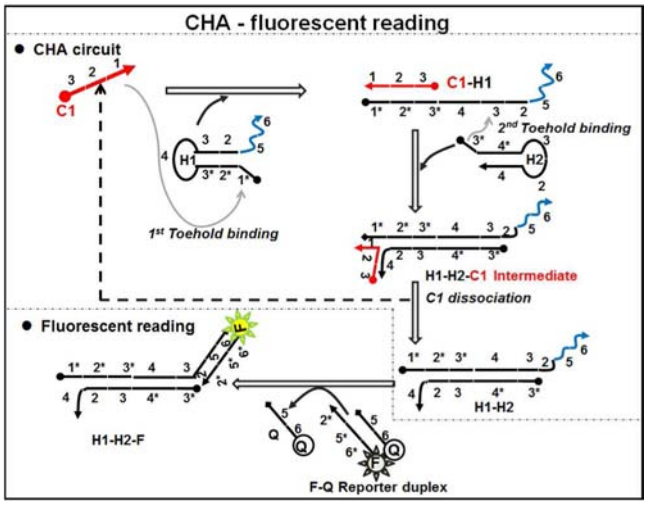

Figure 1. Schematic illustration of the CHA circuit. Complementary domain strands are indicated with an asterisk (e.g., domain 1* complements domain 1).

(Q_{LT}) from a fluorescein amidite (FAM)-labeled oligonucleotide (F_{LT}), allowing the reaction process to be monitored.

At 37 °C, this design showed 50−100 fold amplification within 5−10 h and little background leakage (Figure 2A). While the uncatalyzed reaction rate (background leakage rate) remained low below 50 °C (Figure 2C and Figure S1 in the SI), at 60 °C it increased almost 10-fold (Figure 2A, blue). In parallel, the catalyzed reaction rate decreased sharply with increasing temperature and was reduced almost 10-fold at 60 °C (Figure 2A, green). Thus, the circuit designed to function at 37 °C suffered a near 100-fold decrease in performance at 60 °C.

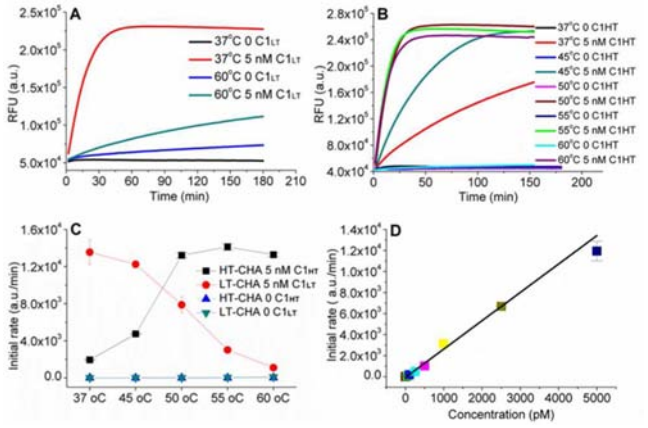

Figure 2. (A) Fluorescence responses of LT-CHA at 37 and 60 °C with and without catalyst. (B) Fluorescence responses of HT-CHA at various temperatures with and without catalyst. (C) Initial rates of LT-CHA and HT-CHA at various temperatures with and without catalyst, as calculated from the data in (A) and (B). (D) Initial rate vs C_{HT} concentration for HT-CHA at 60 °C (r^2 = 0.999). The sequences and conditions used in these experiments are found in Tables S3 and S4, respectively.

Adaptation of the low-temperature CHA circuit (LT-CHA) for high-temperature function required improvements in both the stability of the stem structures (to reduce background) and the binding of the catalyst to the toehold (to increase the catalyzed reaction rate). These conditions could be met by increasing the lengths of the stem and the toehold, respectively. As a basis for design, the predicted free energies for folding and binding at 50 °C (the highest temperature that the original circuit could tolerate well) were used to guide changes in the length and sequence of the modified circuit (Table S2). Three base pairs were added to the two hairpin stems ($H1_{HT}$ and $H2_{HT}$, where "HT" indicates "high temperature") and the reporter duplex (F_{HT}−Q_{HT}), while the length of the toehold was extended from 8 to 9 nucleotides (C_{HT}). A general "four-rule" principle for designing CHA circuits that can function at any temperature is provided in the SI. In short, as the desired temperature for circuit function is increased, the lengths of hairpin stems should be extended to retain the free energy of base pairing; the lengths of toeholds should be extended to maintain efficient catalysis; the hairpin sequence and length should be optimized to avoid misfolding and unintended secondary structures; and the lengths of oligonucleotides should not be extended so far as to accumulate excessive impurities during chemical synthesis.

The augmented CHA circuit (HT-CHA) was assayed at various temperatures. At 60 °C it proved to have a catalytic reaction rate as high as that of LT-CHA at 37 °C. Moreover, it demonstrated 5-fold less background leakage than LT-CHA at 60 °C (Figure 2B,C). There was also an excellent linear relationship (r^2 = 0.999) between the catalyst ($C1_{HT}$) concentration and the initial rate of the circuit reaction at 60 °C (Figures 2D, S2, and S3). As low as 100 pM $C1_{HT}$ could be discriminated from the background (by three standard deviations) within 1 h.

With a functional HT-CHA in hand, the question became whether it could be used as a real-time signal transducer in high-temperature isothermal amplification reactions. A synthetic 77-mer single-stranded DNA (ssDNA) containing an antisense template of the CHA catalyst was used to detect RCA at 60 °C (Figure 3A). Following amplification, each single-stranded monomer should contain the equivalent of the catalyst sequence once, and the catalyst sequence should therefore be present

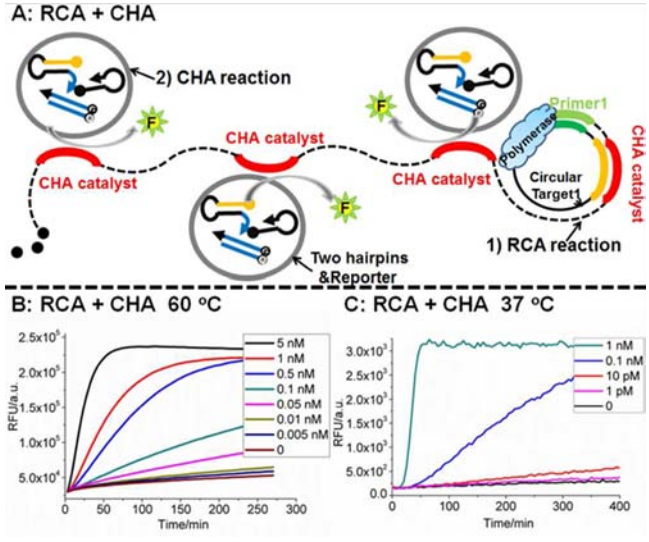

Figure 3. (A) Schematic illustration of CHA as a real-time detector of linear RCA. (B) Concentration dependence of Circular T_{HTRCA} of RCA + CHA at 60 °C in the ThermoPol reaction using EHT-CHA and Bst large-fragment polymerase. (C) Concentration dependence of Circular T_{LTRCA} of RCA + CHA at 37 °C in the Phi29 reaction using EHT-CHA and Phi29 polymerase. The sequences and conditions used in these experiments are found in Tables S3 and S4, respectively.

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thousands of times in each concatemer. The "RCA + CHA" amplification and real-time analysis system was driven by Bst polymerase at 60 °C in the presence of 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100 at pH 8.8 (called the ThermoPol reaction; Table S4). Bst polymerase was chosen because it has previously been widely used for high-temperature RCA reactions.[36,37] The salt concentration in the ThermoPol reaction was lower than that in the TNaK reaction previously used for the design and assay of the HT-CHA circuit. To account for this difference, the interactions between the catalyst sequence and domain 1* of H1 were extended by 1 base pair, yielding $H1_{EHT}$ (where "E" denotes the "extended" toehold). When this redesigned circuit was compared with the original under the new low-salt conditions, HT-CHA showed a 5-fold decrease in catalytic rate but EHT-CHA showed only a 2-fold decrease. While EHT-CHA performed better under low-salt conditions, there was no noticeable difference in performance between EHT-CHA and HT-CHA in the TNaK reaction, indicating that both toeholds are likely saturated at high salt concentrations.

EHT-CHA was able to monitor RCA in real time (Figure 3B) and could detect circular DNA (Circular T_{HTRCA}) concentrations as low as 5 pM (corresponding to 100 amol) within 4.5 h at 60 °C. As was previously the case for LAMP,[30−32] the use of CHA to transduce and report the presence of correct isothermal amplification products leads to much greater specificity of detection than would be obtained just by monitoring the accumulation of DNA (Figure 4). While false amplicons accumulate during RCA even in the absence of template, these amplicons are not reported by the EHT-CHA circuit.

To demonstrate the generality of real-time CHA transduction and detection of isothermal amplification, the same HT-CHA circuit was attempted with a different reaction, SDA (Figure 5A). In this instance, binding of the primer (P_{HTSDA}) initiates an extension reaction by Bst polymerase. The extended duplex is nicked by the enzyme Nb.BsrDI to generate an ssDNA containing the catalyst sequence. A cycle of extension, nicking, and product dissociation leads to the accumulation of multiple ssDNA catalyst sequences. In this "SDA + CHA" system for real-time analysis, the reaction was carried out in 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol at pH 7.9 (called the NEBuffer 2 reaction; Table S4) rather than under the ThermoPol reaction conditions to improve the coordinate performance of the Bst and Nb.BsrDI enzymes. Input T_{HTSDA} concentrations as low as 5 pM (corresponding to 100 amol) could be detected within 1 h (Figure 5B). The performances of HT-CHA and EHT-CHA were similar. As there are almost no real-time sequence-specific detectors for high-temperature linear SDA or RCA, these results potentially open the way for a wider application of nucleic acid circuitry in diagnostics.

With our ability to monitor high-temperature isothermal amplification reactions, the next step was to see whether real-time monitoring of lower-temperature isothermal amplification reactions might be feasible. This was an especially difficult challenge, since single-stranded products form structures that might occlude binding to the CHA circuits. Once again, CHA was used in attempts to monitor both RCA and SDA, but this time at 37 °C. This required another change in the enzymes and hence the reaction conditions used. The RCA reaction used Phi29 polymerase in 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and 4 mM dithiothreitol at pH 7.5 (called the Phi29 reaction; Table S4), while the SDA reaction used Klenow (3′→5′ exo-) polymerase, Nb.BbvCI nicking enzyme, and NEBuffer 2. Both the Phi29 and NEBuffer 2 reactions have high salt concentrations (10 mM Mg^{2+}) similar to that of the TNaK reaction, and thus, the original LT-CHA circuit did not require modification. As shown in Figures 3C and 5C, real-time monitoring of both the RCA and SDA reactions was successful. The detection limit for the RCA target (Circular T_{LTRCA}) was 1 pM (20 amol), which is 40-fold lower than a real-time molecular beacon detector.[38] Additionally,

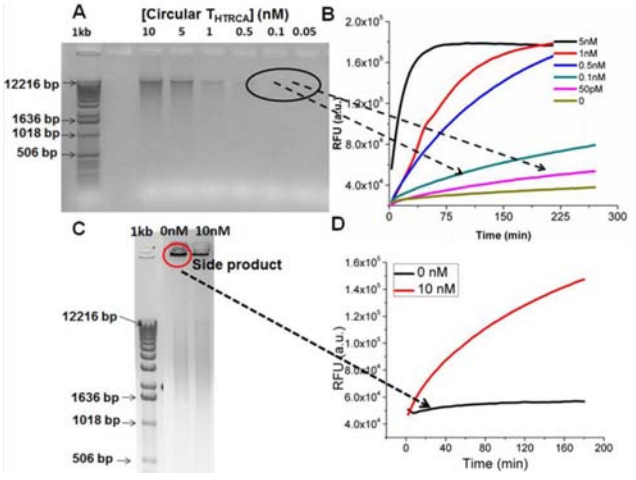

Figure 4. High-temperature CHA transduction is sensitive and specific. (A) 1% agarose gel of the product generated from a 2 h RCA reaction with and without Circular T_{HTRCA}. (B) The RCA products from (A) were diluted 4-fold with EHT-CHA, after which a CHA reaction under ThermoPol reaction conditions was performed at 60 °C. The concentrations listed are those before dilution. (C) 1% agarose gel of the products generated from overnight RCA reactions with and without Circular T_{HTRCA}. (D) The RCA products from (C) were diluted 4-fold with HT-CHA, after which a CHA reaction under ThermoPol reaction conditions was performed at 60 °C. The concentrations listed are those before dilution.

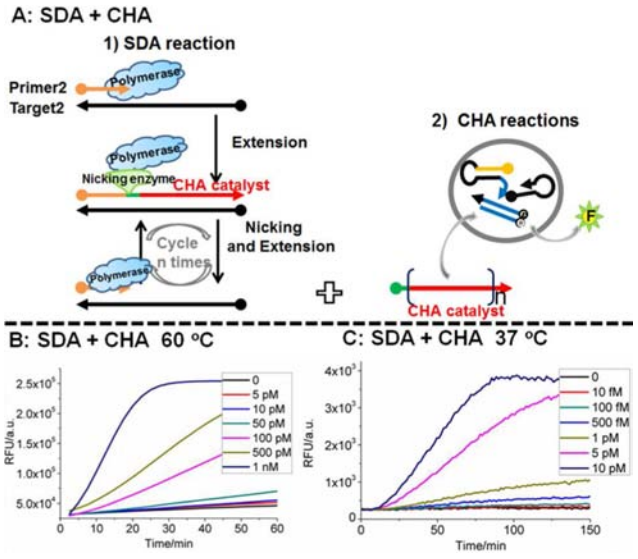

Figure 5. (A) Schematic illustration of CHA as a real-time detector of SDA. (B) Concentration dependence of Circular T_{HTSDA} of SDA + CHA at 60 °C in the NEBuffer 2 reaction using EHT-CHA, Bst large-fragment polymerase, and Nb.BsrDI. (C) Concentration dependence of Circular T_{LTSDA} of SDA + CHA at 37 °C in the NEBuffer 2 reaction using LT-CHA, Klenow (3′→5′ exo-) polymerase, and Nb.BbvCI. The sequences and conditions used in these experiments are found in Tables S3 and S4, respectively.

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the detection limit for the SDA target (T_{LTRCA}) was 100 fM (2 amol), which is 25- to 10000-fold lower than a recently reported real-time detection with molecular beacons[39,40] and was better than even electrochemical detectors.[41] The improved sensitivities of LT-CHA relative to HT-CHA were not due to increased background with the latter but may stem from the improved functionality of the Klenow (3′→5′ exo-) polymerase/Nb.BbvCI enzyme combination over the Bst/Nb.BsrDI system.

In conclusion, we have successfully engineered thermostable CHA circuits for the first time and demonstrated the potential of both high- and low-temperature CHA circuits as real-time monitors of isothermal amplification reactions. Overall, the results strongly support the conclusion that CHA can be readily and rationally adapted to real-time monitoring of a variety of isothermal amplification reactions. It was surprising how purely thermodynamic considerations of hybridization propensities (at different temperatures, in different buffers, and with different enzymes) produced circuit designs that worked almost the first time they were tried. In light of these and others' results, CHA can now be seen to have a number of advantages as a transducer for amplification reactions, including its adaptability to not only temperature and buffer conditions but also different detection modalities[28,30] as well as the incorporation of programmable molecular logic circuits into analytical readouts.[26,27,29]

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, Figures S1−S3, Tables S1−S4, and the design principles of the CHA and HT-CHA circuits. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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