Supporting Information for

Real-time detection of isothermal amplification reactions with thermostable catalytic hairpin assembly

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1. Materials and Methods

Chemicals, oligonucleotides and oligonucleotide complexes. All chemicals were of analytical grade and were purchased from Sigma-Aldrich (MO, USA) unless otherwise indicated. All oligonucleotides were ordered from Integrated DNA Technology (IDT, Coralville, IA, USA). Oligonucleotide sequences are summarized in **Table S1**. All oligonucleotides were stored in 10 μM stocks in H_2O or 1×TE (pH 7.5) at -20 °C. Enzymes used here were Phi29 polymerase, Bst large fragment polymerase, Klenow fragment (3'-5'exo) polymerase, Nb.BbvCI nicking enzyme, Nb.BsrDI nicking enzyme, T4 ligase, ExoI exonuclease, and ExoIII exonuclease. Reaction mixtures used here were:

- 1×TNaK (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, pH 7.5)
- 1×ThermoPol (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH8.8)
- 1×Phi29 (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM Dithiothreitol, pH 7.5)
- 1×NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9)
- 10×NEBuffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0)
- 1×T4 ligation (30 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.8)

Except for 1×TNaK, all the enzymes and reaction mixtures were purchased from New England BioLabs Inc., MA, USA.

Real-time CHA fluorescence kinetic reading. All the hairpins and reporters were annealed at 90 °C for 5 min and cooled down to 25 °C at a rate of 0.1 °C/s before use. CHA's kinetic reading was performed in 1×TNaK reaction conditions with a final concentration of 50 nM H1, 200 nM H2, and 50 nM reporter. The fluorescence signals were recorded every 2 min at different temperatures using the 7300 Real-Time PCR System with MicroAmp® Optical 96-Well Reaction Plates.

Ligation of Circular DNA. The targets of both high temperature RCA and low temperature RCA were ligated with T4 ligation enzymes as per the following protocol. Five tubes were prepared with 'Solution A', each tube containing 1 μM template, 1 μM splint, and 1×ligation buffer for a total of 10 μL per tube. These samples were incubated for 2 min at 95 ℃ and cooled down to 25 ℃ at a rate of 0.1 ℃/s. During the incubation, a mastermix of 'Solution B' was made containing 40 U/μL T4 ligation enzyme and 1×ligation buffer to total 50 μL. After this first incubation finished, 10 μL Solution B was added to each Solution A sample for a total of

20 μL per tube. These A-B solutions were incubated for 6 min at 25 °C. After incubation, the samples were combined into one mixture, purified with a G25 column, and eluted with 50 μL DI water. To further purify the 50 μL sample, we added 2 μL ExoI (40 U/μL), 1 μL ExoIII (100 U/μL), 5 μL 10×NEBuffer 1, and 37 μL DI water for a total 100 μL reaction system. We incubated this at 37 ℃ overnight before purifying the product with a PAGE gel and confirming the concentration of the circular DNA targets with a nanodrop**.**

Real-time reading of RCA-CHA system. High temperature real-time RCA detection was carried out in a 20 μL system consisting of the varying concentrations of circular DNA chosen for this experiment, 100 nM primer, 200 μM dNTPs, 0.4 U/μL Bst, 66 nM H1, 266 nM H2, and 66 nM reporter in the 1×ThermoPol reaction conditions. The fluorescence signals were recorded every 3 min at 60 ℃ using the 7300 Real-Time PCR System with MicroAmp® Optical 96-Well Reaction Plates. Low temperature real-time RCA was carried out in a 20 μL system consisting of the varying concentrations of circular DNA chosen for this experiment, 100 nM primer, 200 μM dNTPs, 0.2 ug/μL BSA, 0.5 U/μL Phi29, 50 nM H1, 200 nM H2, 50 nM reporter, and 1 μM InvtdT (inverted modified oligo dT, see Table S3) in the 1×Phi29 reaction conditions. The fluorescence signals were recorded every 1.5 min at 37 ℃ using the Tecan Safire plate reader with NUNC 384-well Reaction Plates.

Real-time reading of SDA-CHA system. High temperature real-time SDA detection was carried out in the 1×NEBuffer 2 reaction conditions with the varying concentrations of circular DNA chosen for this experiment, 100 nM primer, 200 μM dNTPs, 0.5 U/μL Nb.BsrDI, 0.4 U/μL Bst, 66 nM H1, 266 nM H2, 66 nM reporter, and 1 μM InvtdT. The fluorescence signals for high temperature were recorded every 3 min at 60 ℃ using the 7300 Real-Time PCR System with MicroAmp® Optical 96-Well Reaction Plates. Low temperature real-time SDA was carried out in 1×NEBuffer 2, 0.5 U/μL Nb.BbvCI, 0.25 U/μL Klenow fragment (3'-5'exo), 200 μM dNTPs, 100 nM primer, 50 nM H1, 200 nM H2, 50 nM reporter, and 1 μM InvtdT. The fluorescence signals were recorded every 1.5 min at 37 ℃ using the Tecan Safire plate reader with NUNC 384-well Reaction Plates.

Electrophoresis analysis of RCA reaction by agarose gel. A 20 μL system with the varying concentrations of circular DNA chosen for this experiment, 100 nM primer, 0.4 U/μL Bst, and 1 μM InvtdT in the 1×ThermoPol reaction conditions was incubated at 60 ℃ for 3 h followed by 15 min of incubation at 80 ℃. After this reaction the sample was kept at 4 ℃ until electrophoresis. Each electrophoresis experiment was carried out with a 1% SeaKem® LE agarose gel. Each well had 10 μL of sample and an additional 2 μL 6×Orange loading dye for a total 12 μL system.

2. Supporting Figures and Tables

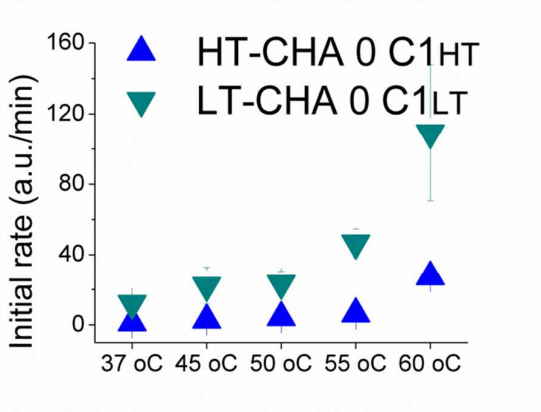


Figure S1: Initial rates of LT-CHA and HT-CHA without catalyst in the lower range of rates.

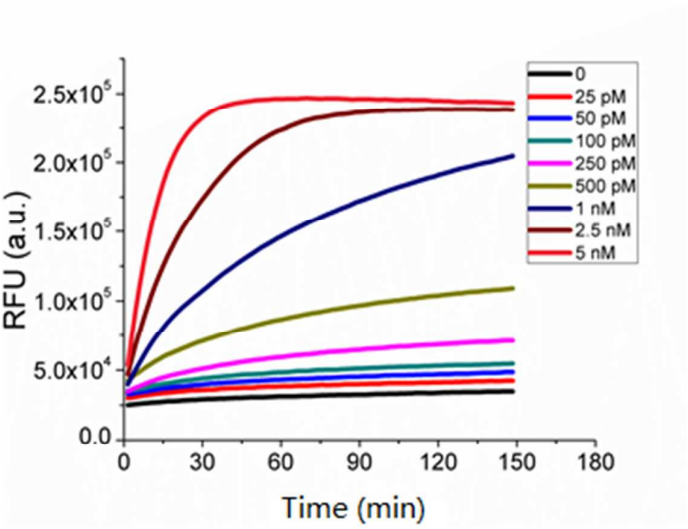


Figure S2: Concentration dependence of C_{HT} with HT-CHA at 60 °C in TNaK reaction. The kinetic traces of different combinations of circuit components are shown in different colors.

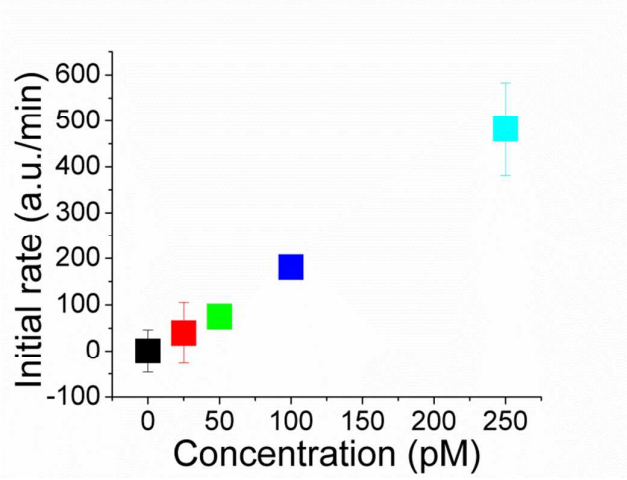


Figure S3: Initial rate vs. the lower range of C_{HT} concentrations for HT-CHA at 60 °C.

	LT-CHA (37 °C)	HT-CHA (60 °C)
Each toehold	8 bases	9 bases
H1 Stem	16 bases	19 bases
H1 Loop	11 bases	14 bases
H2 Stem	11 bases	14 bases
H2 Loop	16 bases	19 bases
Reporter duplex	19 bases	22 bases
Reaction mixture	TNaK: 20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, pH 7.5	

Table S1: Design of the LT-CHA and HT-CHA.

	Components	37 °C	45 °C	50 °C	55 °C	60 °C	65 °C
LT-CHA	**H1**	-14.09	-11.05	-9.14	-7.24	-5.34	-3.44
	H2	-6.95	-5.04	-3.84	-2.64	-1.45	-0.25
	Reporter	-23.22	-19.77	-17.61	-15.46	-13.3	-11.15
HT-CHA	**H1**	-21.41	-17.21	-14.59	-11.96	-9.49	-7.16
	H2	-13.9	-10.34	-8.17	-6.53	-4.9	-3.26
	Reporter	-27.5	-23.62	-21.19	-18.76	-16.33	-13.9
ΔG is calculated with NuPACK using 150 mM NaCl. Temperatures varied.							

Table S2: Comparison of the ΔG value of LT-CHA and HT-CHA components at different temperatures.
ΔG of LT-CHA at 50 °C was chosen as the standard because studies have shown it to be the highest temperature at which LT-CHA functions.

Name		Sequence (5'-3')	Notes
LT-CHA	**$H1_{LT}$**	GTCAGTGA GCTAGGTT AGATGTCG CCATGTGTAGA CGACATCT AACCTAGC CCTTGTCA TAGAGCAC/3InvdT/	Only oligos in the real-time reading system will be modified with /3InvdT/
	$H2_{LT}$	AGATGTCG TCTACACA TGGCGACA TCTAAC CTAGCCCATGTG TAG A/3InvdT/	
	F_{LT}	/56-FAM/CGA GTGCTCTA TGACAAGG GCTAGGTT/3InvdT/	
	Q_{LT}	C CCTTGTCA TAGAGCAC TCG/3IABkFQ/	
	$C1_{LT}$	CGACATCT AACCTAGC TCACTGAC/3InvdT/	
HT-CHA	**$H1_{HT}$**	GTCACGTGA GCTAGCGTT AGCATCGTCG CCATGCTGCTAGCA CGACGATGCT AACGCTAGC CCTTGTCA TACGCAGCAC/3InvdT/	Only oligos in the real-time reading system will be modified with /3InvdT/
	$H2_{HT}$	AGCATCGTCG TGCTAGCAGCATGG CGACGATGCT AACGCTAGC CCATGCTGCTAGCA/3InvdT/	
	F_{HT}	/56-FAM/CGA GTGCTGCGTA TGACAAGG GCTAGCGTT/3InvdT/	

	Q_{HT}	C CCTTGTCA TACGCAGCAC TCG /3IABkFQ/	
	$C1_{HT}$	CGACGATGCT AACGCTAGC TCACGTGAC	
	$H1_{EHT}$	CGTCACGTGA GCTAGCGTT AGCATCGTCG CCATGCTGCTAGCA CGACGATGCT AACGCTAGC CCTTGTCA TACGCAGCAC/3InvdT/	
Invt-dT		TTTTTTTTTTTTTTTTTTT/3InvdT/	
HT-RCA	**T_{HTRCA}**	/5Phos/CTTCCTGT ATTCA CTTGG AGG TAC GTC ACG TGA GCT AGC GTT AGC ATC GTC G AAG TTG AGT CTGTACGTAAGGTTCT	Circular T_{HTRCA} is generated by ligating 5'end and 3'end of T_{HTRCA}
	$Splint_{HT}$	CCAAG TGAAT ACAGGAAGAGAACCTTACGTACAG ACT CAA	
	P_{HTRCA}	CCAAG TGAAT ACAGGAAGAGAACC	
LT-RCA	**T_{LTRCA}**	/5Phos/TGG CTC AGC TGG CTG GTG CCGGTTCTCTTCCTGT ATTCA CTTGG GTC AGT GAG CTA GGT TAG ATG TCG GGT TGT TCT GGT CCA TGA AT	Circular T_{LTRCA} is generated by ligating 5'end and 3'end of T_{LTRCA}
	$Splint_{LT}$	GGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACC	
	P_{LTRCA}	CCAAG TGAAT ACAGGAAGAGAACC	
HT-SDA	**T_{HTSDA}**	TACGTCACGTGAGCTAGCGTTAGCATCGTCG AA GCAATG AAA GCCGATACAGGATCCAACAG	
	P_{HTSDA}	CTG TTG GAT CCT GTA TCG GC	
LT-SDA	**T_{LTSDA}**	GTCAGTGAGCTAGGTTAGATGTCG ATAT CCTCAGC AAA GCCGATACACCAATCCAACAG	
	P_{LTSDA}	CTGTTGGATTGGTGTATCGGC	

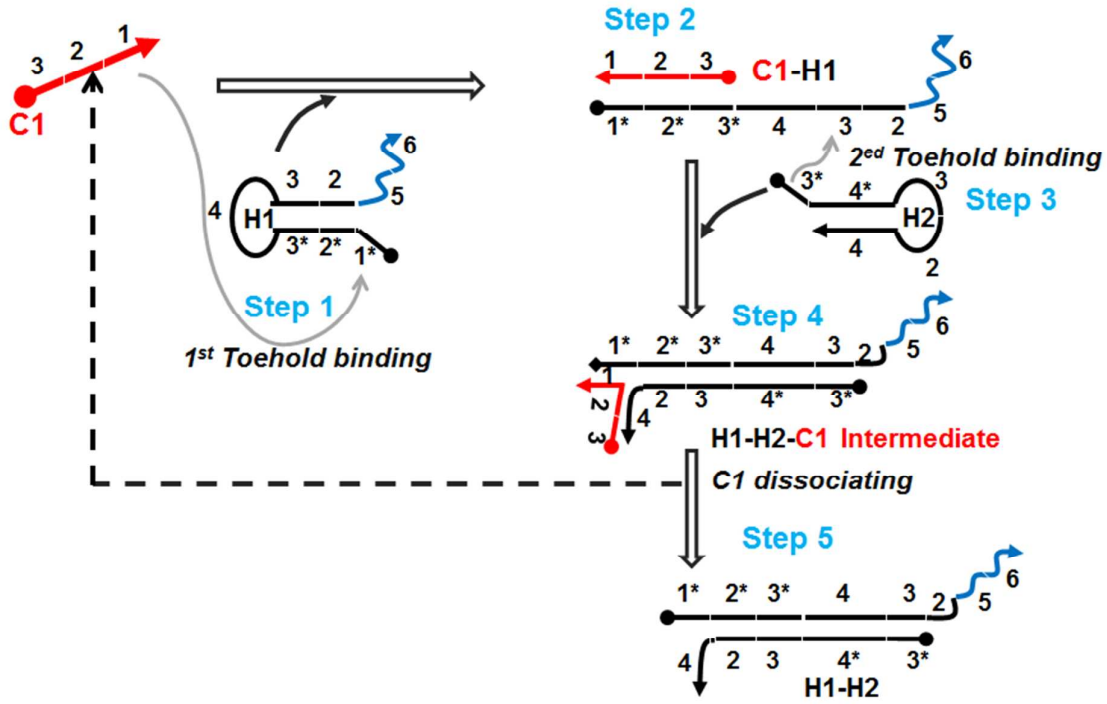
Table S3: Oligonucleotides used in this paper.

Real-time system	Isothermal components		CHA components		Reaction mixtures
LT-CHA (Figure 2A) (Figure 2C)	None		$[H1_{LT}]$=50 nM		TNaK*
			$[H2_{LT}]$=200 nM		
			$[F_{LT}]=1/2[Q_{LT}]$=50 nM		
HT-CHA (Figure 2B) (Figure 2C) (Figure 2D)	None		$[H1_{LT}]$=50 nM		TNaK*
			$[H2_{LT}]$=200 nM		
			$[F_{LT}]=1/2[Q_{LT}]$=50 nM		
60°C HT-RCA (Figure 3B) (Figure 4B) (Figure 4D)	Target	Circular T_{HTRCA}	EHT-CHA	$[H1_{EHT}]$=66 nM (Figure 4B) $[H1_{HT}]$=66 nM (Figure 4D)	ThermoPol*
	Primer	$[P_{HTRCA}]$=100 nM		$[H2_{HT}]$=264 nM	
	Enzyme	Bst large fragment		$[F_{HT}]=1/5[Q_{HT}]$=66 nM	
37 °C LT-RCA (Figure 3C)	Target	Circular T_{LTRCA}	LT-CHA	$[H1_{LT}]$=50 nM	Phi29*
	Primer	$[P_{LTRCA}]$=100 nM		$[H2_{LT}]$=200 nM	
	Enzyme	Phi29 polymerase		$[F_{LT}]=1/2[Q_{LT}]$=50 nM	
60°C HT-SDA (Figure 5B)	Target	T_{HTSDA}	EHT-CHA	$[H1_{EHT}]$=66 nM	NEBuffer2*
	Primer	$[P_{HTSDA}]$=100 nM		$[H2_{HT}]$=264 nM	
	Enzyme	Bst large fragment, Nb. BsrDI		$[F_{HT}]=1/5[Q_{HT}]$=66 nM	
37°C LT-SDA (Figure 5C)	Target	T_{LTSDA}	LT-CHA	$[H1_{LT}]$=50 nM	NEBuffer2*
	Primer	$[P_{LTSDA}]$=100 nM		$[H2_{LT}]$=200 nM	
	Enzyme	Klenow (3'-5'exo-) polymerase, Nb.BbvCI		$[F_{LT}]=1/2[Q_{LT}]$=50 nM	
*TNaK	20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, pH 7.5				
*ThermoPol	20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8				
*Phi29	50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM Dithiothreitol, pH 7.5				
*NEBuffer 2	10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9				

Table S4: Components used in Figure 2 to Figure 5.

3. Design principle of the CHA circuit and HT-CHA circuit.

a. Our CHA circuit workflow is shown below.



As seen in the workflow above, we designed a LT-CHA circuit to work at 37 °C, listed in **Table S1**.

1. Catalyst (C1) and H1 toehold binding
2. Initiating branch migration reaction to open H1, forming C1-H1, and releasing toehold binding site for H2.
3. H2 and H1 toehold binding
4. Initiating branch reaction to open H2, displacing C1 from H1, and finally forming C1-H1-H2 intermediate, in which C1 binds H1 only with toehold part.
5. C1 automatically dissociates from H1, producing H1-H2 product.

	LT-CHA (37°C)
Each toehold	8 bases
H1 Stem	16 bases
H1 Loop	11 bases
H2 Stem	11 bases
H2 Loop	16 bases
Reporter duplex	19 bases
Buffer	20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, pH 7.5

These steps in the LT-CHA circuit correspond to the numbered steps in the workflow above.

b. Four general rules for the design of CHA circuits.

Rule I: Make the ΔG as negative as possible, to assure there is low background leakage.

- The lengths of a duplex or hairpin stem determine the overall stability (ΔG) of the CHA circuit. Under the defined "G+C" ratio, the longer the duplex or hairpin stem is, the more negative the ΔG might be.

As ΔG negativity increases, the background leakage decreases.

Rule II: Determine the optimum toehold length for the overall catalytic reaction rate.

- To simplify the design process, we made all the toeholds the same length. However, determining this single, optimum length required us to balance several factors.
- The catalytic reaction rate as a whole is comprised of the toehold based strand displacement rate (STEPS 1 and 3 of the CHA reaction above) and the catalyst dissociation rate (STEP 5 of the CHA reaction above).
- The strand displacement rate favors the longest toehold possible. The catalyst dissociation rate favors the shortest toehold possible. The optimum length for the entire reaction rate, therefore, lies somewhere in between the two.

Rule III: Minimize the potential for aberrant folding and unintended secondary structures.

- When hairpins misfold or ssDNA form unexpected structures, the catalytic reaction is almost always impaired.
- Software programs like NuPACK, mFOLD, or Circuit Design will help you to carefully consider the appropriate reaction conditions and temperature. This should help to minimize aberrations.

Rule IV: Provided that the requirements for Rule 1 and 2 have been satisfied, minimize the length of the strands.

- This will save you money on materials.
- More importantly, longer lengths of DNA increase the risk for errors or impurities during chemical synthesis. Any impurities in the two hairpins will negatively affect the background leakage rate.
- Thus the minimum length of DNA that stills satisfied Rules 1 and 2 is ideal.

c. Our Design of a HT-CHA

1) Design a LT-CHA according to the previously described rules. Test this standard 37 °C LT-CHA at different temperatures including, 37 °C, 45 °C, 50 °C, 55 °C, and 60 °C.

The results are seen in **Figure 2C**: The catalytic reaction rate consistently decreases with increasing temperature, with a near 10-fold decrease by 60 °C. However, the background leakage rate remains almost unchanged at 45 °C and 50 °C. It only begins to show any significant increase at temperatures over 50 °C. By 60 °C again, the leakage rate increases almost 10-fold. So by 60 °C, we lost almost 100-fold performance.

2) Design a HT-CHA (Table S1 and Table S2), also in accordance with the previously described rules.

After the previous test of the LT-CHA standard, it was hypothesized that to make a 60 °C CHA as effective as one at 37 °C, the toehold length would have to be increased in order to maintain the catalytic rate (**Rule I**) while the hairpin stem would have to be extended in order to decrease background leakage (**Rule II**).

According to a previous study [1], it was suggested that one additional toehold could increase the reaction rate by as much as 10-fold. So the toehold domains were increased from 8 bases to 9 bases (**Rule III**). In addition, adding 3 more base pairs on each hairpin stem could make the ΔG value similar to that of a LT-CHA hairpin at 50 °C (which was chosen as the standard temperature to maintain low background

leakage). Therefore the hairpin stems were changed from 16 bp to 19 bp (**Rule III)**. These were the minimum stem and toehold length increases possible while still meeting **Rule I and II** requirements (**Rule IV**). So our HT-CHA started with a 9-base toehold and a 19-bp stem, which as it turns out functions as well in TNaK reaction conditions as did the original LT-CHA.

Ref. [1] Zhang, D. Y.; Winfree, E. J. Am. Chem. Soc. 2009, 131, 17303.