Supporting information

Probing Spatial Organization of DNA Strands using Enzyme-free Hairpin Assembly Circuits

Bingling Li, Yu Jiang, Xi Chen*, and Andrew D. Ellington*

Institute for Cellular and Molecular Biology, Center for Systems and Synthetic Biology, Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712, USA.

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 1x TE (pH 7.5) at -20 ^oC.

Real-time fluorescence measurements. A typical real time fluorescent reading for a CHA reaction involving **AP**, **H1**, and **H2** is as follows. **AP** complex was formed by mixing indicated concentrations of **OS** (or **mOS**, **OS-Tn**), **TH** (or **THx**, **mTH**) and **BM** in TNaK Buffer [20 mM Tris, pH 7.5; 140 mM NaCl;1 μM $(dT)_{21}$] followed by an annealing process which includes heating at 95 ^oC for 5 min and slowly cooling down to room temperature at a rate of 0.1^oC/s. **Reporter** complex was formed by mixing **S-F** and **S-Q** strands at a 1:2 ratio in TNaK Buffer followed by a similar annealing process. Immediately before the experiment, **H1** and **H2** were similarly annealed in TNaK Buffer. **AP** complex of indicated concentration, 300 nM **H1**, 200 nM **H2**, and 200 nM **Reporter**, all in TNaK buffer, were mixed in a 1:1:1:1 volume ratio into a total volume of 20 uL, followed by adding 1 μL 100 mM $MgCl_2$. Then 17 μL of the final mixture was immediately added into 384-well plate that had been pre-incubated in TECAN Safire plate reader set to experimental temperature (27, 33, or 37^oC). Fluorescent readings over time were taken immediately. Real time fluorescent measurements for CHA reactions triggered by HCR product were performed in a similar way, except that **AP** complex, **H1**, **H2**, and **Reporter** were replaced by HCR product, **H6**, **H5**, and **Reporter2**, respectively. **Reporter2** were obtained by annealing **S-F2** and **S-Q2** at a 1:2 ratio. All CHA reactions triggered by HCR products were performed at 37 ^oC. To calculate the rate of reaction, the raw fluorescent signals (RFUs) were converted to product concentrations using the previously described method.[1] Briefly, the RFU of the quenched reporter alone was noted as RFU_ZERO. Then, a reaction was set up where one CHA hairpin (e.g. **H1** or **H5**) of known concentration was the limiting reactant. The reaction was then allowed to reach

completion at which point the RFU was noted as RFU_FULL. The concentrations of CHA product in other reactions were calculated by linearly interpolating the RFU values between RFU_ZERO and RFU_FULL. The initial rate of a CHA reaction was determined by linear regression of product concentration versus time; when applicable, the turnover rate was calculated by dividing the initial rate of the reaction by the concentration of catalyst.

HCR reactions. A typical two-hairpin HCR reactions (consisting of **Trigger**, **H3**, and **H4**) was setup as follows. Immediate before experiment, **H3**, **H4**, and **Trigger** were annealed in TNaK Buffer without $(dT)_{21}$. 800 nM **H3**, 800 nM **H4**, **Trigger** of indicated concentration, and 2 M NaCl were mixed in 1:1:1:1 volume ratio and incubate at room temperature (~25^{o}C) for roughly 16 hour to 48 hour. To probe the HCR product with the CHA circuit, aliquots of the HCR products were mixed with 1/10 volume of 20 μM **Lock** and incubated at room temperature for ~30 min. The mixtures were then diluted by 12.5-fold using TNaK Buffer before mixing with other components of the CHA reaction as described above. To analyze the HCR product with 8% native PAGE, 20 μL of the HCR products (before **Lock** was added) were mixed with 6 μL 6x Loading Dye (50% Glycerol spiked with tiny amount of Orange G) and loaded on the gel. To analyze the HCR product with 1.5% agarose gel, 4 μM (instead of 800 nM) of **H3** and **H4** were used to set up the HCR reaction. And 10 μL HCR products were mixed with 2 μL 6x Loading Dye and loaded on the gel. Polyacrylamide gels were stained with SYBR gold (Invitrogen) and agarose gels were stained with ethidium bromide.

A typical four-hairpin HCR reaction (consisting of **Trigger2**, **H7**, **H8**, **H9**, and **H10**) was setup as follows. Immediately before experiment, **H7**, **H8**, **H9, H10** and **Trigger2** were individually annealed in TNaK Buffer without $(dT)_{21}$. 1.3 μM **H7**, **H8**, **H9, H10**, **Trigger2** of indicated concentration, and 2 M NaCl were mixed in 3:3:3:3:5 volume ratio and incubate at room temperature (~25^{o}C) for roughly 24 hour.

2. Impact of temperature, toehold length, and other factors on rate of CHA catalyzed by co-localized toehold and branch-migration domain

We systematically studied the impact of toehold (segment '1') length and reaction temperature on the kinetics of the CHA reaction (**Figure 2c and S4**). We observed that: (1) at all three tested temperatures (27^{o}, 33^{o}, and 37^{o} C) the rate of catalysis was positively correlated to toehold length; (2) the rate of the uncatalyzed reaction also positively correlated with temperature; and (3) **AP** complexes with longer toeholds were more efficient catalysts at higher temperatures.

Quantitatively, with a 10-nt toehold (segment '1') and substrate concentrations of 75 nM and 50 nM for **H1** and **H2**, respectively, the **AP** complex can catalyze the formation of **H1:H2** with an apparent turnover rate (rate of product formation divided by the concentration of **AP**) of ~0.2 min^{-1}, roughly 4/5 as fast as our previously reported CHA circuit under similar conditions.[2] We reason that the modestly reduced rate is at least partially caused by the lower rate of strand-displacement ration across a 4-way junction (**Figure S2**, reaction *b*), similar to our previous observation for strand-displacement across a 3-way junction.[3]

We further studied dependence of the reaction rate on the concentration of **OS**. In order to reach an

optimal trade-off between reaction rate and leakage, we decided to use 33° C as the reaction temperature and 10-nt as toehold length in these experiments. As shown in **Figure S3b**, when the concentrations of **TH**, **BM**, **H1** and **H2** were all held constant, a linear relationship between the rate of reaction and the concentration of **OS** (varied from 5 pM to 5 nM) was observed, while higher concentration of **OS** led to saturation of the reaction rate.

The rate of CHA can be influenced by structure and sequence of DNA adjacent to and away from the junction. First, if the surrounding DNA changes the secondary structure of the junction and makes part of the toehold or the branch-migration domain transiently double-stranded, the catalytic activity of the junction structure is expected to be reduced. Second, in all junction structures studied in this work, the two duplexes that co-localize the toehold and the branch-migration domain are not conformationally constrained and represent an ensemble of different tertiary structures. If the surrounding DNA stabilizes a particular tertiary structure, the catalytic activity of the junction structure is expected to be different from the ensemble. The tertiary structure of the junction, in its free form or substrate-bound form (a Holliday junction-like structure, see the intermediate after reaction *b* in **Figure S2**), may also be influenced by its sequence.[4] As more information about DNA circuits continues to accumulate[1,5] it should be increasingly possible to explain and predict these subtle effects on kinetics.

The rate of a CHA reaction also depends on subtleties in the domain organization and sequence of the substrate hairpins. For example, in the CHA reaction shown in **Figure 1b** the catalyst dissociates from the product passively whereas in that shown in **Figure 2b** and **S2** the catalyst is displaced actively. As a result the latter CHA is typically faster than the former, but for unknown reasons also undergoes a faster uncatalyzed reaction.

3. Supporting Figures and Tables

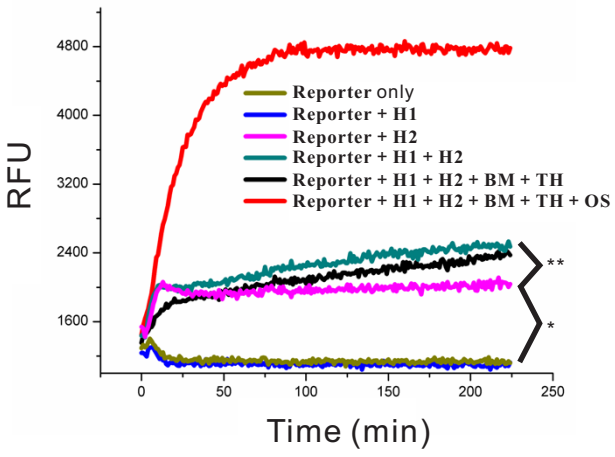

Figure S1. Background analysis of the CHA circuit shown in Figure 2b. The kinetic traces of different combinations of circuit components are shown in different color. Whenever present, the concentrations of the components are: [**OS**] = 12.5 nM, [**TH**] = [**BM**] = 15 nM, [**H1**] = 75 nM, [**H2**] = [**Reporter**] = 50 nM. This analysis illustrates the possible sources of the background (also called circuit leakage). The initial fluorescence increase in the sample '**Reporter + H2**' (denoted with '*') is likely caused by the presence of a small fraction of mis-formed (mis-synthesized and/or mis-folded)

H2 that interacts with **Reporter** directly to cause the separation of the fluorophore-bearing and the quencher-bearing strand. The gradual and persistent fluorescence increase in samples '**Reporter + H1 + H2**' and '**Reporter + H1 + H2 + BM + TH**' (denoted with '**') is likely caused by the uncatalyzed formation of **H1:H2** duplex in the absence of the **AP** complex.

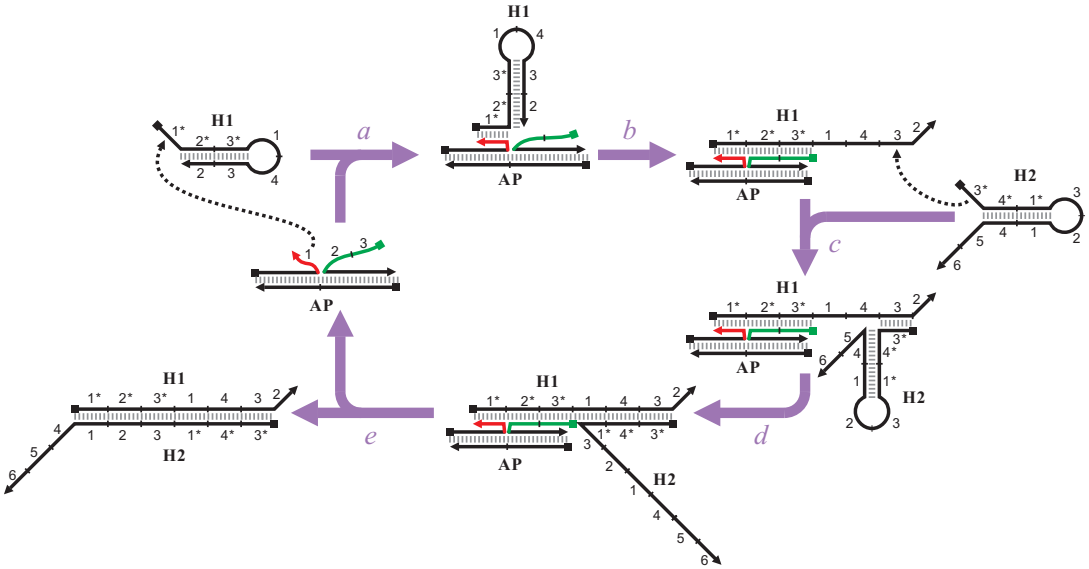

Figure S2. Proposed mechanism of the CHA reaction (**H1** + **H2** → **H1:H2**) catalyzed by the **AP** complex. A subtle difference between this mechanism and the one shown on **Figure 1b** is that here the catalyst (**AP** complex) is actively displaced from **H1** by the **H2** (reaction *e*), whereas in the mechanism shown in **Figure 1b** the catalyst (HCR product) must spontaneously dissociate from the product.

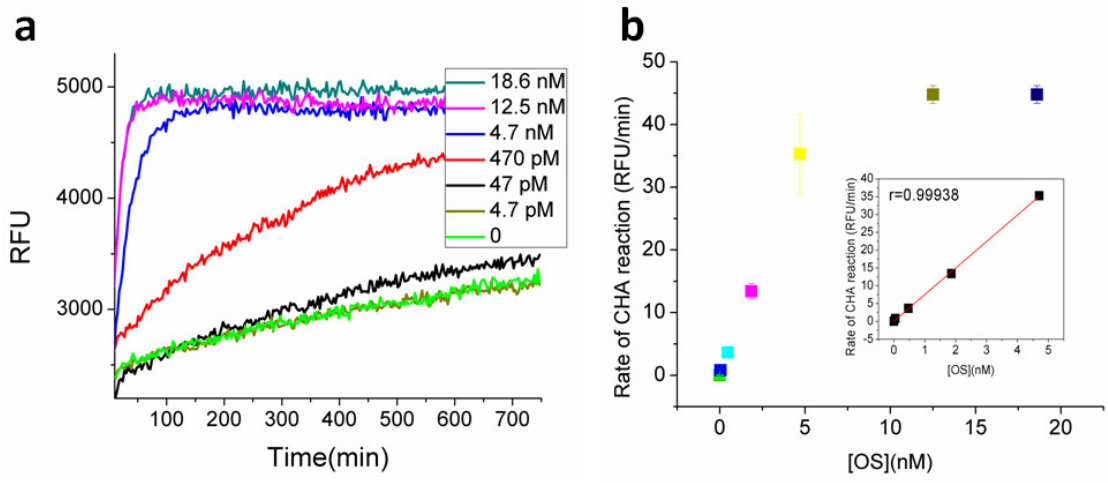

Figure S3. Dependence of the rate of the CHA reaction on the concentration of **OS**, when the concentrations of **TH**, **BM**, **H1**, and **H2** were all held constant. (a) Kinetic traces of the reactions with different concentration of **OS** (inset). (b) Initial rate of fluorescence increase as a function of **OS** concentration. The concentrations of circuit components in these experiments are: [**TH**] = [**BM**] = 15 nM, [**H1**] = 75 nM, [**H2**] = [**Reporter**] = 50 nM.

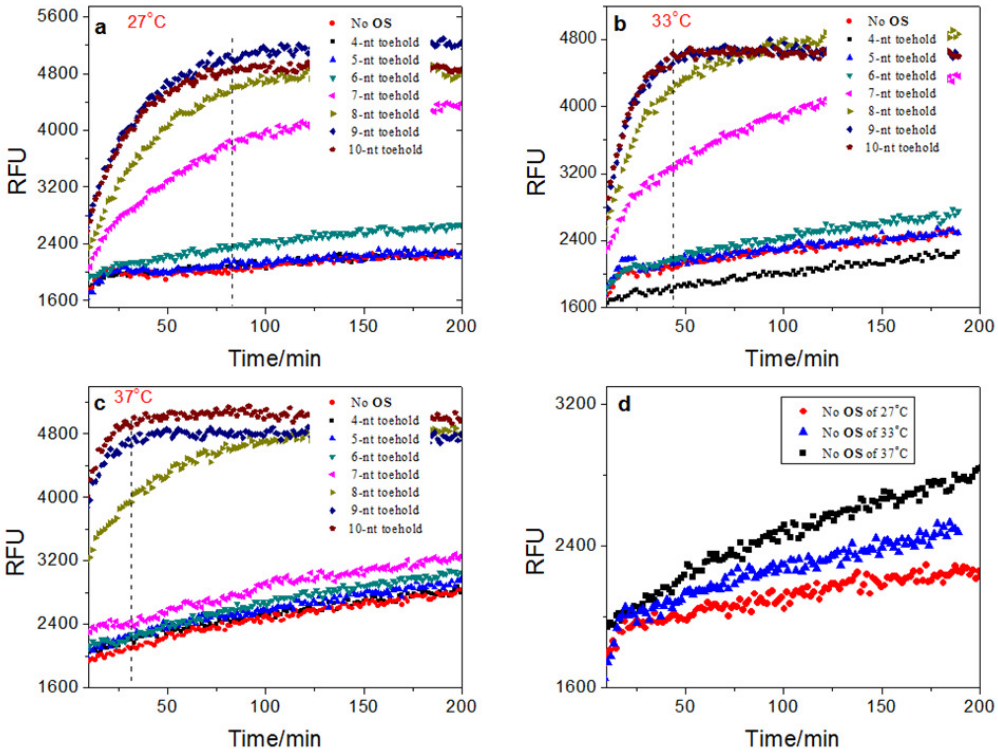

Figure S4. Impact of toehold length and reaction temperature on the reaction kinetics of the CHA reaction. In these experiments **TH** (with 10-nt toehold) was substituted with **TH** analogs with shorter toeholds ($\mathbf{TH_4}$ to $\mathbf{TH_9}$, see **Table S1**). The CHA reactions were carried out at 27 (a), 33 (b) or 37 (c) ^{o}C. The kinetics of fluorescence increase in the absence of **OS** at different temperatures is shown in (d). Whenever present, the concentrations of circuit components in these experiments are: [**OS**] = 12.5 nM, [**TH** (or $\mathbf{TH_x}$, x = 4 to 9)] = [**BM**] = 15 nM, [**H1**] = 75 nM, [**H1**] = [**Reporter**] = 50 nM.

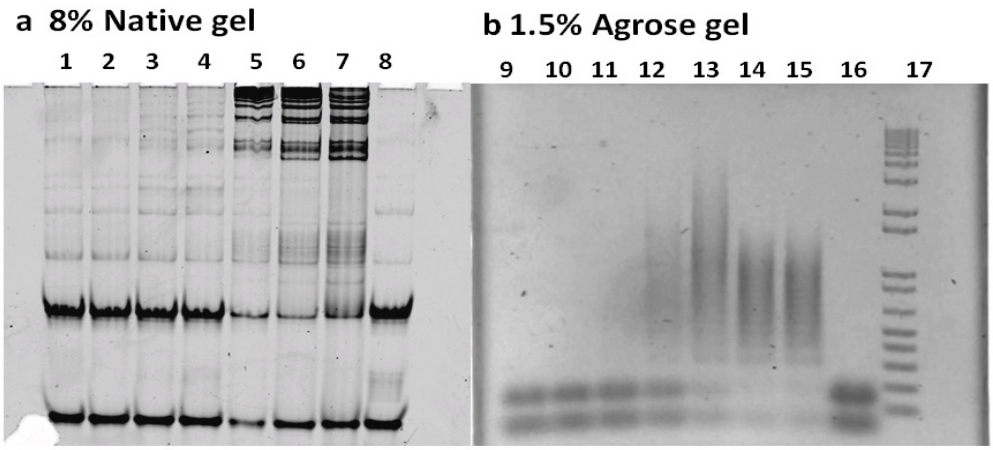

Figure S5. Analysis of HCR product using (a) 8% native polyacrylamide or (b) 1.5% agarose gel electrophoresis. Due to the differences in loading capacity and imaging sensitivity between the two types of electrophoresis, the concentrations of reactants were different. In the experiments shown in (a), [**H3**] = [**H4**] = 200 nM, [**Lock**] = 1 μM. In lanes 1 to 7, **Trigger** concentrations were 0 nM, 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, respectively. Lane 8 shows the mock HCR product where **Lock** was added before 25 nM of **Trigger**. In experiments shown in (b), [**H3**] = [**H4**] = 800 nM. [**Lock**] = 10 μM. In lanes 9 to 15, **Trigger** concentrations were 0 nM, 25 nM, 50 nM, 75 nM, 100 nM, 200 nM, 250 nM, respectively. Lane 16 shows the mock HCR product where **Lock** was added before 100 nM of **Trigger**. Lane 17 shows 1 kb Plus DNA ladder (Invitrogen).

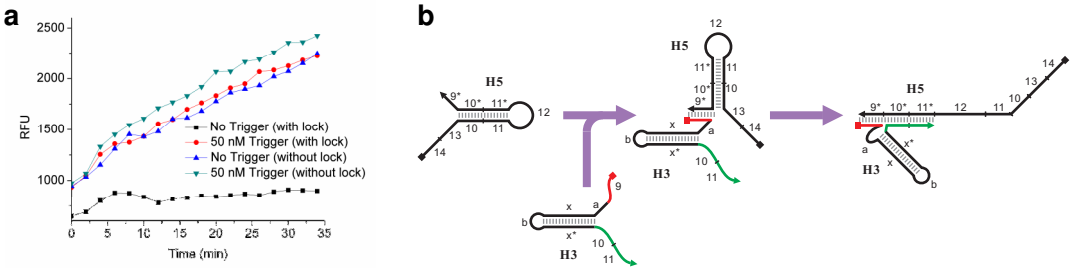

Figure S6. The utility of **Lock** in reducing the background reaction catalyzed by monomeric **H3**. (a) Real-time kinetics of CHA reactions catalyzed by HCR products with and without **Lock**. Final concentrations in a HCR reaction: [**Trigger**] = 50 nM; [**H3**] = [**H4**] = 200 nM. For CHA detection, 9 μL HCR product was mixed with 1 μL 20 μM **Lock** or 1x TNaK buffer and incubated for 30 min. Then 5 μL the mixture was added to a 15 μL solution that contains the remaining components of the CHA reaction. Final concentrations in a CHA reaction: [**H3**] = [**H4**] = 5 nM; [**H5**] = [**Reporter2**] = 50 nM; [**H6**] = 200 nM. (b) Proposed mechanism of monomeric **H3** opening **H5** where domain 9 of **H3** serves as a remote toehold.

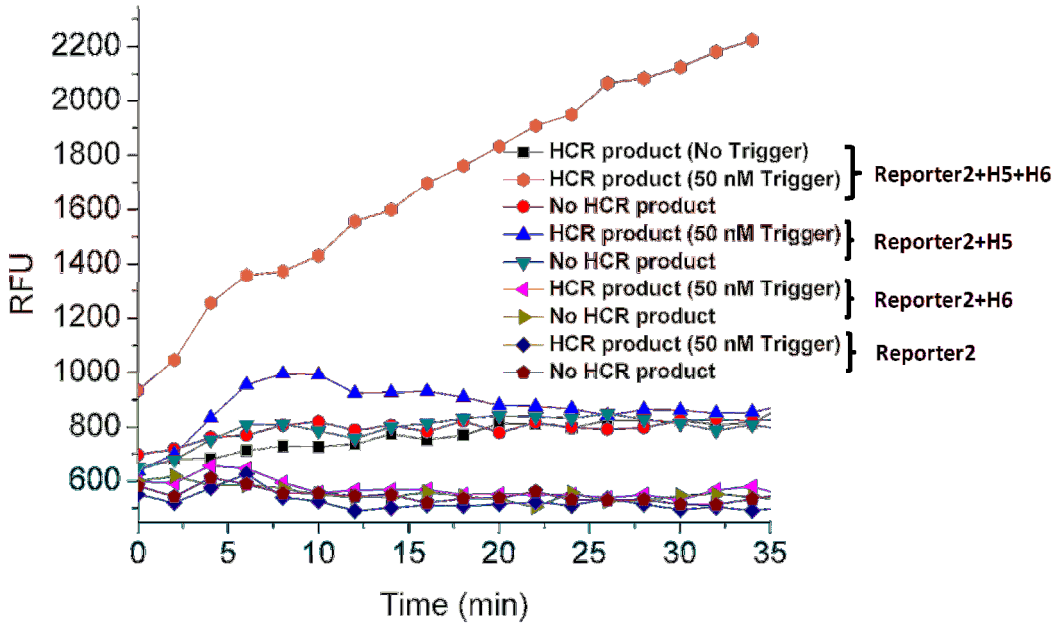

Figure S7. Control experiments to prove that steady increase of fluorescence signal was due to catalysis of the CHA reaction rather than stoichiometric opening of **H5**. Final concentrations in the HCR reaction: [**Trigger**] = 50 nM; [**H3**] = [**H4**] = 200 nM. Before CHA reaction, 9 μL HCR product was mixed with 1 μL of 20 μM **Lock** and incubated for 30 min. Then 5 μL the mixture was added to a 15 μL solution that contains the remaining components of the CHA reaction. Final concentrations in the CHA reaction: [**H5**] = [**Reporter2**] = 50 nM; [**H6**] = 200 nM.

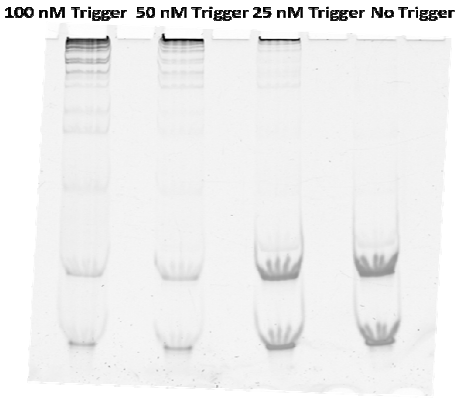

Figure S8. Assembly product of 4-hairpin HCR with different concentration of **Trigger2** analyzed with 8% native PAGE. Final concentrations in the HCR reaction: [**H7**] = [**H8**] = [**H9**] = [**H10**] = 200 nM.

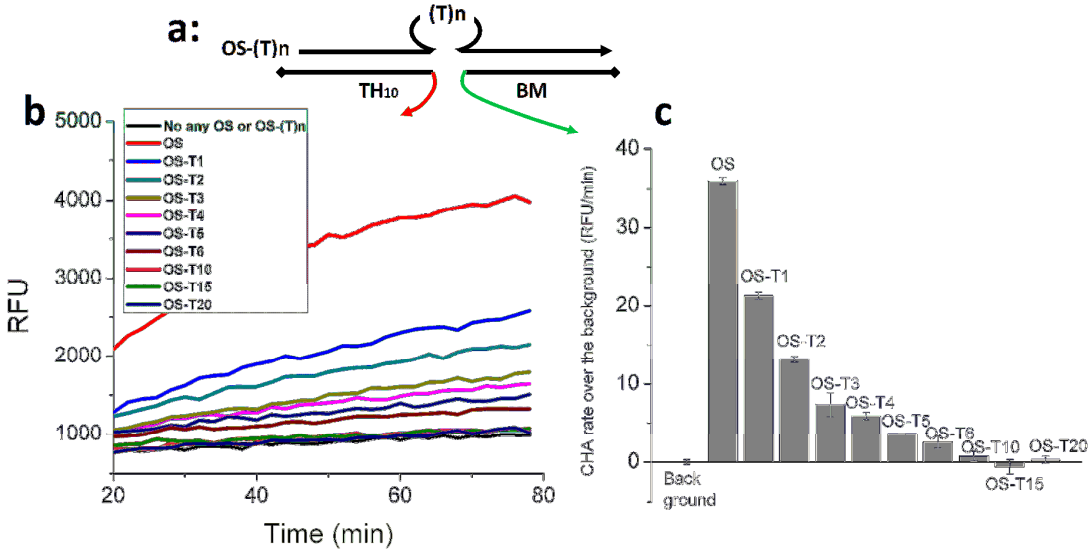

Figure S9. Impact of the distance between the toehold and the branch-migration domain on the kinetics of the CHA reaction. (a) Scheme illustrating where *n* thymidine(s) (*n* = 1 to 20) is/are inserted in the **OS** strand. The resultant strands were named **OS-(T)n**. (b) Real-time kinetics of CHA reactions catalyzed by the modified **AP** complex with **OS-(T)n** being the organizer strand. (c) Bar graph showing the relationship between *n* and the corresponding CHA reaction rate. Note that the background signal caused by leakage has been subtracted. [**OS-(T)n**] = 5 nM, [**TH**] = [**BM**] = 15 nM, [**H1**] = 75 nM, [**H1**] = [**Reporter**] = 50 nM.

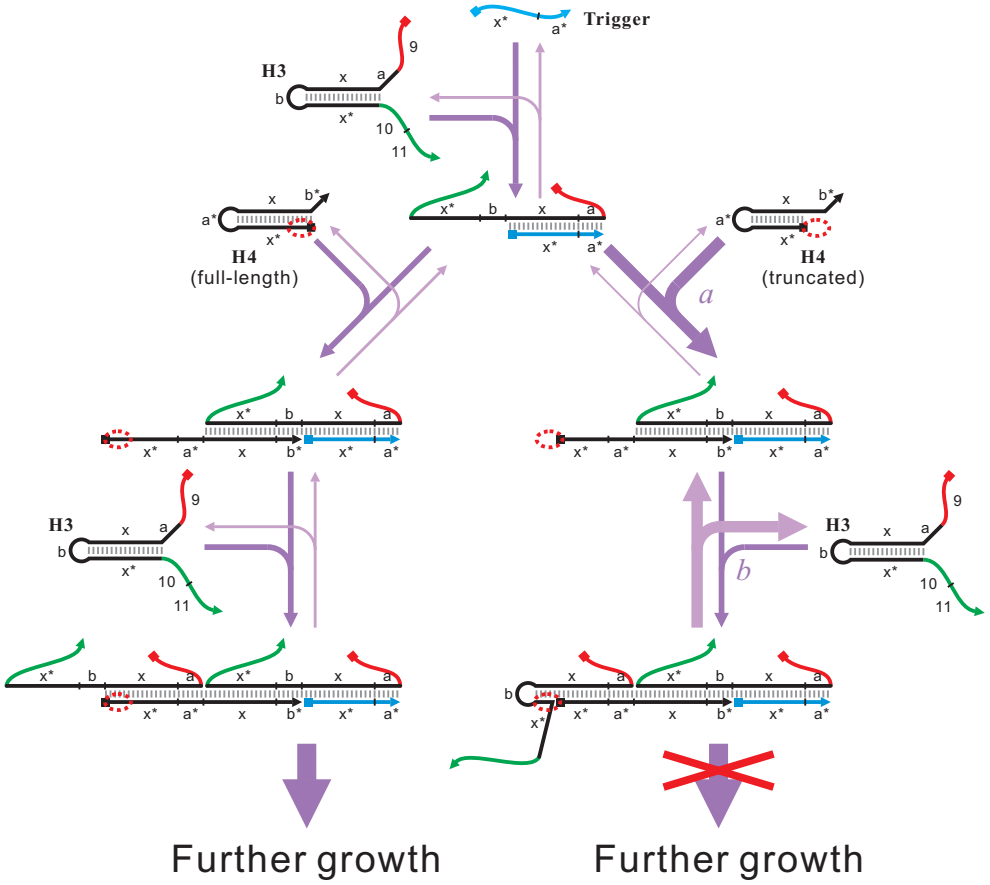

Figure S10: Proposed mechanism for the inhibitor ultrasensitivity (threshold effect). During standard phosphoramidite-based oligonucleotide synthesis the bases are added from 3' to 5'. After every coupling reaction there is a capping reaction to terminate growing oligomers that failed to incorporate the newly introduced base. Due to this procedure, the majority of the impurities in an oligonucleotide synthesis are 5' truncations. Separation of perfectly synthesized oligonucleotides and those with 1- or 2-nt truncations at the 5' end (also called N-1 and N-2 products) is extremely challenging for both PAGE and HPLC purification, especially when hundreds of nanomoles of material is loaded on the gel or column. Therefore, the presence of a small fraction (1 to 10%, which may vary considerably between batches) of N-1 and N-2 product is to be expected. 1- to 2-nt 5' truncation of **H4** effectively increases the toehold length by 1 to 2 nt, which will accelerate the toehold-mediate branch migration by 10- to 100-fold[5a]**.** This means 5'-truncated **H4** will be incorporated into the HCR chain with 10- to 100-fold preference over perfectly formed **H4** (downward reaction of *a*). However, 5'-truncated **H4** cannot fully displace the segment 'x*' of **H3** during its incorporation (downward reaction of *b*) which results in that (1) the dissociation of the newly incorporated **H3** (upward reaction of *b*) is highly favorable and (2) further growth of the HCR chain is impossible because the segment 'x*' of **H3** is not fully exposed to undergo toehold-mediated strand displacement.

Table S1: Oligonucleotides used in this paper.

Name		Sequence (5'-3')	Notes
BM		GATCCCATTCCCATTGGCTCACTGACGCTAGG	
TH		CGACATCTAACCTGG ATGCCTCTGT	
TH substitutes	**TH_9**	CGACATCTAACCTGG ATGCCTCTG	For investigating the length of toehold.
	TH_8	CGACATCTAACCTGG ATGCCTCT	
	TH_7	CGACATCTAACCTGG ATGCCTC	
	TH_6	CGACATCTAACCTGG ATGCCT	
	TH_5	CGACATCTAACCTGG ATGCC	
	TH_4	CGACATCTAACCTGG ATGC	
OS		CCTAGCGTCAGTGAGC CCAGGTTAGATGTCG	
OS substitutes	**OS-T_1**	CCTAGCGTCAGTGAGC T CCAGGTTAGATGTCG	For investigating the distance between toehold domain and branch migration domain.
	OS-T_2	CCTAGCGTCAGTGAGC TT CCAGGTTAGATGTCG	
	OS-T_3	CCTAGCGTCAGTGAGC TTT CCAGGTTAGATGTCG	
	OS-T_4	CCTAGCGTCAGTGAGC TTTT CCAGGTTAGATGTCG	
	OS-T_5	CCTAGCGTCAGTGAGC TTTTT CCAGGTTAGATGTCG	
	OS-T_6	CCTAGCGTCAGTGAGC TTTTTT CCAGGTTAGATGTCG	
	OS-T_{10}	CCTAGCGTCAGTGAGC-$(T)_{10}$- CCAGGTTAGATGTCG	
	OS-T_{15}	CCTAGCGTCAGTGAGC-$(T)_{15}$- CCAGGTTAGATGTCG	
	OS-T_{20}	CCTAGCGTCAGTGAGC-$(T)_{20}$- CCAGGTTAGATGTCG	
mOS		CCTAGCGTCAGTGAGC ATGGTTAGATGTCGC	For detecting defects and developing signal-on mismatch method.
mOS substitutes	**mOS_{MISP1}**	CCTAGCGTCAGTGAGC TTGGTTAGATGTCGC	
	$mOS_{MISP2-A}$	CCTAGCGTCAGTGAGC AAGGTTAGATGTCGC	
	$mOS_{MISP2-G}$	CCTAGCGTCAGTGAGC AGGGTTAGATGTCGC	
	$mOS_{MISP2-C}$	CCTAGCGTCAGTGAGC ACGGTTAGATGTCGC	
	mOS_{MISP3}	CCTAGCGTCAGTGAGC ATCGTTAGATGTCGC	
	mOS_{MISP4}	CCTAGCGTCAGTGAGC ATGCTTAGATGTCGC	
	mOS_{MISP5}	CCTAGCGTCAGTGAGC ATGGATAGATGTCGC	
	mOS_{MISP6}	CCTAGCGTCAGTGAGC ATGGTAAGATGTCGC	
	mOS_{MISP7}	CCTAGCGTCAGTGAGC ATGGTTTGATGTCGC	
	mOS_{MISP8}	CCTAGCGTCAGTGAGC ATGGTTACATGTCGC	
mTH		GCGACATCTAACC**AT GCCTC**	
H1 (Self-PAGE purify)		ACAGAGGCAT CAATGGGA ATGGGATC ATGCCT AACCTAGC GATCCCAT TCCCATTG	CHA circuit used in all experiments using above sequences.
H2(Self -PAGE purify)		ATGGGATC GCTAGGTT AGGCAT GATCCCAT TCCCATTG ATGCCT AACCTAGC CCTTGTCA TAGAGCAC	
S-F		**(5' FAM)**-CGA GTGCTCTA TGACAAGG GCTAGGTT	
S-Q		C CCTTGTC ATAGAGCAC TCG- **(3' IowaBlack FQ)**	
H3(Self -PAGE purify)		**GTCGGTTGCT** GGAATT CGGAGCTAGGTAGGTA GA CATTAC TCTACCTACCTAGCTCCG **TCTCTATCA TTATCTTCC**	HCR circuit 1 (two hairpin assembly; Red: CHA toehold; Green: CHA branch migration domain Underline: HCR toehold
H4(Self -PAGE purify)		TCTACCTACCTAGCTCCG AATTCC CGGAGCTAGGTAGGTAGA GTAATG	
Trigger		TCTACCTACCTAGCTCCG AATTCC	
Lock		AATTCCAGCAAC	
H7(Self -PAGE purify)		**GTCGGTTGCT** GAGTGT CGGAGATGAAGATGAAGC CATCGT GCTTCATCTTCATCTCCG TGGGTTAA	HCR circuit 2 (four hairpin assembly; Red: CHA toehold; Green: CHA branch migration domain; Underline:HCR toehold
H8(Self -PAGE purify)		GCTTCATCTTCATCTCCG GTTTTG CGGAGATGAAGATGAAGC ACGATG	
H9(Self -PAGE purify)		CAAAAC CGGAGATGAAGATGAAGC TTGCCT GCTTCATCTTCATCTCCG **TCTCTATCA TTATCTTCC**	
H10(Self -PAGE purify)		GCTTCATCTTCATCTCCG ACACTC CGGAGATGAAGATGAAGC AGGCAA	
Trigger2		GCTTCATCTTCATCTCCG ACACTC	
H5(Self -PAGE purify)		GACCTCGT CTATCACA TCTCTATC ATTATCTT CCTAGTGTTAAC **AAGATAAT GATAGAG**A **AGCAA CCGAC**	CHA circuit 2 CHA circuit used in detecting both HCR circuit 1 and circuit 2.
H6(Self-PAGE purify)		CCTAGTGTTAAC TCTCTATC ATTATCTT GTTAACACTAGG AAGATAAT	
S-F2		GATAGAGA TGTGATAG ACGAGGTC AAG- **(3' FAM)**	
S-Q2		(**5' IowaBlack FQ**)-CTT GACCTCGT CTATCACA T	

Table S2: Sequences of individual segments.

Name		Sequence (5'-3')	Note
H1 and H2	**Segment 1**	ATGCCTCTGT	'*' represents complementary strand. Eg. Domain x* represents complementary sequence to Domain x.
	Segment 2	TCCCATTG	
	Segment 3	GATCCCAT	
	Segment 4	AACCTAGC	
	Segment 5	CCTTGTCA	
	Segment 6	TAGAGCAC	
H5 and H6	**Segment 9**	GTCGGTTGCT	
	Segment 10	TCTCTATC	
	Segment 11	ATTATCTT	
	Segment 12	CCTAGTGTTAAC	
	Segment 13	CTATCACA	
	Segment 14	GACCTCGT	
H3 and H4	**Segment a**	GGAATT	
	Segment x	CGGAGCTAGGTAGGTA	
	Segment b	CATTAC	
H7, H8, H9, H10	**Segment c**	GAGTGT	
	Segment y	CGGAGATGAAGATGAAGC	
	Segment d	CATCGT	
	Segment e	CAAAAC	
	Segment f	TTGCCT	

4. Reference

(1) Chen, X. *J. Am. Chem. Soc.* **2012**, *134*, 263.

(2) Li, B.; Ellington, A. D.; Chen, X. *Nucleic Acids Res.* **2011**, *39*.

(3) Chen, X. *J. Am. Chem. Soc.* **2012**, *134*, 263.

(4) McKinney, S. A.; Declais, A. C.; Lilley, D. M.; Ha, T. *Nat. Struct. Biol.* **2003**, *10*, 93.

(5) (a) Zhang, D. Y.; Winfree, E. *J. Am. Chem. Soc.* **2009**, *131*, 17303; (b) Genot, A. J.; Zhang, D. Y.; Bath, J.; Turberfield, A. J. *J. Am. Chem. Soc.* **2011**, *133*, 2177.