Single mismatch for Circuit A domain 4

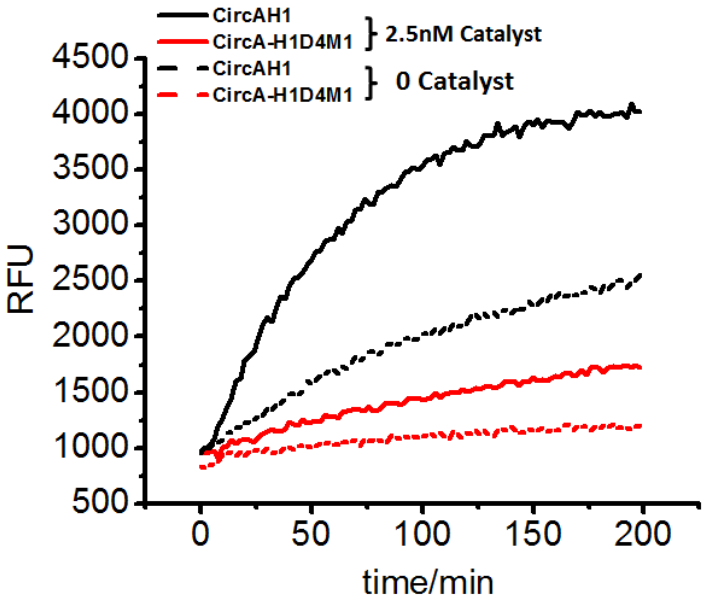


Figure S1. Signal generation in Circuit A with a single mismatch in domain 4. Comparison of wild-type Circuit A to the CircA-H1D4M1 circuit, with and without catalyst. Each CHA reaction was carried out with 50 nM H1 (either wild-type or mismatch), 50 nM CircA-H2, and 50 nM CircA-reporter.

Table S1. Single mismatch sequence for Circuit A: CircA-H1D4M1

Name	Sequence
CircA-H1D4M1	AGAGGCAT CAATGGGA ATGGGATC ATGCCTCT AACCTAGg GATCCCAT TCCCATTG

*Mismatches in red and lowercase

After we determined that the double mismatch on the 3' end of domain 4 will severely compromise the catalytic rate, we tested to see if a single mismatch could return to a higher catalytic rate while maintaining low background leakage. **Figure S1** shows that CircA-H1D4M1 can recover one-fifth of the catalytic rate and background leakage of the original wild-type construct.

Various mismatches on domain 2 of circA-H2

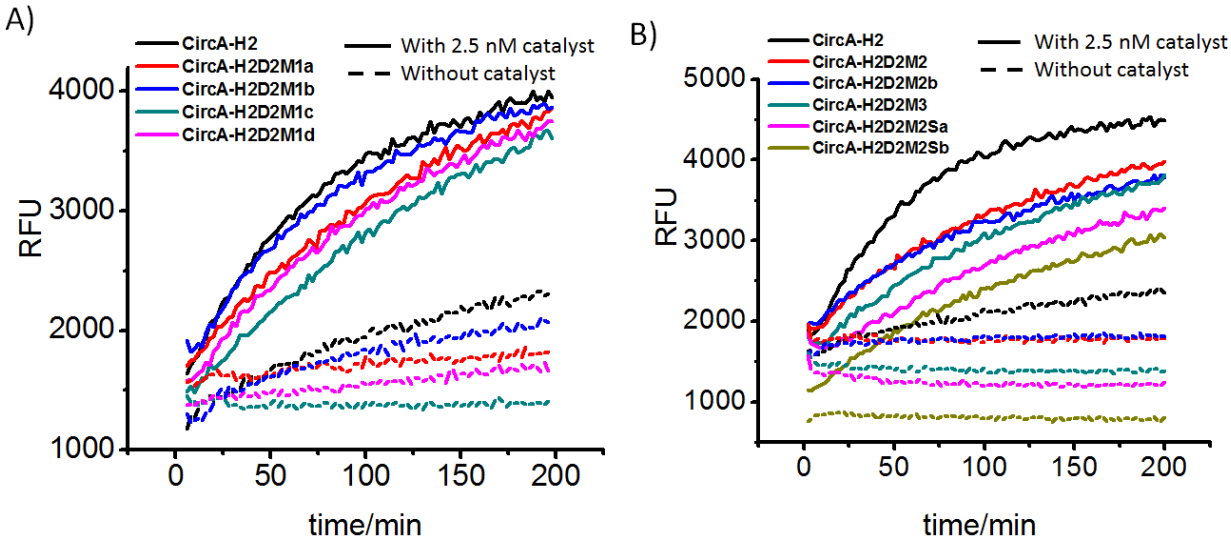


Figure S2. Signal generation in Circuit A with various H2 mismatches. A) Single mismatched H2; B) Double and triple mismatched H2.

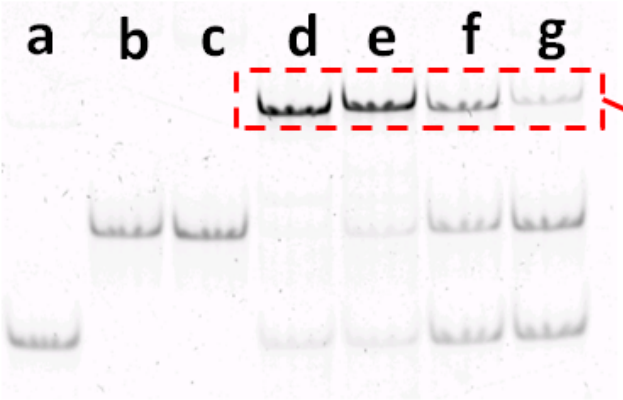
Table S2. Different domain 2 mismatches for Circuit A.

Name	Sequence
CircA-H2D2M1a	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATTa ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M1c	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATTc ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M1d	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATTt ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M1b	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATaG ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M2	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATac ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M2b	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATaa ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M3	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCAaac ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M2a	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCAaTc ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D2M2b	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCtTTc ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC

Native PAGE characterization of the CircA-H2D2M2 reaction

Native polyacrylamide gel electrophoresis was used to further confirm that CHA reactions were occurring similarly irrespective of the absence or presence of mismatches. We prepared hairpins at a concentration of 50nM and incubated them with or without 5nM

catalyst at 37℃ for 3 h prior to loading the reactions (20uL) onto a 10% native polyacrylamide gel. The gel contained 10% acrylamide, 5% glycerol, 0.02M Tris-Boric acid (pH= 8.4), and was poured and polymerized (with 200uL 10% Ammonium Persulfate and 20uLTetramethylethylenediamine). Electrophoresis was carried out at 250v for 1.5h. The fluorescent bands were photographed using a Storm Scanner 840 (Amersham Bioscience, UK) with Excitation 450nM, Emission 520LP, normal sensitivity and PMT Voltage of 800. Fluorescence values were obtained using ImageQuant 5.2 software and the relative fluorescence intensity (R-FI) was determined by subtracting the background (BG) fluorescence and then normalizing to the 'g' band as 1.



bands	R-FI
d	4.5
e	4.1
f	2.2
g	1
BG	0

Figure S3. Native polyacrylamide gel electrophoresis of CHA reactions. a) CircA-H1; b) CircA-H2; c) CircA-H2D2M2; d) CircA-H1+CircA-H2+5nM Catalyst; e) CircA-H1+CircA-H2D2M2+5nM catalyst; f) CircA-H1+CircA-H2; g) CircA-H1+CircA-H2D2M2. The gel was photographed by Storm Scanner 840. The inset Table shows the relative fluorescence intensity (R-FI; derived from ImageQuant 5.2 software) of the bands corresponding to the assembled hairpins for lanes d to g.

Double mismatches at four different breathing positions for Circuit B

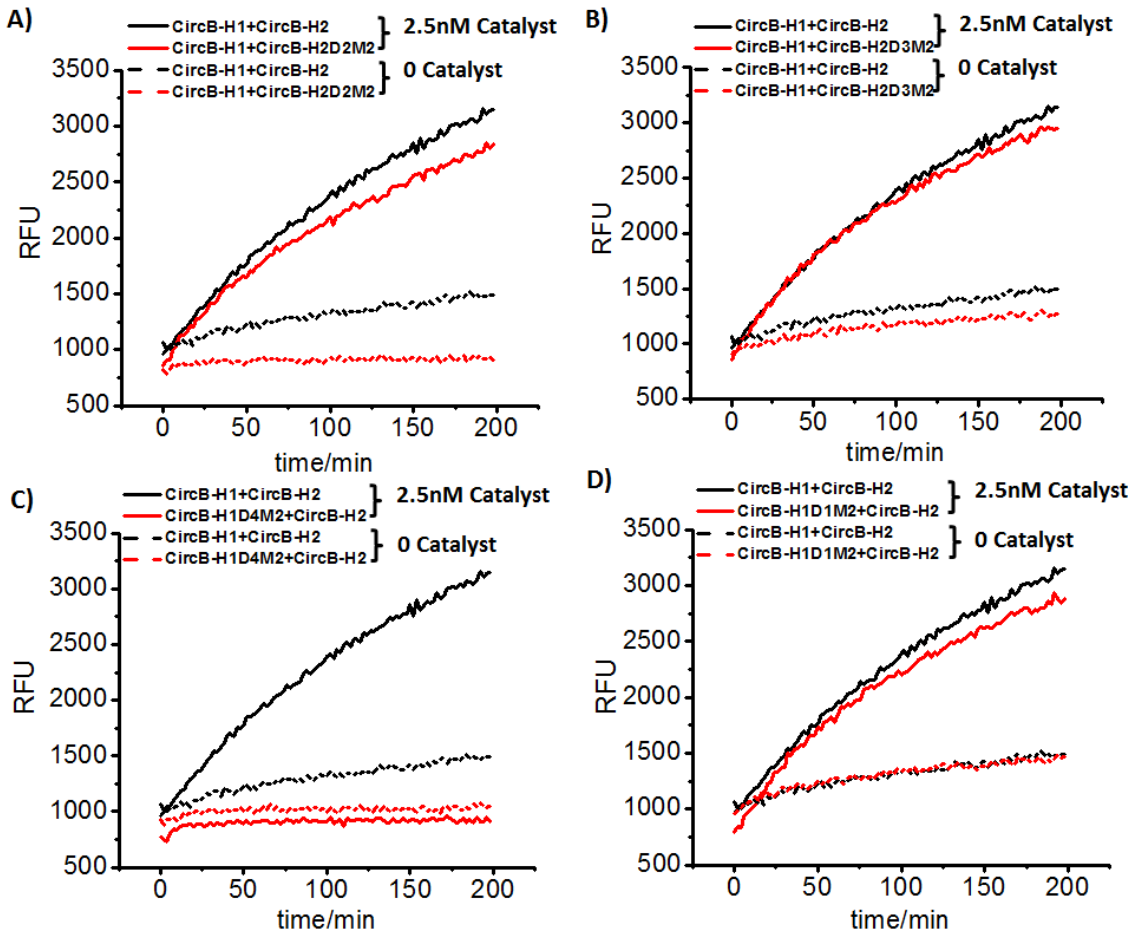


Figure S4. Signal generation with four different mismatches. A) Wild-type circuit and CircB-H2D2M2 circuit; B) Wild-type circuit and CircB-H2D3M2 circuit; C) Wild-type circuit and CircB-H1D4M2 circuit; D) Wild-type circuit and CircB-H1D1M2 circuit. The wild-type data are the same for each comparison; they are simply broken out for ease of viewing. All wild-type and mismatch sequences are based on Circuit B.

Table S3. Wild-type circuit and mismatched sequences*

Name	Sequence
CircB-H1	CAATATCC GAAACGTC CTCCTAAG GGATATTG GGTTTGAG CTTAGGAG GACGTTTC
CircB-H2	CTCCTAAG CTCAAACC CAATATCC CTTAGGAG GACGTTTC GGATATTG GGTTTGAG AGAGTTTC GAGTTCTG
CircB-H2D2M2	CTCCTAAG CTCAAACC CAATATCC CTTAGGAG GACGTca GGATATTG GGTTTGAG AGAGTTTC GAGTTCTG
CircB-H2D3M2	CTCCTAAG CTCAAACC CAATATCC aaTAGGAG GACGTTTC GGATATTG GGTTTGAG AGAGTTTC GAGTTCTG
CircB-H1D4M2	CAATATCC GAAACGTC CTCCTAAG GGATATTG GGTTTGtc CTTAGGAG GACGTTTC
CircB-H1D1M2	CAATATCC GAAACGTC CTCCTAAG tcATATTG GGTTTGAG CTTAGGAG GACGTTTC

In order to generally demonstrate that mismatches on domain 2 can improve signal:noise

ratio, we used NUPACK to generate a second circuit (Circuit B) with the same domain organization as seen in **Figure 1** but with completely new sequences for the hairpin substrates. Specifically, we inserted double mismatches at the same four breathing positions as seen with Circuit A (**Table 1**). **Figure S4** shows results similar to **Figure 3**, in that only CircB-H2D2M2 suppresses background noise without compromising circuit performance.

Single mismatch for Circuit B domain 4

In order to generalize the previous conclusion seen with Circuit A regarding domain 4, we also tested Circuit B with a single mismatch, CircB-H1D4M1. The result was very similar to Circuit A.

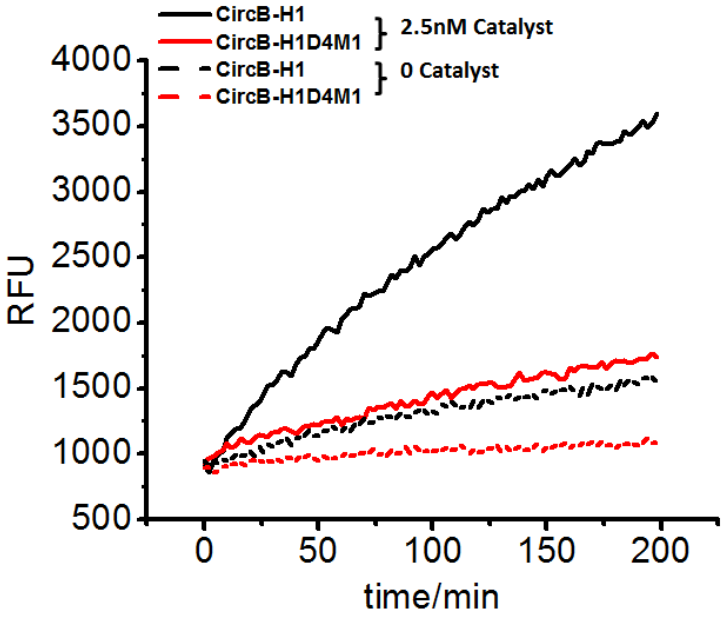


Figure S5. Signal generation in Circuit B with a single mismatch in domain 4. Comparison of wild-type Circuit B to the CircB-H1D4M1 circuit, with and without catalyst. Each CHA reaction was carried out with 50 nM H1 (either wild-type or mismatch), 50 nM CircB-H2, and 50 nM CircB-reporter.

Table S4. Single mismatch sequence for Circuit B: CircB-H1D4M1

Name	Sequence
CircB-H1D4M1	CAATATCC GAAACGTC CTCCTAAG GGATATTG GGTTTGTa CTTAGGAG GACGTTTC

Single and double mismatch for Circuit B on domain 2

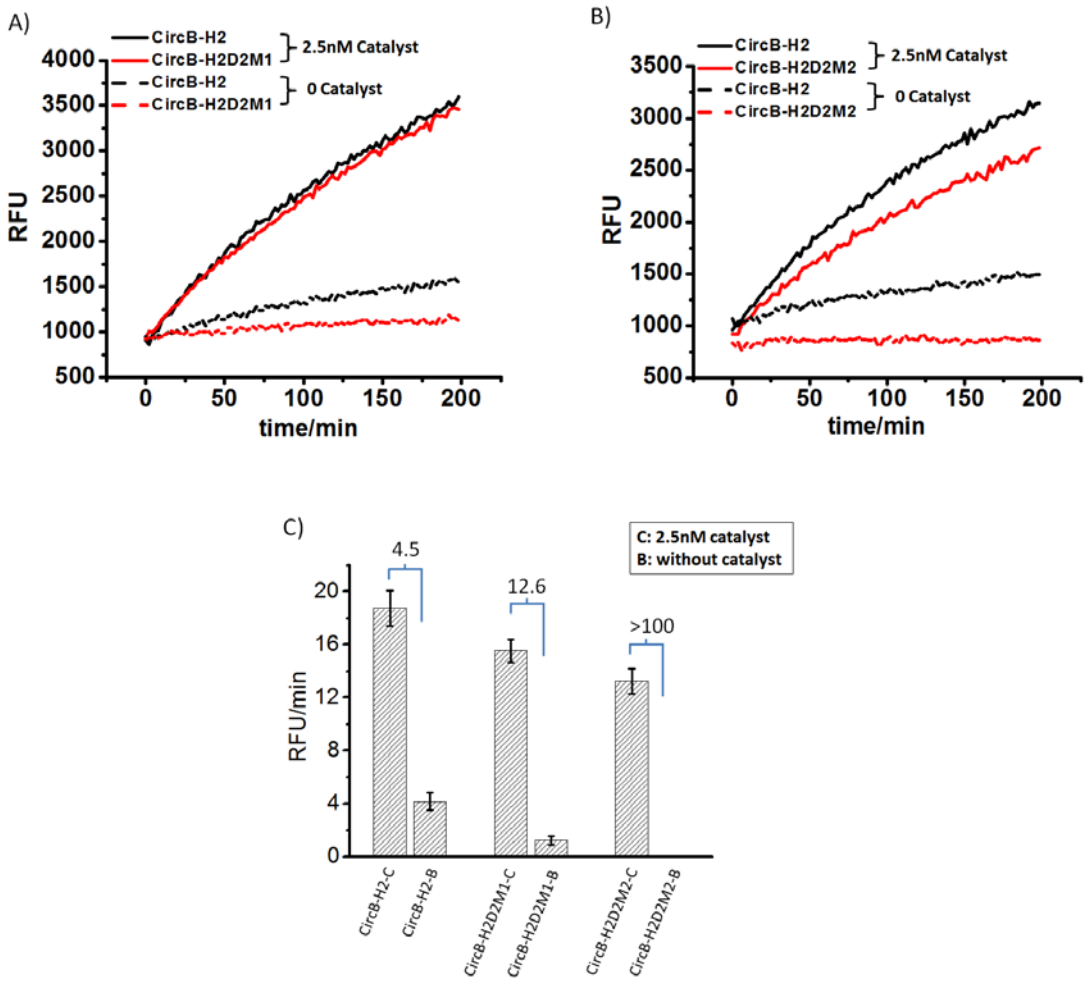


Figure S6. Signal generation in Circuit B with domain 2 mismatches. A) Wild-type circuit and CircB-H2D2M1 circuit; B) Wild-type circuit and CircB-H2D2M2 circuit; C) Calculated signal:background ratios from A) and B). Each CHA reaction was carried out with 50 nM of H2 (either wild-type or mismatch), 50 nM CircB-H1, and 50 nM CircB-Reporter.

Table S5. Different domain 2 mismatches for Circuit B.

Name	Sequence
CircB-H2D2M1	CTCCTAAG CTCAAACC CAATATCC CTTAGGAG GACGTTTa GGATATTG GGTTTGAG AGAGTTTC GAGTTCTG
CircB-H2D2M2	CTCCTAAG CTCAAACC CAATATCC CTTAGGAG GACGTTca GGATATTG GGTTTGAG AGAGTTTC GAGTTCTG

Reporter used in the fluorescence reading

Table S6. Reporter sequences

Name	Sequence
CircA-ReporterF	/56-FAM/CGA GTGCTCTA TGACAAGG GCTAGGT

CircA-ReporterQ	C CCTTGTC ATAGAGCAC TCG/3IABkFQ/
CircB-ReporterF	/56-FAM/-CAC AGAACTC GAAACTCT CTCAAACC
CircB-ReporterQ	G AGAGTTTC GAGTTCT GTG-/3IABkFQ/

CircA-Reporter was a mixture of CircA-ReporterF and CircA-ReporterQ with a ratio of 1:2. For example, 50 nM CircA-Reporter contains 50 nM CircA-ReporterF and 100 nM CircA-ReporterQ. Similarly, CircB-Reporter was a mixture of CircB-ReporterF and CircB-ReporterQ with a ratio of 1:2 where 50 nM CircB-Reporter contains 50 nM CircB-ReporterF and 100 nM CircB-ReporterQ.

Materials and Methods

All the chemicals used in our experiments were of analytical grade and were purchased from Sigma-Aldrich (MO, USA) unless otherwise indicated. All the oligonucleotides were ordered from Integrated DNA Technology (IDT, Coralville, IA, USA). All the hairpins were purified by 12% polyacrylamide gel electrophoresis (PAGE). All hairpins and duplexes were annealed at 95 ^{o}C for 5 min and cooled down to 25 ^{o}C by 0.1 ^{o}C/s before use. The buffer used in the annealing step and the CHA reaction was Tris-HCl buffer with 20 mM Tris-HCl (pH 7.5, 25 ^{o}C), 140 mM NaCl, 5 mM KCl. A 20 uL CHA reaction sample contained 50 nM H1 (either wild-type or mismatch), 50 nM H2 (either wild-type or mismatch), 50 nM Reporter (which consisted of ReporterF:ReporterQ in a 1:2 ratio), and either 2.5 nM catalyst or no catalyst at all. All the kinetic readings were carried out with a 384-well plate from Thermo Fisher Scientific (Rochester, NY) in a TECAN Safire plate reader; each kinetic reading proceeded for 3 h.