Template Name	Sequence	Trigger
LS2	5' - TCCGGA *GAAT* TAATGACTCT TCCGG ***A*** *GAAT* - 3' NH_2	5' - ATTCTCCGGA - 3'
LS3	5' - CGCGCG *GTTTGG* TAATGACTCT CGCGCG *GTTTGG* - 3' NH_2	5' - CCAAACCGCGCG - 3'
Long random sequence in the loop - Increase the loop opening ΔG, Decrease the elongated trigger:template ΔG		
LS2 lrs-1	5' - TCCGGA *GAAT* TAATGACTCT GCTT TCCGG ***A*** *GAAT* - 3' NH_2	5' - ATTCTCCGGA - 3'
LS2 lrs-4	5' - TCCGGA *GAAT* TAATGACTCT GCTTAGTCAG TCCGG ***A*** *GAAT* - 3' NH_2	5' - ATTCTCCGGA - 3'
LS3 lrs-2	5' - CGCGCG *GTTTGG* TAATGACTCT GCTTAG CGCGCG *GTTTGG* - 3' NH_2	5' - CCAAACCGCGCG - 3'
LS3 lrs-4	5' - CGCGCG *GTTTGG* TAATGACTCT ACTTGGCTTA CGCGCG *GTTTGG* - 3' NH_2	5' - CCAAACCGCGCG - 3'
LS2 lowtG lrs1	5' - TCCGGA *TAAT* TAATGACTCT GCTT TCCGG ***A*** *TAAT* - 3' NH_2	5' - ATTATCCGGA - 3'
LS2 lowtG lrs2	5' - TCCGGA *TAAT* TAATGACTCT ACTTGGCTTACG TCCGG ***A*** *TAAT* - 3' NH_2	5' - ATTATCCGGA - 3'
LS3 lt-1 lrs1	5' - CGCGCG *GTTTGGACG* TAATGACTCT GCTT CGCGCG *GTTTGGACG* - 3' NH_2	5' - CGTCCAAACCGCGCG - 3'
LS3 lt-1 lrs2	5' - CGCGCG *GTTTGGACG* TAATGACTCT GCTTAGTCAG CGCGCG *GTTTGGACG* - 3' NH_2	5' - CGTCCAAACCGCGCG - 3'
Vary palindrome length and GC content - change palindrome ΔG and elongated trigger:template ΔG		
LS3 lp*††	5' - CGCGCGCG *GTTTG-* TAATGACTCT CGCGCGCG *GTTTG-* - 3' NH_2	5' - CAAACCGCGCGCG - 3'
LS3 lowpG3	5' - TCCGGA *GTTTGG* TAATGACTCT TCCGG ***A*** *GTTTGG* - 3' NH_2	5' - CCAAACTCCGGA - 3'
LS3 sp	5' - CGCG *GTTTGG* TAATGACTCT CGCG *GTTTGG* - 3' NH_2	5' - CCAAACCGCG - 3'
LS3 lowpG2	5' - TAGCTA *GTTTGG* TAATGACTCT TAGCTA *GTTTGG* - 3' NH_2	5' - CCAAACTAGCTA - 3'
LS2 hpG1*††	5'- GCCGGC *GAAT* TAATGACTCT GCCGGC *GAAT* - 3' NH_2	5' - ATTCGCCGGC - 3'
LS2 lp*††	5' - GTCCGGAC *GAAT* TAATGACTCT GTCCGGAC *GAAT* - 3' NH_2	5' - ATTCGTCCGGAC - 3'
LS2 sp†	5' - CCGG *GAAT* TAATGACTCT CCGG *GAAT* - 3' NH_2	5' - ATTCCCGG - 3'
Vary toehold length and GC content - change toehold ΔG and elongated trigger:template ΔG		
LS3 lt1	5' - CGCGCG *GTTTGGACG* TAATGACTCT CGCGCG *GTTTGGACG* - 3' NH_2	5' - CGTCCAAACCGCGCG - 3'
LS3 htG††	5' - CGCGCG *GTGCGG* TAATGACTCT CGCGCG *GTGCGG* - 3' NH_2	5' - CCGCACCGCGCG - 3'
LS3 lowtG	5' - CGCGCG *GTTTAT* TAATGACTCT CGCGCG *GTTTAT* - 3' NH_2	5' - ATAAACCGCGCG - 3'
LS2 htG2	5' - TCCGGA *GCGC* TAATGACTCT TCCGG ***A*** *GCGC* - 3' NH_2	5' - GCGCTCCGGA - 3'
LS2 lt3	5' - TCCGGA *GAATGATC* TAATGACTCT TCCGG ***A*** *GAATGATC* - 3' NH_2	5' - GATCATTCTCCGGA - 3'
LS2 st‡	5' - TCCGGA *GA* TAATGACTCT TCCGG ***A*** *GA* - 3' NH_2	5' - TCTCCGGA - 3'
LS2 lowtG	5' - TCCGGA *TAAT* TAATGACTCT TCCGG ***A*** *TAAT* - 3' NH_2	5' - ATTATCCGGA - 3'
No 5' toehold - lock the 5' toehold using added nucleotides in the loop - decrease loop ΔG and elongated trigger:template ΔG, remove cooperative binding of trigger		
LS2 no5'*††	5' - TCCGGA GAAT TAATGACTCT ATTC TCCGG ***A*** *GAAT* - 3' NH_2	5' - ATTCTCCGGA - 3'
LS2 no5' lrs3*††	5' - TCCGGA GAAT TAATGACTCT GTATAGCT ATTC TCCGG A *GAAT* - 3' NH_2	5' - ATTCTCCGGA - 3'
Linear templates		
Ran2**†	5' - GGGGAAATAG GTGAGACTCT GGGGAAATAG - 3' NH2	5' - CTATTTCCCC - 3'
EXPAR1**†	5' - CTCACGCTAC GGACGACTCT CTCACGCTAC - 3' PO_3	5' - GTAGCGTGAG - 3'

† = did not enter second phase, †† = did not have a measureable first phase, * = *ab initio* synthesis after approximately 80 min, ** = *ab initio* synthesis after approximately 30 min, ‡ = no amplification

Table SI 1: Template and trigger sequences. Bases in italics compromise the toehold, bases in bold are the palindrome, and underlined bases show the nickase recognition site. A nucleotide that is both bold and italic represents a base in the palindrome that is not predicted to be bound by mfold, making it both a part of the palindromic sequence and a part of the toehold. These free nucleotides were considered part of the toehold when calculating the toehold ΔG. Blue bases show added complimentary sequences that bind the 5' toehold.

Template Name	3' toe ΔG, kcal/mol	5' toe ΔG, kcal/mol	Palindrome ΔG, kcal/mol	Palindrome T_m	Loop ΔG, kcal/mol	Loop T_m	Trigger:Template ΔG, kcal/mol	Trigger:Template T_m	Elongated Trigger:Template ΔG, kcal/mol	Elongated Trigger:Template T_m
LS2	-0.4	0.2	-3.9	27.0°C	-1.6	67.1°C	-6.9	47.5°C	-13.0	65.5°C
LS3	-2.0	-2.0	-5.4	42.2°C	-3.66	76.7°C	-11.6	65.4°C	-18.5	74.7°C
Long random sequence in the loop - Increase the loop opening ΔG, Decrease the elongated trigger:template ΔG										
LS2 lrs-1	-0.4	0.2	-3.9	27.0°C	-1.14	63.7°C	-6.9	47.5°C	-16.8	70.8°C
LS2 lrs-4	-0.4	0.2	-3.9	27.0°C	-0.49	58.8°C	-6.9	47.5°C	-21.4	74.5
LS3 lrs-2	-2.0	-2.0	-5.4	42.2°C	-3.25	73.5°C	-11.7	65.6°C	-24.0	79.1°C
LS3 lrs-4	-2.0	-2.0	-5.4	42.2°C	-2.8	71.2°C	-11.9	66.1°C	-26.0	79.0°C
LS2 lowtG lrs1	0.4	0.4	-3.9	27.0°C	-1.06	63.2°C	-5.8	42.2°C	-15.7	68.7°C
LS2 lowtG lrs2	0.4	0.4	-3.9	27.0°C	-0.26	56.9°C	-5.8	42.2°C	-22.2	74.3°C
LS3 lt-1 lrs1	-5.2	-5.2	-5.4	42.2°C	-3.03	72.7°C	-14.8	71.8°C	-25.5	80.3°C
LS3 lt-1 lrs2	-5.2	-5.2	-5.4	42.2°C	-2.41	68.5°C	-14.9	71.9°C	-30.5	82.3°C
Vary palindrome length and GC content - change palindrome ΔG and elongated trigger:template ΔG										
LS3 lp*††	-0.7	-0.7	-8.6	60.3°C	-6.97	86.5°C	-13.5	69.9°C	-20.3	77.0°C
LS3 lowpG3	-2.8	-2.0	-3.9	27.0°C	-1.35	65.4°C	-8.9	56.7°C	-15.4	69.2°C
LS3 sp	-2.0	-2.0	-2.2	7.0°C	-0.46	58.9°C	-8.4	55.0°C	-15.3	70.0°C
LS3 lowpG2	-2.8	-2.0	-2.2	12.7°C	0.73	49.2°C	-6.8	49.3°C	-13.0	64.3°C
LS2 hpG1*††	0.2	0.2	-5	38.3°C	-3.56	78.9°C	-9.1	58.1°C	-16	71.9°C
LS2 lp*††	0.2	0.2	-5	41.9°C	-3.56	73.7°C	-9.1	57.6°C	-16	70.5°C
LS2 sp†	0.2	0.2	-1.7	-4.7°C	-0.15	56.4°C	-5.6	38.3°C	-12.4	65.1°C
Vary toehold length and GC content - change toehold ΔG and elongated trigger:template ΔG										
LS3 lt1	-5.2	-5.2	-5.4	42.2°C	-3.45	75.3°C	-14.8	71.8°C	-21.7	77.8°C
LS3 htG††	-4.3	-4.3	-5.4	42.2°C	-3.66	76.7°C	-13.9	72.4°C	-20.8	78.9°C
LS3 lowtG	-0.5	-0.5	-5.4	42.2°C	-3.66	76.7°C	-10.1	61.1°C	-17	72.8°C
LS2 htG2	-3.6	-2.4	-3.9	27.0°C	-1.56	67.1°C	-9.8	60.6°C	-16.2	72.0°C
LS2 lt3	-3.4	-2.8	-3.9	27.0°C	-1.14	63.7°C	-9.4	58.6°C	-16	70.3°C
LS2 st‡	0.1	1	-3.9	27.0°C	-1.67	68.0°C	-5.9	40.4°C	-12.4	65.2°C
LS2 lowtG	0.4	0.4	-3.9	27.0°C	-1.48	66.6°C	-5.8	42.2°C	-11.9	63.0°C
No 5' toehold - lock the 5' toehold using added nucleotides in the loop - decrease loop ΔG and elongated trigger:template ΔG, remove cooperative binding of trigger										
LS2 no5'*††	-0.4	n/a	-3.9	27.0°C	-4.05	73.9°C	-6.9	47.5°C	-15	67.4°C
LS2 no5' lrs3*††	-0.4	n/a	-3.9	27.0°C	-3.31	70.3°C	-6.9	47.5°C	-20.4	72.1°C
Linear templates										
Ran2**†	n/a	n/a	n/a	n/a	n/a	n/a	-5.1	41.2°C	-13.9	67.4°C
EXPAR1**†	n/a	n/a	n/a	n/a	n/a	n/a	-6.5	47.4°C	-16.7	73.3°C

† = did not enter second phase, †† = did not have a measureable first phase, * = *ab initio* synthesis after approximately 80 min, ** = *ab initio* synthesis after approximately 30 min, ‡ = no amplification

Table SI 2: Template Thermodynamics: Thermodynamics of the most probable looped template structures. All templates contained two open toeholds and a palindromic loop, with the exception of the no5' toehold templates. Thermodynamics are also give for trigger:template association and long trigger:template association, where the long trigger is the original trigger with the elongated nickase recognition site.

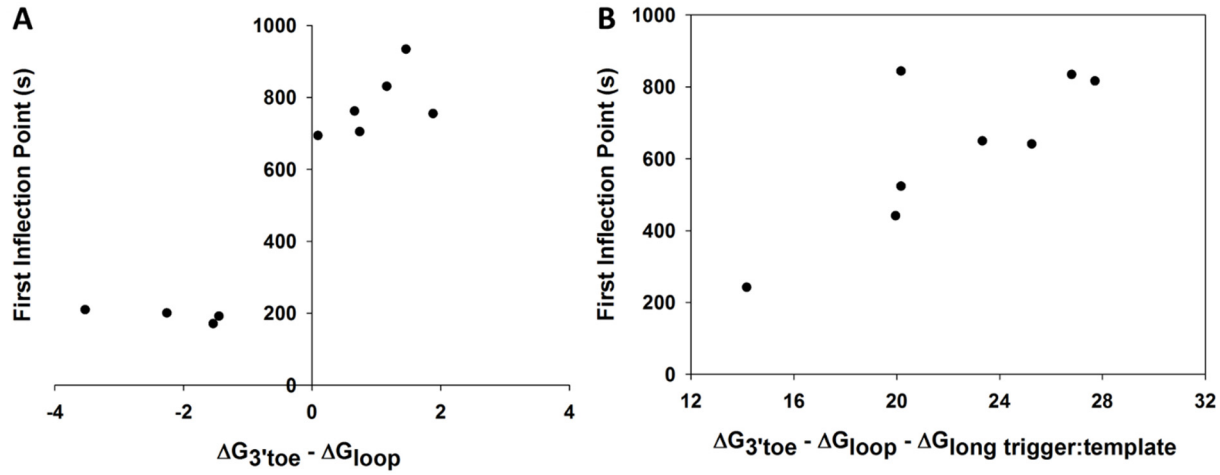


Figure SI 1: Correlation between kinetics of the first phase and the template and trigger thermodynamics. The 3' toehold region dominates kinetics of loop opening[1] to form an amplifiable molecule, while a strong template loop will prevent a stable open template. The first inflection point serves as a surrogate for reaction kinetics (see Fig SI 5 for details), with a lower first inflection point indicating faster first phase kinetics. A) For type I templates, there are two distinct clustered groups of inflection points: high inflection points when the 3' toehold association ΔG exceeds the template loop ΔG (positive x-axis), and low inflection points when the loop ΔG exceeds the ΔG of the 3' toehold (negative x-axis) (Wilcoxon Rank Sum Test, $p<0.01$). B) Type II templates appear to slow when the long trigger containing the extended nickase recognition site is strengthened, likely due to the template poisoning phenomena that has previously been suggested in the literature[2]. The first inflection shows only a modest correlation with DNA template thermodynamics (Spearman's Rho = 0.6587). A larger value on the x axis represents a template has stronger loop structure and long trigger association when compared to new trigger association to the 3' end of the template, resulting in reduced first phase kinetics. These correlations are stronger than correlations between trigger:template association thermodynamics and reaction speed in the original EXPAR reaction ($R = 0.4072$)[3]. This is likely due to the closed template loop; thermodynamics of DNA association largely dominate the reaction kinetics, contrasting the sequence dependence seen in traditional EXPAR.

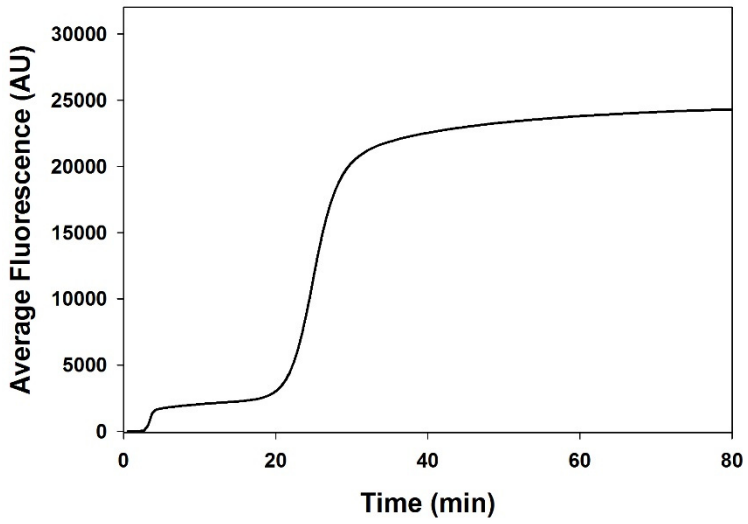


Fig SI 2: Output of biphasic templateLS3 lowpG2. This template has a palindromic region but is unstable at the reaction temperature (Tm = 49.2°C); the resulting amplification was biphasic.

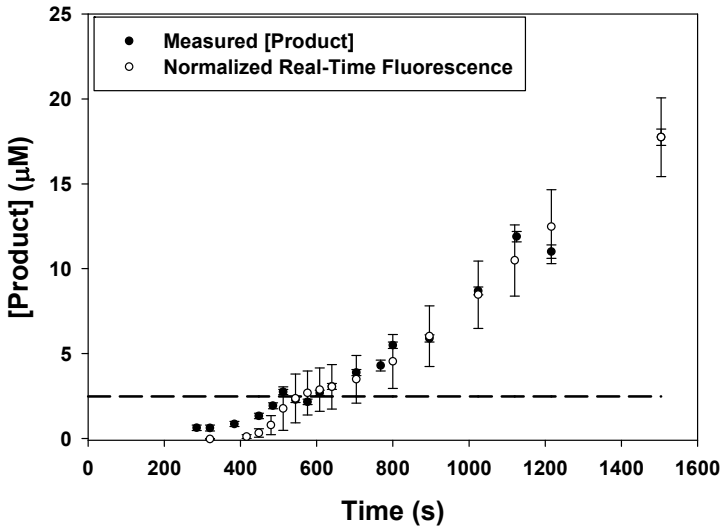
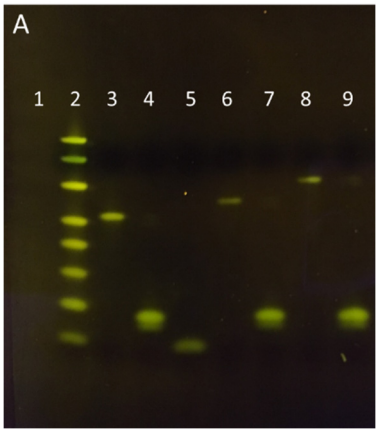
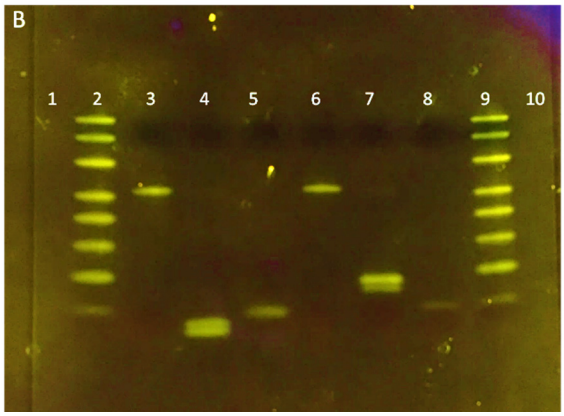


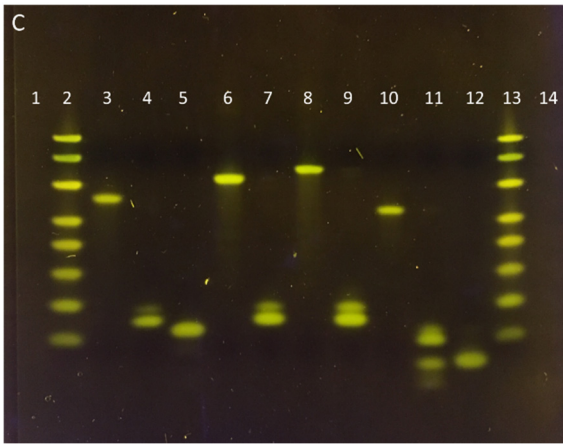
Figure SI 3: Product quantification time course. The filled circles show product concentration in LS3 reactions that were removed and quantified at different times. The open circles are an average of n = 18 runs of LS3 reaction fluorescence taken in a real-time PCR instrument, which have been normalized to match the final 1504 second quantified product. The dotted line shows the level of the first plateau (2.5 ± 0.2 µM), averaged over 3 time points. The error bars on the filled points show the standard deviation of the product quantification measurement.



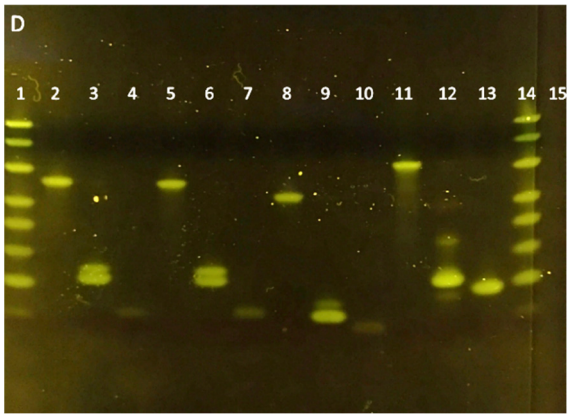
Well number	Sample name	Sample concentration
1	Empty	-
2	Ladder	5.00 ng/µl
3	LS2 template	0.25 µM
4	LS2 product	3.28 µM
5	LS2 trigger	5.00 µM
6	LS2 lrs-1 template	0.10 µM
7	LS2 lrs-1 product	3.20 µM
8	LS2 lrs-4 template	0.10 µM
9	LS2 lrs-4 product	3.50 µM



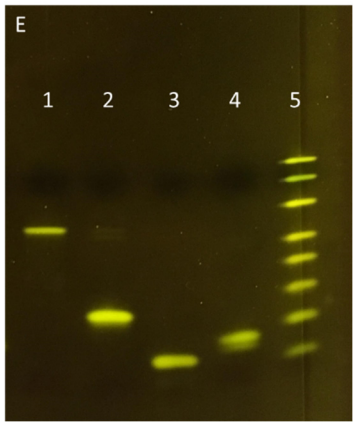
Well number	Sample name	Sample concentration
1	Empty	-
2	Ladder	5.00 ng/µl
3	LS2 htG-2 template	0.25 µM
4	LS2 htG-2 product	2.92 µM
5	LS2 htG-2 trigger	2.50µM
6	LS2 lowtG template	0.25 µM
7	LS2 lowtG product	2.77 µM
8	LS2 lowtG trigger	5.00 µM
9	Ladder	5.00 ng/µl
10	Empty	-



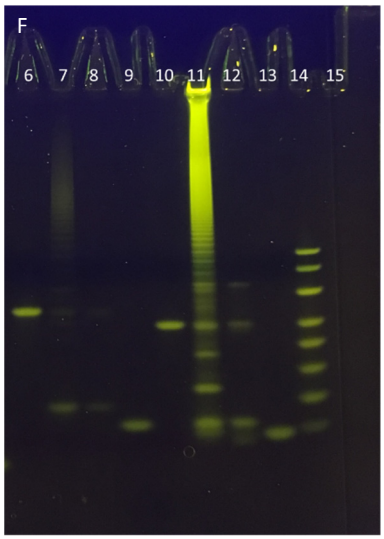
Well number	Sample name	Sample concentration
1	Empty	-
2	Ladder	5.00 ng/µl
3	LS3 template	0.50 µM
4	LS3 product	2.70 µM
5	LS3 trigger	5.00 µM
6	LS3 lrs-2 template	1.00 µM
7	LS3 lrs-2 product	7.65 µM
8	LS3 lrs-4 template	1.00 µM
9	LS3 lrs-4 product	7.09 µM
10	LS3 htG template	0.50 µM
11	LS3 htG product	4.79 µM
12	LS3 htG trigger	2.50 µM
13	Ladder	5.00 ng/µl
14	Empty	-
15	Empty	-



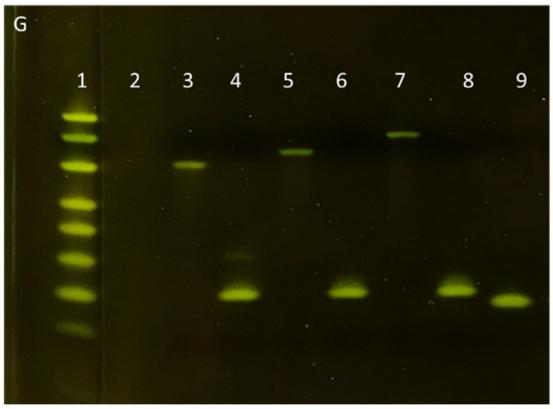
Well number	Sample name	Sample concentration
1	Ladder	5.00 ng/µM
2	LS3 lowpG-2 template	0.5 uM
3	LS3 lowpG-2 product	3.66 µM
4	LS3 lowpG-2 trigger	10.00 µM
5	LS3 lowpG-3 template	0.50 µM
6	LS3 lowpG-3 product	4.02 µM
7	LS3 lowpG-3 trigger	10.00 µM
8	LS3 sp template	0.50 µM
9	LS3 sp product	4.59 µM
10	LS3 sp trigger	20.00 µM
11	LS3 lt-1 template	0.50 µM
12	LS3 lt-1 product	3.02 µM
13	LS3 lt-1 trigger	2.50 µM
14	Ladder	5.00 ng/µM
15	Empty	-



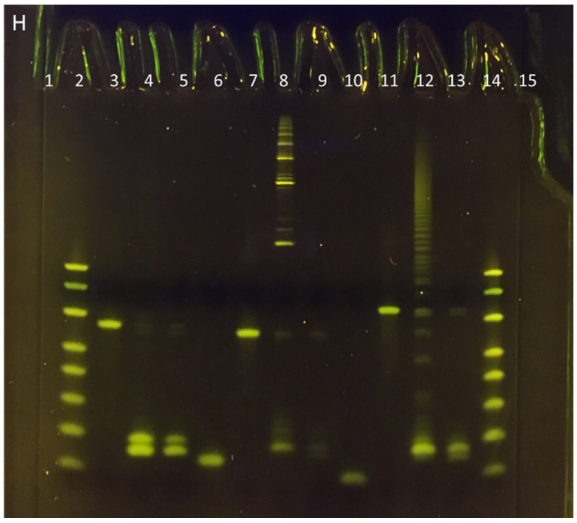
Well number	Sample name	Sample concentration
1	LS3 lowtG template	0.50 µM
2	LS3 lowtG product	12.77 µM
3	LS3 lowtG trigger	5.00 µM
4	LS2 product	3.28 µM
5	Ladder	5 ng/µl



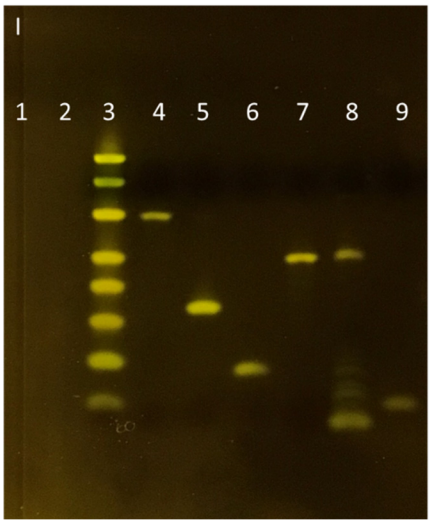
Well number	Sample name	Sample concentration
6	LS2 lp template	0.50 µM
7	LS2 lp product	n/a
8	LS2 lp product*	2.43 µM
9	LS2 lp trigger	2.50 µM
10	LS2 hpG-1 template	0.50 µM
11	LS2 hpG-1 product	n/a
12	LS2 hpG-1 product*	5.33 µM
13	LS2 hpG-1 trigger	5.00 µM
14	Ladder	5.00 ng/µl
15	Empty	-



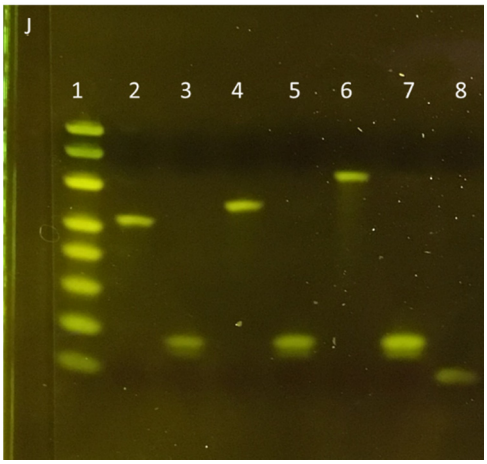
Well number	Sample name	Sample concentration
1	Ladder	5.00 ng/uL
2	Empty	-
3	LS3 lt1 template	0.25 µM
4	LS3 lt1 product	2.52 µM
5	LS3 lt1 lrs1 template	0.25 µM
6	LS3 lt1 lrs1 product	2.16 µM
7	LS3 lt1 lrs2 template	0.25 µM
8	LS3 lt1 lrs2 product	1.96 µM
9	LS3 lt1 trigger	2.5 µM



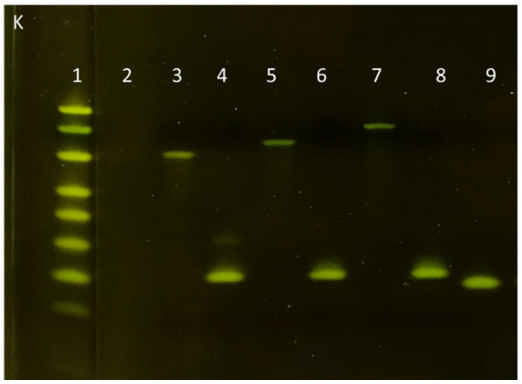
Well number	Sample name	Sample concentration
1	Empty	-
2	Ladder	5.00 ng/µl
3	LS3 lp ptemplate	0.50 µM
4	LS3 lp product	N/A
5	LS3 lp product*	2.77 µM
6	LS3 lp trigger	2.50 µM
7	LS2 no5' template	0.50 µM
8	LS2 no5' product	N/A
9	LS2 no5' product*	0.78 µM
10	LS2 trigger	50 µM
11	LS2 no5 lrs-3 template	0.50 µM
12	LS2 no5 lrs-3 product	N/A
13	LS2 no5 lrs-3 product*	3.34 µM
14	Ladder	5.00 ng/µl
15	Empty	-



Well number	Sample name	Sample concentration
1	Empty	-
2	Empty	-
3	Ladder	5 ng/µl
4	LS2 lt-3 template	0.25 µM
5	LS2 lt-3 product	3.81 µM
6	LS2 lt-3 trigger	2.50 µM
7	EXPAR1 template	0.50 µM
8	EXPAR1 product**	5.58 µM
9	EXPAR1 trigger	5.00 µM



Well number	Sample name	Sample concentration
1	Ladder	5.00 ng/µL
2	LS2 lowtG template	0.25 µM
3	LS2 lowtG product	3.25 µM
4	LS2 lowtG lrs1 template	0.25 µM
5	LS2 lowtG lrs1 product	3.10 µM
6	LS2 lowtG lrs2 template	0.25 µM
7	LS2 lowtG lrs2 product	3.45 µM
8	LS2 lowtG trigger	5.00 uM



Well number	Sample name	Sample concentration
1	Ladder	5.00 ng/uL
2	Empty	-
3	LS3 lt1 template	0.25 μM
4	LS3 lt1 product	2.52 μM
5	LS3 lt1 lrs1 template	0.25 μM
6	LS3 lt1 lrs1 product	2.16 μM
7	LS3 lt1 lrs2 template	0.25 μM
8	LS3 lt1 lrs2 product	1.96 μM
9	LS3 lt1 trigger	2.5 μM

Figure SI 4: PAGE gel analysis. Templates, triggers, and reaction products are shown for the listed DNA amplification reactions. All looped templates form products in the same size range as their triggers, with no evidence of *ab initio* synthesis before 4800s in biphasic amplification. Templates that showed multiple large bands characteristic of *ab initio* synthesis were rerun for shorter reaction durations: * = *ab initio* synthesis detected at 9632 s, rerun for 4800 s. ** = *ab initio* synthesis detected after 4800 s, rerun for 1800 s. The ladder shown is the 10/60 oligonucleotide length standard (Integrated DNA Technologies, Coralville, IA), which ranges from 10 to 60 nucleotide ssDNA. (A) LS2 with added long random sequences and long toehold templates, triggers, and reaction products. (B) LS2 high and low toehold ΔG templates, triggers, and reaction products. (C) LS3 with added long random sequences and LS3 high toehold ΔG templates, triggers, and reaction products. (D) LS3 long toehold, LS3 short palindrome, LS3 low palindrome ΔG templates, triggers, and reaction products. (E) LS3 low toehold ΔG templates, triggers, and reaction products. (F) LS2 long palindrome and high palindrome ΔG templates, triggers, and reaction products. (G) LS3 long toehold with added long random sequences templates, trigger, and reaction products. (H) LS3 long palindrome and LS2 templates with no open 5' toehold templates, triggers, and reaction products. (I) LS2 long toehold and traditional linear EXPAR templates, triggers, and reaction products. (J) LS2 low toehold ΔG with added long random sequences templates, triggers, and reaction products.

PAGE analysis of reaction products.

PAGE protocol. Novex™ TBE Running Buffer (5X) was diluted to a 1X final concentration in milliQ ultrapure water. Templates, triggers, reaction products and ladder were diluted to concentrations specified in 2X TBE-Urea Sample Buffer. Oligo length standard 10/60 was used as the ladder. The working solution for loading the samples to the gel was prepared using 1uL of the sample, 1X TBE-Urea Sample Buffer and 1X TE buffer to bring the final volume to 6 µL. The samples were denatured by incubating at 80˚C in a thermocycler (iCycler, Bio-Rad, Hercules, CA) for 5 minutes and immediately put on ice for storage until loading to the gel. The 1X Novex™ TBE Running Buffer was heated to approximately 50˚C before filling the Bolt® Mini Gel Tank, purchased from Thermo Fisher Scientific (Waltham, MA). Novex™ TBE-Urea Gels, 15%, 10 or 15 well, were used according to the manufacturer's protocol with the voltage set to 180V for approximately 25 minutes. Gel staining solution was prepared by diluting 10,000X SYBR® Gold Nucleic Acid Gel Stain to 1X in 1X Novex™ TBE Running Buffer. Immediately after the run, gels were stained and imaged using UltraBright LED Transilluminator (Maestrogen, Atkinson, NH) at a 470nm wavelength excitation.

Analysis of product lengths. We and others[4] hypothesized that an elongated trigger forms due to trigger dimerization and subsequent polymerase extension, which would lead to the main end product of the

reaction to be the elongated trigger when reactions are run until they plateau (Figure 2). The PAGE gels in Figure SI 3 show that many of the reaction products contain two different bands; one of the bands matches the hypothesized size of the elongated trigger and the other differs by one or two nucleotides from the size of the elongated trigger. The bands that are one or two nucleotides shorter than the elongated trigger (in the case of LS3 lowpG-2, LS2 lowpG-3 and LS3 sp templates, Figure SI 3D) may possibly be formed when the nicking enzyme cleaves the complementary template strand by displacing the polymerase before it is done with full polymerization of the template[5]. These short oligonucleotides would still have the palindrome sites that would cause the production of elongated triggers, but they would be one or two nucleotides shorter than the original elongated trigger. Other template products have a second band that is one or two nucleotides longer than the elongated trigger band, and in some cases, there is only one band that is the same size with the hypothesized elongated trigger band (LS2 lt-3 (Figure SI 3A) and LS3 lowtG templates(Figure SI 3E)). Other have products that match the size of their triggers. For the templates L LS2 hpG-1 (Figure SI 3G) and LS3 lp (Figure SI 3H), it is possible they do not have elongated trigger products because their short reaction time to prevent *ab initio* synthesis may not allow the elongation step to fully occur. The templates and the long triggers that contain a restriction endonuclease sequence are not seen in most of the gel images. Most of the products were diluted between 1:10 and 1:40 to increase resolution of the high concentrated products, such that the low concentrated products cannot be detected. Products with a visible template band were not diluted; these were poorly amplified samples with low reaction product concentrations (Table SI 3).

Some templates have *ab initio* synthesis after 1800s (traditional EXPAR templates EXPAR1, Ran2 (Figure SI 3I)) or 4800s (LS2 hpG1, LS2 lp (Figure SI 3G), LS3 lp, LS2 no5', LS2 no5' lrs3 (Figure SI 3H)), characterized by many bands above the initial trigger size[6]. If the reaction time is reduced from 9632s to 4800s, we did not detect *ab initio* synthesis in the DNA biphasic amplification reactions.

Template	Estimated [Product] (μM)	SD (μM)
LS3 lp*	27.7	± 4.2
LS3	80.9	± 4.6
LS3 lrs-2	76.5	± 4.5
LS3 lrs-4	70.9	± 4.5
LS3 lt1	90.7	± 5.0
LS3 htG	95.9	± 4.9
LS3 lowtG	63.9	± 4.2
LS3 lt1 lrs-1	54.1	± 4.2
LS3 lt1 lrs-2	49.3	± 4.1
LS2 hpG1*	5.3	± 1.0
LS2 lp*	12.2	± 1.0
LS2	82.0	± 7.5
LS2 lrs-1	80.1	± 7.5
LS2 lrs-4	87.4	± 8.7
LS2 lt3	114.3	± 7.8
LS2 htG2	116.9	± 9.5
LS2 lowtG	83.0	± 7.5
LS2 no5'*	7.8	± 1.0
LS2 no5' lrs3*	33.4	± 1.1
LS3 lowpG3	80.4	± 4.5
LS2 lowtG lrs1 product	62.3	± 4.6
LS2 lowtG lrs2 product	69.8	± 4.5
LS3 sp	91.7	± 5.4
LS3 lowpG2	73.2	± 4.6
EXPAR1**	5.6	± 0.3

**ab initio* synthesis detected, quantified after 4800s

***ab initio* synthesis detected, quantified after 1800s

Table SI 3: Endpoint concentrations of trigger, quantified at 9632 seconds unless otherwise indicated. Samples were taken from reaction endpoints and quantified using calibrated SYBR fluorescence. Errors represent standard deviation of the endpoint measurement (see product quantification and data analysis and statistics below).

Product quantification. NanoDrop 3300 Fluorospectrometer (Thermo Scientific, Wilmington, DE) was used for measuring the reaction product concentrations. The standards (ssDNA oligos, Eurofins Genomics, Louisville, KY) and the reaction products were diluted in 1X TE buffer (1mM Tris-HCl, 0.5 mM EDTA) if needed. Nucleic acid stains were diluted in 1X TE Buffer. 1X SYBR® Gold Nucleic Acid Gel Stain (for low concentration samples) or 2.5X SYBR® Green II RNA Gel Stain (for high concentration samples), and 1.2 μL of the sample were brought to a final volume of 12 μL with 1X TE buffer. The standards were prepared in the same way as the reaction products with the addition of mock reaction product (reaction components

without enzymes or trigger) and triggers diluted in 1X TE Buffer. Samples were excited with blue light (470 ± 10 nm) with autogain on. The fluorescence peaks of the dyes were determined to be 512 nm for SYBR® Green II RNA Gel Stain and 536 nm for SYBR® Gold Nucleic Acid Gel Stain for the specific salt conditions, and the average fluorescence of 5 replicate measurements was used to determine the product concentrations of the reaction products. 1X TE buffer was used as the blank measurement.

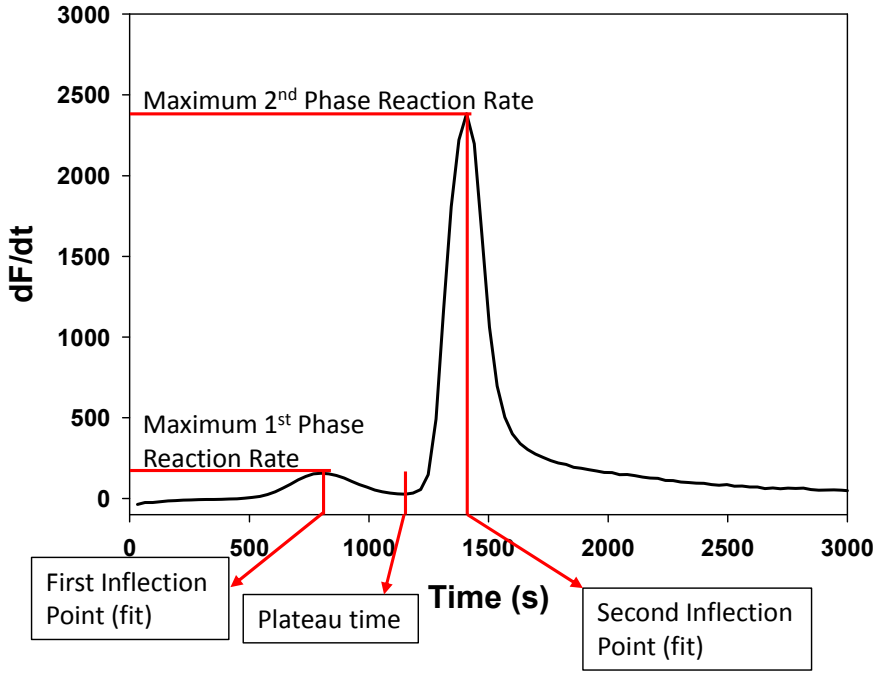


Figure SI 5: Retrieving reaction parameters. The parameters calculated for each amplification reaction were: first and second inflection points (seconds), maximum reaction rates for the first and second phase (d(Fluorescence)/dt), and first plateau time (seconds).

Data analysis and statistics:

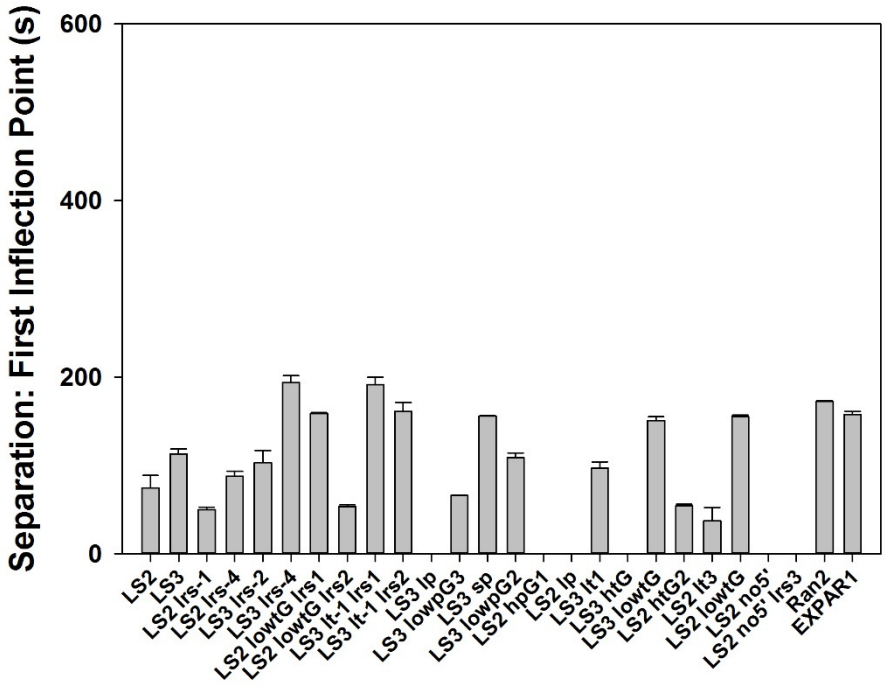
Custom matlab data analysis software. Inflection points were defined as the peaks in the first derivative of the fluorescence with respect to time. To determine exact inflection points, the top of the peak was fit to a quadratic function dF/dt = at2+bt+c where F is fluorescence and t is time. The inflection point was defined as the zero of the second derivative (tIF = 2at+b). The first plateau was defined as the fluorescence corresponding to the time of the lowest point between the first and second peaks in the first derivative. The maximum reaction rates were defined as the top of the peaks in the first derivative (maximum dF/dt). (Figure SI 4).

Statistics. Weighted averages (x_{wav}) from average experimental triplicates (x_i) standard deviations (σ_i) were calculated when appropriate as follows:

$$x_{wav} = \frac{\sum w_i x_i}{\sum w_i}\ , w_i = \frac{1}{\sigma_i^2}, and\ \sigma_{wav}^2 = \frac{1}{\sum w_i}$$

Standard curves relating fluorescence to trigger DNA concentration were fit to a simple linear regression model using the statistical computing software RStudio. The mean trigger concentration predictions of the unknowns were obtained using the "inverse.predict" function of RStudio, under the package "chemCal". Standard deviations were computed using the function "stdev" in the electronic spreadsheet program Microsoft Excel. Standard error from the predicted concentration was converted to standard deviation and the cumulative standard deviation of the trigger DNA concentration was calculating using the formula[7]:

$$SD_{total} = \sqrt{SD_{prediction}{}^2 + SD_{sample\ mean\ of\ the\ replicates}{}^2}$$



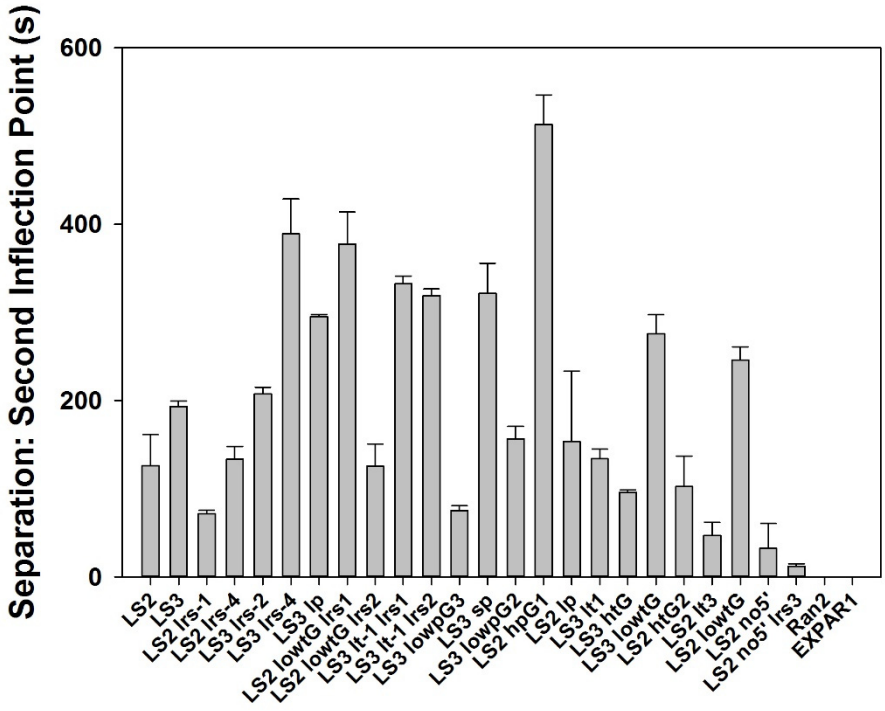


Figure SI 6: **Separation of inflection points between negative and positive controls.** Separation is defined as the time between the inflection point of positive controls initiated with 10pM of trigger and negative controls with no trigger initially present. All reactions initiated with 10pM of trigger DNA had inflection points that were distinct from negative controls, with the exception of templates with bound 5' toeholds (LS2 no5' and LS2 no5' lrs3). Separation times for the first amplification phase were on the same order as the original EXPAR templates Ran2 and EXPAR 1. Error bars show the standard deviation of experimental triplicates.

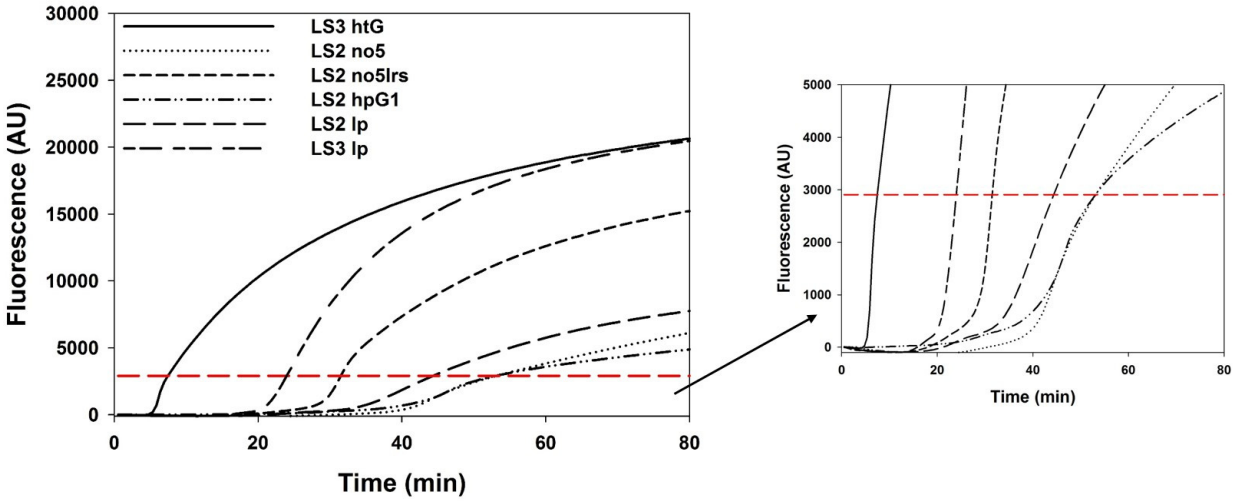


Figure SI 7: Templates without a measurable first phase. Several templates did not have a measureable first phase. Many of these templates had strong template loops when compared trigger association: LS2 no5 and LS2 no5lrs were designed with extra nucleotides that bound the 5' toehold (ΔG_{loop}: LS2 > LS2 no5' lrs3 > LS2 no5'), which made them inefficient molecular switches. LS2 hpG1 and LS2 lp both contained stronger palindromes and loop structures than the base template LS2, and as a result also gave inefficient amplification kinetics. LS3 htG and LS3 lp could efficiently switch on, but had vanishingly short plateaus. These templates all appear to enter the second phase of amplification. The red dotted line represents the average plateau output fluorescence of two standard EXPAR reactions using a linear template. The traces show representative reactions, which were initiated by 10pM of trigger.

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