Sequence Dependence of Isothermal DNA Amplification via EXPAR

Supplementary Data

SUPPLEMENTARY METHODS:

EXPAR template design – thermodynamic criteria: We calculated the melting temperature (T_M) values for template/trigger and template/template hybridization and determined the number of bonds involved in template-template self-hybridization via the Zuker-Turner algorithm(55), through the UNAFold application(55), using as input parameters a DNA type set to oligo, a temperature of 55 °C, with a DNA concentration of 0.05 μM, and cation concentrations of [Na^+] at 1M, [Mg^{2+}] at 0 M, which although different from the electrolyte type and concentration used in EXPAR (30mM [K^+], 3 mM [Mg^{2+}]) yields good agreement between experimental and calculated data (Supplementary Results Figure S2). The trigger/template T_M was calculated based on predicted ΔH and ΔS values and the concentration of the EXPAR template present in the reaction, using equation (1), which is valid if one strand (here the template) is present in excess in the reaction.

$$T_M = \frac{\Delta H^0}{\Delta S^0 - R\ln[template]} \qquad \textbf{(1)}$$

By contrast, the T_M for template/template self-hybridization was calculated using equation (2) implemented in UNAfold, which assumes that the total DNA concentration represents both strands present in equal concentration.

$$T_M = \frac{\Delta H^0}{\Delta S^0 - R\ln([DNA]/4)} \qquad \textbf{(2)}$$

Data Analysis: To analyze the EXPAR real-time fluorescence amplification curves, we developed a MATLAB program called EXPAR Data Analysis Tool (EDAT), which performs a nonlinear least-squares curve fit of the real-time fluorescence data to a sigmoidal hyperbolic tangent (tanh) function given by:

$$\boldsymbol{yfit(t) = ymin + 0.5 * \Delta y * \left[1 + \tanh\left(\frac{t-to}{\tau}\right)\right]} \qquad \textbf{(3)}$$

Here, y_{fit} is the fitted value of normalized fluorescence at each time point t. There are four fitting parameters: y_{min} is a normalized baseline value; Δy is a multiplicative scaling factor; t_{o} denotes the point of inflection (POI); and τ is a measure of steepness of the sigmoidal function near the POI. Prior to fitting the above function, the raw fluorescence data is normalized to range from 0 to 1 by defining:

$$\hat{R} = (R-R_{\text{min}})/(R_{\text{max}}-R_{\text{min}}). \qquad \textbf{(4)}$$

Here, $\hat{R}$ is the normalized data while R is the raw fluorescence measurement. R_{min} and R_{max} are the minimum and maximum values of the measured fluorescence. Our least squares curve fit method uses the "trust-region-reflective" algorithm (65), to find the parameters t_{o}, τ, y_{min} and Δy that provide the best fit by the function (3) to the normalized data (5), over the entire set of discrete times t.

For each curve, EDAT determines and records the time required for the fitted data to reach 10% and 90% of the maximum plateau, designated as P10 and P90 for positive, trigger containing reactions, and N10 and N90 for negative, no trigger controls. The program also records the quality of data fitting (resnorm). Curves with no apparent amplification and curves for which the fluorescence intensity only started to increase above background at the very end of the amplification time were classified as "No Amplification" (NA) and "Late Amplification" (LA), respectively (Supplementary Results Figure S1A and B), and were not evaluated further.

Likewise, curves for which the amplification is clearly non-sigmoidal were classified as "Non-Sigmoidal" (NS, Supplementary Results Figure S1C), and were not further evaluated. If a curve amplified in a sigmoidal fashion and nearly reached the final plateau at the end of experiment (Supplementary Results Figure S1D), then we padded the curve at the end with the last detected fluorescence intensity value, which provides a reasonable approximation of the actual value. These curves were designated as "Fit-with-padding." Some curves have a dip in fluorescence intensity in the first minute of amplification related to instrument performance. For these curves, the initial inconsistent data was replaced with the fluorescence intensity at an operator selected "cut time", and the curves were designated as "cutting start" (Supplementary Results Figure S1F). Likewise, for some curves the fluorescence intensity continues to increase or decrease slightly (Supplementary Results Figure S1E). Some templates further exhibit a second exponential rise, likely due to "ab initio" DNA synthesis as previously reported (54), which is not the process of interest herein. Since both phenomena distort the curve fit of the initial sigmoidal rise, the data following a given operator selected "cut time" (selected ~ 5 minutes after the first sigmoidal curve has reached the plateau) was replaced with the fluorescence intensity at the cut time, and the curves were designated as "cutting end".

Position Weight Matrix classification method:

The PWMs for low P90 and large positive Diff were constructed as follows: The first PWM was generated based on templates in the training set whose P90 was in the lowest 30% of the P90 value range. The second PWM was generated using templates in the training set whose Diff was in the highest 40% of the Diff value range. In each case, for the 14 variable positions within the EXPAR template sequence, we defined the probability to find any of the four nucleotides at position n as $p_i^{(n)}$, where n is the position index ($1<n<14$), and i is the nucleotide index ($0<i<3$).

$p_0^{(n)}$ denoted the highest probability for any $p_i^{(n)}$ occurring in position n. Then, the "energies" for the position n were defined as:

$$\varepsilon_i^{(n)} = \ln \frac{p_0^{(n)}}{p_i^{(n)}} \qquad \textbf{(5)}$$

The resulting position weight matrix $\varepsilon_i^{(n)}$ consists of 4 rows for the 4 nucleotides, and 14 columns for the 14 variable positions within the template. We then applied these two position weight matrices to calculate scores for all other template sequences. First, we generated a sequence matrix $S_i^{(n)}$, where n is the position index, and i is the nucleotide index, as before. If position n contains the nucleotide with index i, then $S_i^{(n)}=1$, otherwise $S_i^{(n)}=0$.

The P90 and Diff scores of sequence s are then calculated according to:

$$E(s) = \sum_{n=1}^{14} \sum_{i=0}^{3} \varepsilon_i^{(n)} S_i^{(n)} \qquad \textbf{(6)}$$

SUPPLEMENTARY RESULTS:

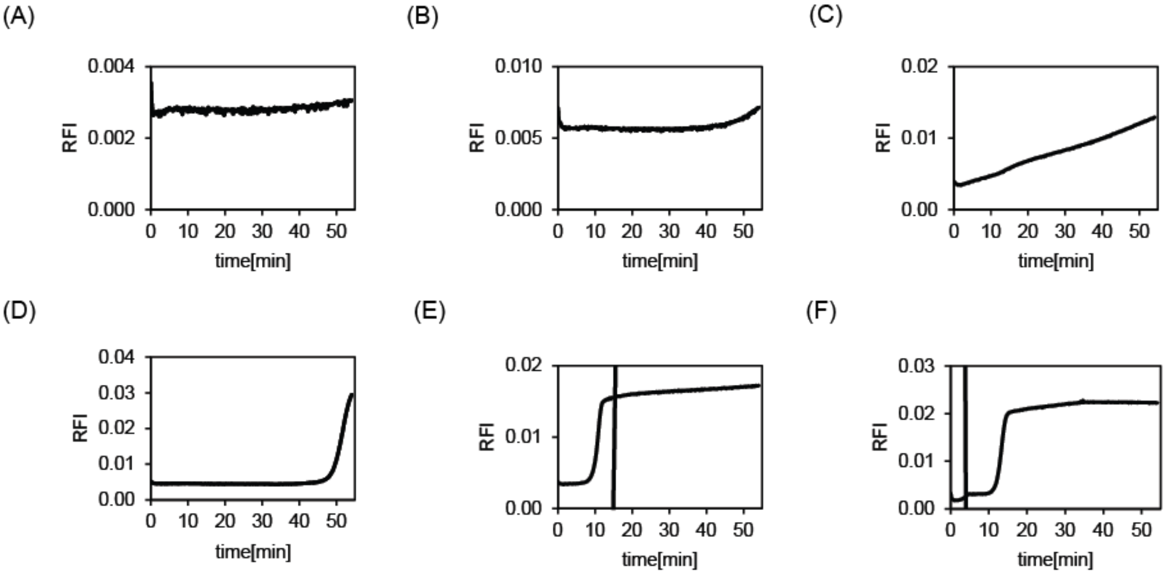

Figure S1: Examples of non-conventional curves: (A) No amplification (B) Late amplification (C) Non sigmoidal (D) Fit with padding (E) Fit with cutting end (E) Fit with cutting start

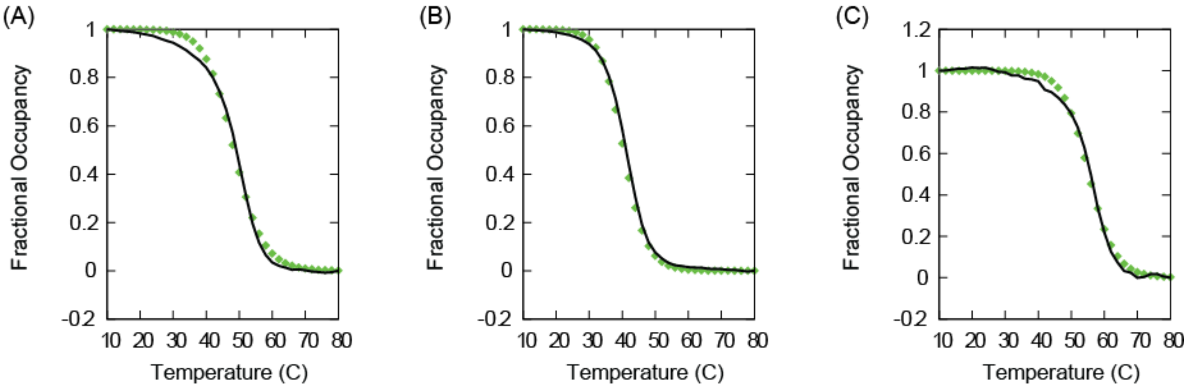

Figure S2: Demonstration of reasonable agreement between calculated and experimentally determined trigger-template T_M values and melting curves for three selected EXPAR template / trigger pairs: (A) Seq ID # 1, (B) Seq ID # 35, and (C) Seq ID # 21. Experimental melting curves (dotted green line) were determined on a Bio-Rad Opticon I thermocycler in EXPAR buffer, using 50 nM 5' FAM labeled trigger and 500 nM 3' BHQ labeled template. Calculated melting curves (solid black line) and T_M values are based on ΔH and ΔS values predicted via the Zuker-Turner algorithm(55), through the UNAFold application (55).

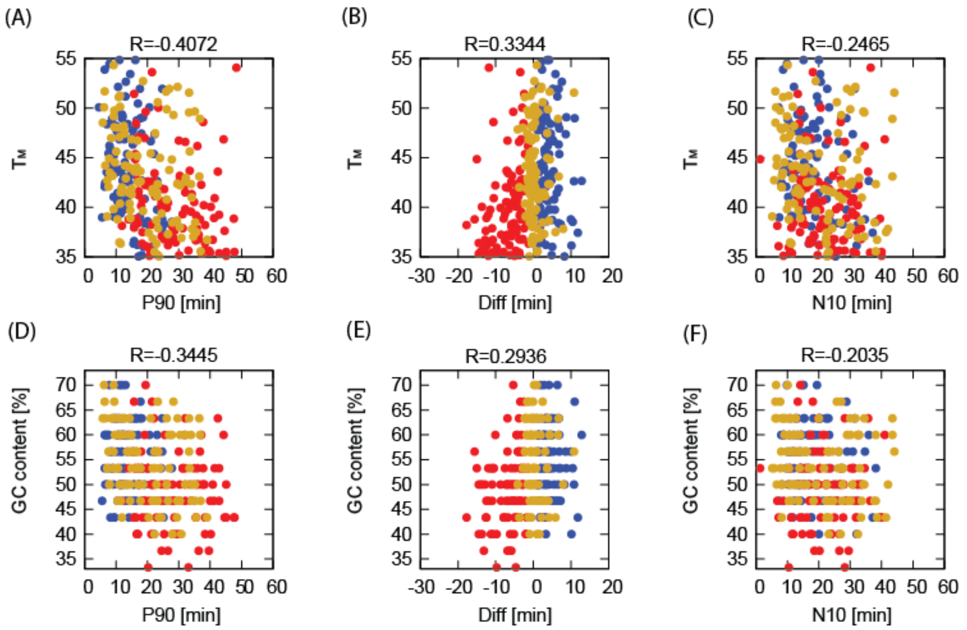

Figure S3. Plots and Spearman correlation coefficients R for (A)-(C) the trigger-template melting temperature versus P90, Diff, and N10, and (D)-(F) the template GC content versus P90, Diff, and N10. Blue: well performing class I templates; red: poorly performing class II templates; orange: class III templates with intermediate performance.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	Diff
1	CCTACGACTGAACAGACTCTCCTACGACTG	43	15	6	CI	12.6	16.6	4.0
2	CCTACGACTTAACAGACTCTCCTACGACTT	39	-25	2	CI	16.3	21.4	5.1
3	CCTACGACGGAACAGACTCTCCTACGACGG	48	-25	2	CI	15.4	16.5	1.2
4	CCTACGAGTGAACAGACTCTCCTACGAGTG	43	-25	2	CIII	17.8	17.8	0.1
5	CCTACGGCTGAACAGACTCTCCTACGGCTG	48	2	6	CIII	12.5	12.0	-0.5
6	CCTACTACTGAACAGACTCTCCTACTACTG	36	15	6	CI	18.8	23.8	5.0
7	CCTAGGACTGAACAGACTCTCCTAGGACTG	41	45	20	NR,CE	0.0	0.0	0.0
8	CCTGCGACTGAACAGACTCTCCTGCGACTG	49	15	6	CI	11.9	13.2	1.3
9	CCAACGACTGAACAGACTCTCCAACGACTG	45	15	6	CIII	24.0	27.7	3.7
10	CGTACGACTGAACAGACTCTCGTACGACTG	44	49	20	NR,LA,NA	0.0	0.0	0.0
11	GCTACGACTGAACAGACTCTGCTACGACTG	44	15	6	NR,CE	0.0	0.0	0.0
12	CCTGTGCATCCCTAGACTCTCCTGTGCATC	42	-12	4	CIII	7.6	8.0	0.5
13	GGGGAAATAGGTGAGACTCTGGGGAAATAG	36	9	6	CI	15.5	16.8	1.3
14	GCTACCTTCAACTCGACTCTGCTACCTTCA	40	-41	2	CIII	18.7	19.1	0.4
15	TGGCGTGAAAAACGGACTCTTGGCGTGAAA	44	-27	3	CI	11.4	14.0	2.6
16	GGAGGAGATGCACAGACTCTGGAGGAGATG	40	14	8	CII	18.0	13.8	-4.1
17	GCACATGGATGTTTGACTCTGCACATGGAT	41	-2	8	CIII	23.0	24.6	1.6
18	CTTGTGCTGGTGGCGACTCTCTTGTGCTGG	44	-34	2	CI	7.6	9.0	1.4
19	GACCCGTCTACGGCGACTCTGACCCGTCTA	45	23	4	CIII	21.0	20.0	-1.0
20	ATCCACCCCTGACGGACTCTATCCACCCCT	45	-35	3	CIII	14.4	14.2	-0.3
21	CCCCCGCACTGGTGGACTCTCCCCCGCACT	54	15	8	SD	0.0	0.0	0.0
22	AGTATGCGAAGAGCGACTCTAGTATGCGAA	40	4	8	CII	33.9	30.6	-3.3
23	TTGGACACCAGTTCGACTCTTTGGACACCA	41	-40	3	CI	9.2	10.3	1.0
24	CAATGAGGTCTAGCGACTCTCAATGAGGTC	35	19	8	CII,LA	37.4	22.9	-14.5
25	TCATGGTGTCGGAAGACTCTTCATGGTGTC	36	27	10	CIII	11.1	10.8	-0.3
26	ACATCGTCTGCGGCGACTCTACATCGTCTG	41	19	4	CIII	23.1	26.3	3.2
27	AGCGTGCAGCGTGGGACTCTAGCGTGCAGC	53	1	6	SD	0.0	0.0	0.0
28	CTCCAGGACCCTGCGACTCTCTCCAGGACC	45	21	8	SD	0.0	0.0	0.0
29	CTTGCGACCTCCGGGACTCTCTTGCGACCT	45	5	4	CI	9.2	12.5	3.4
30	CCGTGGCAACCGACGACTCTCCGTGGCAAC	49	-26	3	CIII	28.3	27.2	-1.1
31	ACGTGTGGGCGGCGGACTCTACGTGTGGGC	51	15	4	CI	13.0	19.4	6.4
32	TAGCACCATGTAAAGACTCTTAGCACCATG	42	-2	8	CIII	11.9	8.2	-3.7
33	AGTGGGTAATTCGCGACTCTAGTGGGTAAT	35	16	4	CII	20.7	8.7	-12.0
34	CGTTTGGCACTTGTGACTCTCGTTTGGCAC	46	12	6	NA,LA,NS	0.0	0.0	0.0
35	GATGGTCATTCCGAGACTCTGATGGTCATT	35	-81	8	CII	28.5	24.9	-3.7
36	GACGCAGCCCCGTGGACTCTGACGCAGCCC	53	-14	6	CI	13.1	20.7	7.6
37	GCCTAGGCCGGGCTGACTCTGCCTAGGCCG	51	54	22	NA	0.0	0.0	0.0
38	TCTTGCCCGTCGCCGACTCTTCTTGCCCGT	49	24	8	CI	12.0	15.4	3.4
39	GGTGGCAGCGATAAGACTCTGGTGGCAGCG	55	12	8	CIII	14.4	12.5	-1.9

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
40	CTGGGCTATTGTTAGACTCTCTGGGCTATT	39	-71	2	CI	5.5	7.4	1.9
41	CGTCTAAAGCGGAAGACTCTCGTCTAAAGC	39	-19	4	SD,LA	0.0	0.0	0.0
42	GCCGAGGCTGGATTGACTCTGCCGAGGCTG	52	20	6	NA	0.0	0.0	0.0
43	TACCCTAGGCGCCAGACTCTTACCCTAGGC	42	39	6	CII	31.9	21.8	-10.1
44	GGGTGCGGTGCACGGACTCTGGGTGCGGTG	53	34	12	CIII	18.7	19.0	0.3
45	ATGACTCAACCGATGACTCTATGACTCAAC	37	-40	2	NA	0.0	0.0	0.0
46	AGCGTACCCAACTAGACTCTAGCGTACCCA	47	-3	4	CIII	22.5	26.1	3.6
47	GGGGAGCACTGTGAGACTCTGGGGAGCACT	49	20	6	CIII	5.7	6.1	0.4
48	CCCCCAATAGGTGAGACTCTCCCCCAATAG	40	25	10	CIII	14.3	12.1	-2.2
49	GGGGAAATAGGGTGGACTCTGGGGAAATAG	36	-42	5	CII	18.3	16.9	-1.3
50	GGGGAGCACTGGTGGACTCTGGGGAGCACT	49	15	8	NR,CE	0.0	0.0	0.0
51	CCCCCAATAGGGTGGACTCTCCCCCAATAG	40	8	8	CIII	11.3	10.4	-0.9
52	CCCCCGCACTGTGAGACTCTCCCCCGCACT	54	20	6	CI	7.9	9.8	1.9
53	GGGGAAGGTCGTGAGACTCTGGGGAAGGTC	44	9	6	CIII	12.8	12.7	-0.1
54	CAATGAATAGGTGAGACTCTCAATGAATAG	29	16	10	NA	0.0	0.0	0.0
55	GGGGAAATAGTAGCGACTCTGGGGAAATAG	36	11	8	CIII	22.8	22.5	-0.4
56	GGGGAAGGTCTAGCGACTCTGGGGAAGGTC	44	-34	2	CIII	6.6	6.5	-0.1
57	GCACAAATAGGTGAGACTCTGCACAAATAG	35	-15	3	CII,LA	47.8	39.9	-7.9
58	GCACATGGATGTGAGACTCTGCACATGGAT	41	34	10	CII	32.0	26.9	-5.1
59	GGGGAGCTGGGTGAGACTCTGGGGAGCTGG	50	-19	4	CIII	6.1	6.3	0.2
60	GGGGAGCTGGTGGCGACTCTGGGGAGCTGG	50	-19	4	CI	4.6	7.2	2.7
61	CTTGTAATAGTGGCGACTCTCTTGTAATAG	28	13	8	NA	0.0	0.0	0.0
62	CCTACGACTGAACAGACTCCCCTACGACTG	43	15	6	SD	0.0	0.0	0.0
63	CCTACGACTGGGAAGACTCTCCTACGACTG	41	-25	2	CII	22.5	18.5	-4.0
64	CCTACGACTGAACAGACTCTCCTACGACTGCTC	45	15	6	CII,NS	16.2	1.2	-15.0
65	CCCACGGATTAGCCGACTCTCCCACGGATT	46	-4	3	NR,CE	0.0	0.0	0.0
66	GCTCCTCGACGATGGACTCTGCTCCTCGAC	47	-3	4	CII	32.1	28.3	-3.8
67	GTCAACGTCGGCGCGACTCTGTCAACGTCG	45	18	4	LA,NS	0.0	0.0	0.0
68	GAATGTCGGTTTAAGACTCTGAATGTCGGT	38	-116	2	CI	15.7	17.5	1.8
69	CTGTCCGGCGAGACGACTCTCTGTCCGGCG	54	17	4	SD	0.0	0.0	0.0
70	GCAGCCTTCCTGACGACTCTGCAGCCTTCC	48	17	6	CIII	9.4	9.4	0.0
71	AGTCTCTCTGTATGGACTCTAGTCTCTCTG	38	-15	4	CIII	30.3	30.1	-0.2
72	ACTAAGCTTATTGCGACTCTACTAAGCTTA	35	17	6	NA,NS	0.0	0.0	0.0
73	TTCCTTGGGACATTGACTCTTTCCTTGGGA	41	-46	2	CII	24.5	17.3	-7.2
74	CCCCCCCAAGAATGGACTCTCCCCCCCAAG	52	-37	3	CIII	6.4	6.2	-0.1
75	GACATAGGTGTGGAGACTCTGACATAGGTG	37	-28	6	CII,LA	25.3	22.3	-3.0
76	TGGGGGCCAAACGCGACTCTTGGGGGCCAA	51	22	4	NA,NS	0.0	0.0	0.0
77	TGTCGCACCTTGAGGACTCTTGTCGCACCT	48	15	8	CI	10.0	11.5	1.5
78	CTTCTGTGCCGGGCGACTCTCTTCTGTGCC	43	20	8	CIII	8.9	8.3	-0.6

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

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ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
79	TATCGAGAGGCCCCGACTCTTATCGAGAGG	39	1	4	CII	36.8	21.2	-15.6
80	CTGACCAGTTTGATGACTCTCTGACCAGTT	39	-162	3	NR,CE	0.0	0.0	0.0
81	AGCCTATCTGTACCGACTCTAGCCTATCTG	39	-40	2	CIII	14.5	14.1	-0.4
82	CCATCATTTGTCTGGACTCTCCATCATTTG	35	14	8	CIII	23.6	22.6	-1.1
83	GCGATGCGAGGCGGGACTCTGCGATGCGAG	50	-34	2	CII	19.4	14.1	-5.3
84	CTGAAGCCATAGTAGACTCTCTGAAGCCAT	41	-68	3	SD	0.0	0.0	0.0
85	CACAATAGGAAATTGACTCTCACAATAGGA	36	-19	4	CII,NA	39.6	26.6	-13.1
86	TGCTTGGGTTGTTCGACTCTTGCTTGGGTT	42	-41	2	CI	6.5	8.1	1.7
87	ATGACATACGAACCGACTCTATGACATACG	38	-25	2	CII,NA,LA	44.6	32.0	-12.7
88	GACACCTATGTTGTGACTCTGACACCTATG	37	-18	3	CII	28.1	24.5	-3.6
89	GGCATTTATGCGGGGACTCTGGCATTTATG	35	17	8	CII	21.9	10.9	-11.0
90	AGCGTCACGACATCGACTCTAGCGTCACGA	50	7	6	CII	23.5	20.6	-2.9
91	TTCAAACGGAAACCGACTCTTTCAAACGGA	39	-1	3	CII,LA	47.5	39.5	-7.9
92	GGAAGACTCTTAGCGACTCTGGAAGACTCT	37	-34	2	CII	40.8	28.0	-12.8
93	CCACGACTCCGGTTGACTCTCCACGACTCC	46	5	4	CI	15.4	22.2	6.8
94	GCAGCACTACCGGGGACTCTGCAGCACTAC	44	17	6	CII,LA	42.5	35.7	-6.8
95	CTGACCTGTTAGGCGACTCTCTGACCTGTT	39	-10	6	CI	11.4	14.4	2.9
96	CCCATTCCACGCATGACTCTCCCATTCCAC	42	-3	4	NA,LA	0.0	0.0	0.0
97	TGCGAGTGGAGCGGGACTCTTGCGAGTGGA	47	-15	4	CIII	12.6	11.0	-1.6
98	CCTCTAAGGTATCAGACTCTCCTCTAAGGT	37	-62	3	NR,CE	0.0	0.0	0.0
99	CGCGTATTGATCGTGACTCTCGCGTATTGA	44	11	4	CII	33.4	26.3	-7.1
100	TGGGCACTCCAGGGGACTCTTGGGCACTCC	50	-8	8	CIII	9.4	7.7	-1.6
101	TAGTGGTGTATAGTGACTCTTAGTGGTGTA	38	-18	6	CII	17.0	11.9	-5.1
102	TGATACCGAAGGTGGACTCTTGATACCGAA	35	-40	2	CII,LA	40.0	36.3	-3.7
103	AAGTCTCCGTTTCAGACTCTAAGTCTCCGT	41	-43	5	CIII	34.0	33.7	-0.3
104	CCCGCCTTCCCCGAGACTCTCCCGCCTTCC	52	-40	2	CIII	25.9	30.3	4.4
105	CGCAATCTCTTCATGACTCTCGCAATCTCT	41	-7	4	CI	12.9	14.4	1.6
106	TCTTGTGGAGAAATGACTCTTCTTGTGGAG	42	-18	3	CII	22.8	16.1	-6.7
107	TAGTGCATTTTACAGACTCTTAGTGCATTT	36	-27	6	NA,LA	0.0	0.0	0.0
108	CTATTCTGCCAATTGACTCTCTATTCTGCC	38	15	6	CII	16.4	14.7	-1.7
109	TCGCTTCATAGCTGGACTCTTCGCTTCATA	40	-3	4	CII	32.0	25.6	-6.5
110	CCTGGATGAAGCCTGACTCTCCTGGATGAA	39	-4	4	CII	31.2	23.5	-7.6
111	ATTCCGGCATAAGGGACTCTATTCCGGCAT	44	-4	4	NA,LA	0.0	0.0	0.0
112	AGTTGTTCCTGATGGACTCTAGTTGTTCCT	38	-88	3	CII	18.4	14.0	-4.4
113	CTACAAGCAAAGCTGACTCTCTACAAGCAA	37	-26	4	CIII,LA	38.9	38.5	-0.5
114	GGCTGCAAAGTACTGACTCTGGCTGCAAAG	45	2	6	CIII	16.8	16.7	-0.1
115	CCTAGTGGGCTTATGACTCTCCTAGTGGGC	45	-64	3	NR,CE	0.0	0.0	0.0
116	AAGCCCTCACAAGAGACTCTAAGCCCTCAC	46	17	8	CII	36.0	34.6	-1.4
117	GCGATAACACAACCGACTCTGCGATAACAC	41	-34	2	CI	19.1	22.5	3.4

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

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ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
118	CGGTCTAATGGTGAGACTCTCGGTCTAATG	38	-15	4	CII	35.8	33.5	-2.4
119	GGACCGTACTCCACGACTCTGGACCGTACT	42	-34	3	CII,LA	37.1	32.6	-4.5
120	AACGATGGACGACCGACTCTAACGATGGAC	38	-25	2	CI	20.3	27.5	7.2
121	CAGCGGGTTGCCCTGACTCTCAGCGGGTTG	49	-29	4	NR,CE	0.0	0.0	0.0
122	ATACAGGTGCTTTTGACTCTATACAGGTGC	39	-30	2	CI	9.4	11.2	1.8
123	CGTAACGGTCGGTGGACTCTCGTAACGGTC	42	6	6	CIII,LA	30.5	30.1	-0.4
124	CGGTAAGAACCCTCGACTCTCGGTAAGAAC	38	-41	2	NA,LA	0.0	0.0	0.0
125	TGCTATGAGACGCGGACTCTTGCTATGAGA	36	18	4	CII	38.2	24.7	-13.5
126	GAAGCTGAACTACGGACTCTGAAGCTGAAC	37	-26	4	CII	36.2	33.4	-2.8
127	CCAGCGCAGCCTGGGACTCTCCAGCGCAGC	54	18	4	SD	0.0	0.0	0.0
128	CGGAGACACCACTCGACTCTCGGAGACACC	47	14	6	CIII	8.4	8.9	0.6
129	GGCCCACTGTAATAGACTCTGGCCCACTGT	50	3	4	CI	10.6	14.3	3.7
130	CGTTTGATGCCTGGGACTCTCGTTTGATGC	42	-4	6	CII	17.1	14.6	-2.6
131	CCTAGTCACCTATGGACTCTCCTAGTCACC	40	-12	4	NR,CE	0.0	0.0	0.0
132	ACACTACGGAGTCAGACTCTACACTACGGA	43	-2	8	NA,LA	0.0	0.0	0.0
133	CTGTGCGGTACTTAGACTCTCTGTGCGGTA	48	-8	4	CIII	10.6	7.8	-2.8
134	TGGTTTCGGCTGTCGACTCTTGGTTTCGGC	47	24	6	CIII	16.7	16.2	-0.5
135	TAGCATAGCGGTTCGACTCTTAGCATAGCG	43	-41	2	CIII	32.0	30.6	-1.3
136	TTTGGAGCTACCCGGACTCTTTTGGAGCTA	38	8	4	CII	21.4	15.5	-5.8
137	TCTGGATCAAGACCGACTCTTCTGGATCAA	35	6	8	CII	42.8	31.8	-11.0
138	CCGAGCCGACCGCCGACTCTCCGAGCCGAC	52	-28	4	NA	0.0	0.0	0.0
139	TGTCTGGTGTTATCGACTCTTGTCTGGTGT	41	-25	4	CI	10.4	12.2	1.8
140	TAACAGGGAGGTCAGACTCTTAACAGGGAG	40	6	6	CII	26.1	17.4	-8.7
141	TCGCCCCAACGGTAGACTCTTCGCCCCAAC	50	-43	4	CIII	26.2	30.0	3.8
142	TCTCTGAACGACGGGACTCTTCTCTGAACG	43	-25	2	CII	20.2	14.3	-5.8
143	CAGTGCCGATCCCAGACTCTCAGTGCCGAT	46	-110	3	LA	0.0	0.0	0.0
144	GTCTACTTCCAGATGACTCTGTCTACTTCC	37	-24	3	CIII	21.6	22.2	0.6
145	GTGACGCCCGAAATGACTCTGTGACGCCCG	55	-40	2	CI	16.2	20.0	3.7
146	CCATTACGAGCGACGACTCTCCATTACGAG	38	1	4	CII	31.2	20.3	-10.9
147	ACACGCGGACTTTAGACTCTACACGCGGAC	52	18	4	CIII,NA	33.2	44.1	10.8
148	CCGGCTTACGCCTCGACTCTCCGGCTTACG	48	-4	4	NR,CE	0.0	0.0	0.0
149	TTCGGACCTGTGGGGACTCTTTCGGACCTG	44	-37	2	CI	7.0	8.6	1.7
150	ACTTTACCTGGTTTGACTCTACTTTACCTG	36	12	6	CIII	22.3	23.2	0.9
151	CTGTAACTCGGGAAGACTCTCTGTAACTCG	38	-23	6	SD	0.0	0.0	0.0
152	CTGCACCTGGATATGACTCTCTGCACCTGG	49	4	4	CI	8.7	10.1	1.4
153	CAGGAACTTGATTGGACTCTCAGGAACTTG	40	-41	4	CIII	14.5	13.5	-1.0
154	CCTGCCTCACTTAAGACTCTCCTGCCTCAC	47	17	6	CI	21.0	26.4	5.3
155	GATCTCCACCACCCGACTCTGATCTCCACC	40	-40	2	CI	12.4	17.0	4.6
156	CCATGACAGGGGACGACTCTCCATGACAGG	42	-7	4	CIII	14.9	13.2	-1.7

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
157	GGCAACGCCCGCAGGACTCTGGCAACGCCC	54	-12	3	CII,NA	48.4	36.6	-11.8
158	GCGACAAATGGGCGGACTCTGCGACAAATG	42	-34	2	CIII	22.2	22.9	0.8
159	ACTCACTTACATCGGACTCTACTCACTTAC	36	-39	2	SD,LA	0.0	0.0	0.0
160	TACCCCGCGAAATCGACTCTTACCCCGCGA	54	15	4	CI	6.3	8.6	2.3
161	GTACCTCACCGCGAGACTCTGTACCTCACC	41	15	4	CI	10.4	14.2	3.8
162	CCTCCACTCTGTATGACTCTCCTCCACTCT	42	-68	2	CI	6.4	9.7	3.3
163	TCGCAGGATAATTGGACTCTTCGCAGGATA	44	-18	4	CIII	17.8	18.3	0.5
164	ACTCGGGGTAAAATGACTCTACTCGGGGTA	48	-13	8	CIII	12.4	8.3	-4.1
165	GCAGGCTCCTCCCGGACTCTGCAGGCTCCT	49	8	4	CI	11.9	14.7	2.8
166	GGTTAGGTCGATCAGACTCTGGTTAGGTCG	43	3	8	NR,CE	0.0	0.0	0.0
167	ATGAAGTGCCGCCGGACTCTATGAAGTGCC	42	17	6	CI	11.8	12.8	1.1
168	TACCACTCTTGTGGGACTCTTACCACTCTT	38	24	8	CI	19.0	25.9	6.8
169	ATACTCGCCTCGGGGACTCTATACTCGCCT	40	14	8	CI	11.6	15.8	4.1
170	TTCGTGTCTTAGGGGACTCTTTCGTGTCTT	36	-19	3	CIII	11.5	11.3	-0.2
171	ACCCTGAGACAGGTGACTCTACCCTGAGAC	44	1	4	CII	15.5	11.9	-3.6
172	AGGCCAATAAGCCCGACTCTAGGCCAATAA	37	11	4	CIII	34.6	34.5	-0.1
173	CCAAATTGGGTCCCGACTCTCCAAATTGGG	39	21	8	CII,LA	42.9	30.8	-12.0
174	TAGATTGCATGTATGACTCTTAGATTGCAT	36	-40	4	CII	20.2	10.5	-9.7
175	GGACCCGCAACCTGGACTCTGGACCCGCAA	51	4	4	CIII	23.3	25.8	2.5
176	GTAATTGCGCCTTCGACTCTGTAATTGCGC	42	10	4	NS	0.0	0.0	0.0
177	AACGAGCCGAGTGGGACTCTAACGAGCCGA	49	-25	2	CIII,NA,LA	36.8	43.4	6.6
178	TGTAAGGCGTATGGGACTCTTGTAAGGCGT	43	-77	4	CIII	8.0	8.6	0.7
179	CGGTATCTGCGCCCGACTCTCGGTATCTGC	43	10	4	CI	18.2	23.0	4.8
180	CAGAATACGACTCAGACTCTCAGAATACGA	36	-25	2	NR,LA	0.0	0.0	0.0
181	GGACAAGGGCATCAGACTCTGGACAAGGGC	48	14	8	CI	12.0	18.9	6.9
182	GCTGACAAAGGTGCGACTCTGCTGACAAAG	40	-37	8	CIII	9.2	9.9	0.7
183	TCGCCCTCAAAGTAGACTCTTCGCCCTCAA	47	-114	3	CI	18.1	22.7	4.6
184	ATGCTACGCTCATTGACTCTATGCTACGCT	43	-44	2	CII	30.3	24.6	-5.7
185	ATGTGAACCAAGCAGACTCTATGTGAACCA	39	-29	2	CII	35.5	31.9	-3.6
186	AGCGGCTCCCGGTTGACTCTAGCGGCTCCC	54	13	6	CIII	9.2	10.1	0.9
187	GGGTTTTACCGCTAGACTCTGGGTTTTACC	38	-8	4	CII	27.9	22.0	-5.9
188	CTCAACGATATCCAGACTCTCTCAACGATA	38	-25	2	CII	15.7	11.2	-4.5
189	CCATCAGAACGGTAGACTCTCCATCAGAAC	38	-43	4	CIII,NA,LA	34.2	42.1	7.9
190	GCATCCTCCTTTGCGACTCTGCATCCTCCT	43	-34	2	NR,CE	0.0	0.0	0.0
191	GGGAAACTGCATGGGACTCTGGGAAACTGC	44	-43	4	NR,CE	0.0	0.0	0.0
192	TTACACGGTTAGTTGACTCTTTACACGGTT	36	-11	8	CI	16.8	20.0	3.3
193	CGCTACAGCTATGTGACTCTCGCTACAGCT	46	-34	4	NS	0.0	0.0	0.0
194	CCTTGCTGTGACCCGACTCTCCTTGCTGTG	45	-40	2	CI	7.6	10.7	3.1
195	GCTTATACGACTAGGACTCTGCTTATACGA	37	-25	2	CII,NA	28.6	25.0	-3.7

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
196	GTGCAGTCAGATTAGACTCTGTGCAGTCAG	45	14	8	CIII	7.8	6.6	-1.2
197	CCACGATCACGTGCGACTCTCCACGATCAC	43	19	6	CIII,LA	32.3	32.8	0.5
198	TCGCCTCTCAACAGGACTCTTCGCCTCTCA	46	-63	3	CI	7.9	11.7	3.8
199	CGCTCGGTGACTCCGACTCTCGCTCGGTGA	51	-13	3	CII	15.8	13.3	-2.5
200	CGCTGAGAACTTGTGACTCTCGCTGAGAAC	44	19	8	CIII	27.0	27.3	0.3
201	GGACGGCTGGATTTGACTCTGGACGGCTGG	53	-13	7	CI	14.5	20.4	5.9
202	ATTTCACACGCTTGGACTCTATTTCACACG	39	-44	2	SD,NA	0.0	0.0	0.0
203	GTCGCTTACCGCCCGACTCTGTCGCTTACC	43	21	8	CIII,NA,LA	36.6	43.4	6.7
204	TCTGAAGGGCCCGTGACTCTTCTGAAGGGC	44	25	6	NS	0.0	0.0	0.0
205	GATCATCGCACCAGGACTCTGATCATCGCA	42	-10	4	CIII	21.7	21.7	0.0
206	TCGGTGTCGTCCGCGACTCTTCGGTGTCGT	49	15	4	CI,NA,LA	16.6	20.2	3.7
207	GAGATGACGTCTACGACTCTGAGATGACGT	36	24	6	NS	0.0	0.0	0.0
208	TCCTCCTGAATACAGACTCTTCCTCCTGAA	39	7	8	CII,LA	41.2	28.7	-12.5
209	GGAGGGTGATAATCGACTCTGGAGGGTGAT	43	14	8	CIII	7.2	5.8	-1.4
210	ATCCAACTTAACAAGACTCTATCCAACTTA	36	-44	3	CII,LA	33.0	28.3	-4.7
211	GTCACTCCGCACAAGACTCTGTCACTCCGC	48	-116	2	CI	13.7	18.2	4.5
212	CGCCCGACCTTAGCGACTCTCGCCCGACCT	55	22	8	CI	11.0	15.1	4.1
213	TGTTCTCTTCATACGACTCTTGTTCTCTTC	36	-25	2	CI	22.0	32.1	10.1
214	AAGAGCTGGTTGTTGACTCTAAGAGCTGGT	41	14	8	SD	0.0	0.0	0.0
215	CTGCCAGCATCCCCGACTCTCTGCCAGCAT	46	-40	2	NR,NA,LA	0.0	0.0	0.0
216	TCGTTTTGAGATCAGACTCTTCGTTTTGAG	40	-4	4	CII	40.2	26.6	-13.6
217	ACTTACCGTTAGGGGACTCTACTTACCGTT	38	-4	8	CI	25.3	32.5	7.3
218	AGGTCGGGCAAACTGACTCTAGGTCGGGCA	52	-5	5	CI	10.6	12.1	1.5
219	CCGAATACAATGAGGACTCTCCGAATACAA	37	-40	2	LA	0.0	0.0	0.0
220	ACGAGTGAATGCCTGACTCTACGAGTGAAT	38	-25	2	CIII,NA,LA	35.2	37.7	2.5
221	TGCATAGGGGTTTGGACTCTTGCATAGGGG	43	-40	4	CII	16.9	14.0	-2.8
222	CCGCCAAGGACTACGACTCTCCGCCAAGGA	51	-25	2	CIII	10.9	8.6	-2.3
223	AGGGGCCGACTCATGACTCTAGGGGCCGAC	54	-8	4	CII	21.5	18.1	-3.4
224	CGTTTTCCCCTTTTGACTCTCGTTTTCCCC	44	-38	2	CI	11.6	13.5	1.9
225	CGTTAGGAGCCGGGGACTCTCGTTAGGAGC	43	8	4	CII	22.0	19.8	-2.1
226	GTCCTACATAGGGGGACTCTGTCCTACATA	36	22	8	CII	45.0	31.0	-14.0
227	TTGACCGTGTCTTCGACTCTTTGACCGTGT	43	-41	2	CI	12.5	16.7	4.2
228	CTGATACGGCGGAGGACTCTCTGATACGGC	43	19	4	CI	12.0	14.5	2.6
229	CCGCTATTCTGCCAGACTCTCCGCTATTCT	40	-5	6	CI	12.1	14.0	1.9
230	TGGGTGATCAATATGACTCTTGGGTGATCA	40	-8	6	NS	0.0	0.0	0.0
231	ATCGTTCTCATACGGACTCTATCGTTCTCA	39	-25	3	CIII	29.4	33.5	4.1
232	AGCCCCATGTAAAAGACTCTAGCCCCATGT	48	-116	2	CI	11.6	14.7	3.1
233	TGTGAGAAGGGCAGGACTCTTGTGAGAAGG	39	-17	3	CII	14.0	10.7	-3.3
234	ATACGCTAGAAGTGGACTCTATACGCTAGA	38	-8	4	CII	38.4	20.6	-17.8

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

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ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
235	GCCTTGTGACTTCGGACTCTGCCTTGTGAC	44	-43	2	CII,NA	15.5	12.0	-3.5
236	GGGGGAGCCACGATGACTCTGGGGGAGCCA	53	-25	2	NR,SD,NA	0.0	0.0	0.0
237	CCTGCTTCCCAGTAGACTCTCCTGCTTCCC	47	-118	3	CI	11.0	14.3	3.3
238	GACATACAGACAATGACTCTGACATACAGA	35	-18	3	CII	21.2	11.5	-9.7
239	AACTCGCCTGCCTAGACTCTAACTCGCCTG	45	-12	4	CI	13.6	17.7	4.2
240	AGTCCTGCGAGATCGACTCTAGTCCTGCGA	48	14	6	CII	16.3	13.7	-2.6
241	CAAGCACGCCGTTTGACTCTCAAGCACGCC	50	-35	6	CI	20.6	23.3	2.7
242	CGGGTTGATGGCGCGACTCTCGGGTTGATG	44	18	4	CI	9.8	10.9	1.0
243	TCGCCTTTATCGACGACTCTTCGCCTTTAT	39	4	6	NA	0.0	0.0	0.0
244	ACCATCCAGCTACAGACTCTACCATCCAGC	45	-34	4	CII,NA	16.2	14.0	-2.2
245	GACTGCGAAAGTACGACTCTGACTGCGAAA	43	-25	2	CI,LA	21.4	32.2	10.8
246	GCGGGCTATCGCATGACTCTGCGGGCTATC	47	24	8	CIII,NA	11.0	10.5	-0.5
247	CGTACCAAGAACCCGACTCTCGTACCAAGA	41	-40	2	CII	41.5	31.1	-10.5
248	GTTTCGCCACCCTAGACTCTGTTTCGCCAC	45	-12	4	CI	22.1	29.0	6.9
249	TCAGCTTGATTTACGACTCTTCAGCTTGAT	38	-64	4	CIII,LA	28.0	27.0	-1.0
250	TAGCTTGTCGTAGAGACTCTTAGCTTGTCG	42	19	8	CIII	29.5	29.7	0.1
251	CGTTAGCCGATCTAGACTCTCGTTAGCCGA	47	-4	6	CIII	15.0	13.8	-1.2
252	GCCGACAAGACTTTGACTCTGCCGACAAGA	47	-31	6	CII,NS	17.3	15.8	-1.5
253	ATGGGTACGGTTGAGACTCTATGGGTACGG	44	9	6	CI	8.7	10.5	1.8
254	TCTGTCACACTCAGGACTCTTCTGTCACAC	41	-16	3	CIII,LA	26.1	27.5	1.4
255	ATCAGGGATTACACGACTCTATCAGGGATT	36	-25	2	NR,CE	0.0	0.0	0.0
256	ATGTGGGTTTGATGGACTCTATGTGGGTTT	39	-86	4	CI	9.5	11.6	2.2
257	ATGAGTGGGAAGTGGACTCTATGAGTGGGA	43	-12	4	CIII	17.0	16.1	-0.9
258	GCCCAAATAAAGGGGACTCTGCCCAAATAA	36	-11	3	CIII,LA	37.0	38.1	1.1
259	GGCTTATGGCGCTTGACTCTGGCTTATGGC	44	14	4	CIII	16.0	15.2	-0.8
260	GCCGCGATAACGAGGACTCTGCCGCGATAA	47	15	4	CII,LA	44.2	41.0	-3.2
261	CTGCCCTGACCCACGACTCTCTGCCCTGAC	47	-25	2	NA,LA	0.0	0.0	0.0
262	AGCCCGGTATGTAGGACTCTAGCCCGGTAT	47	5	4	CII	30.0	23.1	-6.9
263	AAAGTCAACCAGAAGACTCTAAAGTCAACC	37	-25	4	SD,LA	0.0	0.0	0.0
264	GAATGCTAGAAAATGACTCTGAATGCTAGA	36	-8	4	CII	26.6	20.3	-6.3
265	AGATGGCTTCACAAGACTCTAGATGGCTTC	39	-13	4	CIII	10.4	10.5	0.2
266	TAGACACTAGATGGGACTCTTAGACACTAG	36	-3	4	CII	19.7	7.2	-12.5
267	GGGGCTACTACTGGGACTCTGGGGCTACTA	45	-36	8	CIII,NA	9.7	9.0	-0.7
268	TCATTGGGATTATTGACTCTTCATTGGGAT	37	-307	2	SD,LA	0.0	0.0	0.0
269	AGTTTGGCAGCTTAGACTCTAGTTTGGCAG	42	-64	4	CII	19.3	17.0	-2.2
270	CGATCCGCTGTAGGGACTCTCGATCCGCTG	48	-41	2	CI	16.4	18.3	1.9
271	TCCCAATCGTCGCAGACTCTTCCCAATCGT	43	-120	2	LA	0.0	0.0	0.0
272	AAGCAACGAGTACAGACTCTAAGCAACGAG	41	-25	2	CII	34.0	29.0	-5.0
273	GGATACGTCTGGGTGACTCTGGATACGTCT	39	-18	4	CI	20.1	23.7	3.6

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

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ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
274	ACAGCCGTGCCCAAGACTCTACAGCCGTGC	52	-116	2	CI	17.5	22.7	5.2
275	GTCGACTAATGCCAGACTCTGTCGACTAAT	35	24	6	NA,LA	0.0	0.0	0.0
276	GCTTAGAGCTCGCTGACTCTGCTTAGAGCT	38	13	6	NS	0.0	0.0	0.0
277	TGCATTGGAGCCTTGACTCTTGCATTGGAG	40	-40	4	CII	37.9	32.5	-5.4
278	CGACAACTTCTAATGACTCTCGACAACTTC	37	-41	2	CI	25.2	37.1	11.9
279	CAGCGGCCAAACAAGACTCTCAGCGGCCAA	52	9	4	CIII	27.0	29.0	2.0
280	TCATAGCTCACTGGGACTCTTCATAGCTCA	35	-24	4	CII	23.2	19.6	-3.6
281	CGGAAGGGACAGTTGACTCTCGGAAGGGAC	47	-42	8	CII	19.1	17.4	-1.8
282	CCTCCCAGTAGCTAGACTCTCCTCCCAGTA	44	-5	4	CIII	13.6	11.5	-2.1
283	GCATACAGCATCAGGACTCTGCATACAGCA	42	-43	4	CII	21.1	13.9	-7.3
284	ATACCTACGAATAAGACTCTATACCTACGA	37	-25	2	CII	36.4	29.5	-6.9
285	AGACCTCCCAAGCCGACTCTAGACCTCCCA	45	-13	4	CIII	27.8	32.2	4.4
286	ATCGCCCGATGATTGACTCTATCGCCCGAT	49	12	8	CII	37.7	27.0	-10.6
287	TGCGTTTTGTGCCAGACTCTTGCGTTTTGT	42	-120	2	CIII	23.3	27.3	4.0
288	ACAGAGCTTGCATGGACTCTACAGAGCTTG	41	-45	4	CII	17.2	13.0	-4.2
289	GATCGTGACTTCTAGACTCTGATCGTGACT	37	-13	4	CI	15.8	18.0	2.2
290	ACGGAGCAAATGCAGACTCTACGGAGCAAA	45	16	6	CI	15.7	19.4	3.7
291	TTCGCTTCAGATGGGACTCTTTCGCTTCAG	43	-120	2	CII	22.3	15.6	-6.7
292	TGATGACCCCTATGGACTCTTGATGACCCC	43	-42	2	CI	7.7	12.1	4.4
293	GTTATCCAGCTAGTGACTCTGTTATCCAGC	38	-34	4	CII	11.9	6.7	-5.3
294	CACTGTGCTACAAGGACTCTCACTGTGCTA	43	9	6	CIII	13.1	11.7	-1.5
295	CGATGATAACATCCGACTCTCGATGATAAC	35	-8	4	CII,LA	45.1	40.1	-5.0
296	ATGACTCAGTAAGTGACTCTATGACTCAGT	36	-58	6	CII	38.4	23.9	-14.5
297	TTGGGAGCCCGTTAGACTCTTTGGGAGCCC	48	5	8	NR,CE	0.0	0.0	0.0
298	ACCACATAACATTCGACTCTACCACATAAC	37	-41	2	CIII	30.9	32.4	1.6
300	ATGCTCCAATACGGGACTCTATGCTCCAAT	39	-42	2	CIII	22.9	22.8	-0.1
299	CATACGGACTGAGTGACTCTCATACGGACT	39	22	8	NR,LA	0.0	0.0	0.0
301	TAGAACGGTGTTTGGACTCTTAGAACGGTG	41	-38	4	CIII	21.6	24.4	2.8
302	GCTTATGTCTGAGCGACTCTGCTTATGTCT	35	-65	4	CI	18.2	23.0	4.9
303	TCCTGCTCGTAATTGACTCTTCCTGCTCGT	48	-18	4	CI	7.1	13.7	6.6
304	TGGGGGTCGAGACAGACTCTTGGGGGTCGA	50	-41	2	NR,NS	0.0	0.0	0.0
305	CGATCGTCCGACGTGACTCTCGATCGTCCG	50	22	6	CIII	33.3	34.5	1.2
306	CCGCAGCATTTACAGACTCTCCGCAGCATT	49	-7	4	CI	18.5	27.2	8.8
307	GAATCCGCTTTGCCGACTCTGAATCCGCTT	42	-40	2	CI,NA	24.4	31.2	6.8
308	ACGTGCTGCCCAACGACTCTACGTGCTGCC	52	0	4	CI	24.7	31.4	6.8
309	AGACAGACAACGAAGACTCTAGACAGACAA	38	-25	2	NA,LA	0.0	0.0	0.0
310	AAGATCATGCAATAGACTCTAAGATCATGC	37	-20	4	CII	24.5	18.7	-5.8
311	AGTCCGTTAAATCTGACTCTAGTCCGTTAA	37	-18	4	CII	28.0	17.2	-10.8
312	AATCAACGTCTGATGACTCTAATCAACGTC	37	-62	4	NA	0.0	0.0	0.0

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
313	GTAAGGTTCACTATGACTCTGTAAGGTTCA	35	-75	2	CII	30.1	24.0	-6.2
314	TTCCCATGTAGAGCGACTCTTTCCCATGTA	37	-17	4	CIII,NA	26.5	29.4	2.9
315	GACATCTCCTACGTGACTCTGACATCTCCT	35	0	4	CI,NS	17.1	25.4	8.3
316	GACACCCTTGTGCCGACTCTGACACCCTTG	42	-40	2	CIII,LA	33.8	38.2	4.4
317	GACCGAGTAGATCTGACTCTGACCGAGTAG	41	13	6	CII	34.9	30.2	-4.8
318	GATGTTTGGAGCTTGACTCTGATGTTTGGA	38	-45	4	SD	0.0	0.0	0.0
319	ACCTAGCCTCGGTTGACTCTACCTAGCCTC	41	-31	3	CIII	14.9	13.9	-1.0
320	CAGTGTCCCGCGATGACTCTCAGTGTCCCG	47	15	4	CI	11.0	15.3	4.4
321	TCACGAATCCATTAGACTCTTCACGAATCC	43	-25	2	CI	14.2	17.6	3.4
322	CCCTACGCTAGTTAGACTCTCCCTACGCTA	46	-88	3	CIII	10.0	10.4	0.5
323	CCTATCCTGACCGCGACTCTCCTATCCTGA	38	15	4	CIII	23.4	24.3	0.9
324	ACGGTTGGGCCTATGACTCTACGGTTGGGC	51	7	4	CIII	11.2	10.5	-0.7
325	CATGCCTGATCTCGGACTCTCATGCCTGAT	40	-20	4	CII	32.0	20.5	-11.5
326	AGGGGAACCGAACAGACTCTAGGGGAACCG	50	-4	8	CI	17.0	19.9	2.9
327	GATGGACGAACTCTGACTCTGATGGACGAA	41	-25	2	CI	13.4	19.0	5.6
328	TGAACGCCAGTACAGACTCTTGAACGCCAG	46	-4	4	CIII	33.4	33.0	-0.4
329	ACAAAGCCTTTCTGGACTCTACAAAGCCTT	40	-27	8	NR,LA	0.0	0.0	0.0
330	GCTGGTCCCATGCTGACTCTGCTGGTCCCA	49	-20	4	SD,NA,LA	0.0	0.0	0.0
331	AAGAAAACGGTACTGACTCTAAGAAAACGG	36	16	8	NA	0.0	0.0	0.0
332	AGTCTTGCTCGCCGGACTCTAGTCTTGCTC	39	12	4	CI	24.7	34.7	10.0
333	TGGTGCGACTGTGTGACTCTTGGTGCGACT	48	-34	2	CI	8.6	10.7	2.1
334	GATTGCCCCGAGTAGACTCTGATTGCCCCG	50	-16	4	CIII	11.3	11.6	0.4
335	CCATCATGTAATCCGACTCTCCATCATGTA	37	-40	2	CIII	20.4	22.5	2.1
336	AAAGGTTTGCACTGGACTCTAAAGGTTTGC	39	-40	4	CIII	24.5	23.3	-1.2
337	CCGCATCATATCCCGACTCTCCGCATCATA	44	-40	2	CI	10.7	16.1	5.3
338	TAAACCGACTGTCCGACTCTTAAACCGACT	40	8	6	NR,LA	0.0	0.0	0.0
339	GATGACCGCCTCTAGACTCTGATGACCGCC	47	-13	4	CI	12.7	17.8	5.1
340	ACTCTCCTGCTGTCGACTCTACTCTCCTGC	44	24	6	CIII	14.3	14.1	-0.2
341	TGGGATCGGCTATTGACTCTTGGGATCGGC	49	-27	4	CIII	9.3	9.8	0.5
342	CTGGGGTAAACTTGGACTCTCTGGGGTAAA	39	-72	4	CI	17.6	18.9	1.2
343	ACATCGACGCCTCAGACTCTACATCGACGC	47	-25	4	CI	22.2	30.8	8.6
344	TCAGAAGACTAACCGACTCTTCAGAAGACT	37	21	8	CII,LA	35.4	27.8	-7.6
345	AGCAGCGGAGACAGGACTCTAGCAGCGGAG	52	8	7	CIII	29.6	31.7	2.1
346	GGTCGTTTGTTACTGACTCTGGTCGTTTGT	41	-34	4	CI	13.8	19.9	6.2
347	GACCAAGTCGTGGTGACTCTGACCAAGTCG	43	0	9	NR,CE	0.0	0.0	0.0
348	TCGATCAGTAACCGGACTCTTCGATCAGTA	38	16	4	CII,NA,LA	28.6	23.1	-5.5
349	CAAACGCGACGGTAGACTCTCAAACGCGAC	46	22	2	NR,NA,LA	0.0	0.0	0.0
350	AATTCCCCTGGCTGGACTCTAATTCCCCTG	39	-17	4	CI	27.7	38.1	10.4
351	ATATCCGTCAGAACGACTCTATATCCGTCA	38	-49	3	CIII	35.6	41.4	5.8

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.

Table S1: Template Sequences, Thermodynamic Parameters, and Performance Metrics

ID #	Template Sequence	T_M1	T_M2	# Bonds	Results	P90	N10	DIFF
352	TCGGTTGCTTAATAGACTCTTCGGTTGCTT	46	-71	2	CI	8.3	11.3	3.0
353	ACACTTGTACCTTAGACTCTACACTTGTAC	36	-13	4	CII	28.9	17.7	-11.2
354	AAGTTGGCCCGAGAGACTCTAAGTTGGCCC	44	19	8	CIII	16.6	15.9	-0.7
355	TTGCCTGGTAGTTAGACTCTTTGCCTGGTA	43	-21	6	CI	15.0	18.9	4.0
356	CTCACGCTACGGACGACTCTCTCACGCTAC	43	2	4	CI	23.0	35.9	12.9
357	CCTCCTGTGACTCGGACTCTCCTCCTGTGA	44	-47	2	CI	14.2	16.9	2.8
358	TGCGGACCTCCTGTGACTCTTGCGGACCTC	48	-39	2	CI	10.2	13.2	3.0
359	TAGAGCATGTTTCTGACTCTTAGAGCATGT	39	18	8	CII	16.6	14.7	-1.9
360	GTGGTGAAGGGCGTGACTCTGTGGTGAAGG	42	-77	4	CI	6.5	8.0	1.5
361	CGGGAAATGTACCAGACTCTCGGGAAATGT	42	-40	6	CIII	13.9	14.2	0.3
362	TGATTCCCGCACCTGACTCTTGATTCCCGC	47	-31	2	CI	15.1	19.6	4.6
363	GTACGGTAGGTGGGGACTCTGTACGGTAGG	41	7	8	CII	25.6	21.2	-4.3
364	AGCGCAGTTGAATAGACTCTAGCGCAGTTG	50	18	4	CIII,LA	35.1	34.2	-1.0
365	TCTGCGAATGGGGTGACTCTTCTGCGAATG	43	-34	2	CII	19.9	16.5	-3.4
366	GGCACTTAAAAGCGGACTCTGGCACTTAAA	37	-36	4	CII	26.9	18.4	-8.5
367	CTTCTGTCTAACGCGACTCTCTTCTGTCTA	36	22	4	SD	0.0	0.0	0.0
368	CCGCTATGTTGAACGACTCTCCGCTATGTT	41	-25	2	CI	11.1	12.3	1.2
369	GATGATCCGACTAAGACTCTGATGATCCGA	41	-40	2	CII	18.4	13.4	-4.9
370	ACAAATGCGTAAACGACTCTACAAATGCGT	42	16	6	CII	25.4	23.4	-2.0
371	GAACCCCCGTTGCGGACTCTGAACCCCCGT	49	15	6	CI	17.8	28.7	10.9
372	AACGTTCAGTAGTAGACTCTAACGTTCAGT	39	11	6	NA,NS	0.0	0.0	0.0
373	AGAACCAGACCAATGACTCTAGAACCAGAC	40	-13	4	LA	0.0	0.0	0.0
374	CCCCTTCTAGTAACGACTCTCCCCTTCTAG	39	-25	2	CIII	7.2	5.2	-2.0
375	TTGGAACGAGTGCTGACTCTTTGGAACGAG	40	-25	2	CII	24.3	19.7	-4.6
376	ATTGCACAACGGTGGACTCTATTGCACAAC	40	13	6	CI	23.0	28.7	5.7
377	TAACTACGGCGGTTGACTCTTAACTACGGC	40	19	4	CII	26.5	19.8	-6.7
378	AACCGTACAAACAGGACTCTAACCGTACAA	36	-6	4	NR,LA	0.0	0.0	0.0
379	ATTAGCCAGCCGATGACTCTATTAGCCAGC	41	-40	2	CIII	11.5	12.4	0.8
380	GTAGGCGATGATGAGACTCTGTAGGCGATG	44	-34	2	CI	9.0	11.1	2.2
381	TCATGGACATCTGCGACTCTTCATGGACAT	37	-34	2	NR,NA,LA	0.0	0.0	0.0
382	GCGTTCTAACACGCGACTCTGCGTTCTAAC	41	22	4	NA,LA	0.0	0.0	0.0
383	ACCAAAAGGCTAGCGACTCTACCAAAAGGC	43	14	6	CII	30.3	27.0	-3.2
384	GCCCTCACATTTACGACTCTGCCCTCACAT	45	-25	2	CI	12.8	15.4	2.6

T_M1: template-trigger T_M in °C; T_M2: template-template T_M in °C; # bonds: number of bonds for template self hybridization. T_M values and # of bonds determined via the program UnaFold. Results: summary of data analysis and classification results; CI: Class I, well performing; CII: Class II, poorly performing; CIII: Class III, intermediate performance. NR: sequence excluded because not enough replicates of decent quality; CE: Cut End; SD: sequence excluded because standard deviation in P90 or DIFF > 30% of the mean. Additional annotation for sequences with some / all replicates outside of the "normal" expected sigmoidal response. LA: Late Amplification; NA: No Amplification; NS: Non-Sigmoidal. For the 307 sequences with qualified data analysis, P90, N10, and Diff values are listed in minutes.