Supplementary Information

Contents

Materials and Methods

Buffers and cocktails

1 (cleavage buffer, no divalent metals) 50 mM sodium cacodylate, 200 mM NaCl, 1 mM EDTA, pH 7.4. **2** (wash buffer, TEN) 50 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5. **3** (template stripping buffer) 0.1 M NaOH, EDTA 1 mM. **4** (NaOH neutralization buffer) 25 mM sodium cacodylate pH 6. **5** (elution buffer) 1% $LiClO_4$ in 10 mM Tris-HCl pH 8.0. **6** (pH variance buffers) 50 mM Tris-HCl pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 Tris-HCl, 1 mM EDTA, 200 mM NaCl. Cocktail for the first amplification of selection products (5x First Amp Cocktail): 32.6 μM Primer 3, 39.1 μM Primer 4, 1.5 mM each dATP, dGTP, dCTP, TTP, 5 mM $MgSO_4$, and 5x Thermopol Buffer (New England Biolabs). Cocktail for the second amplification for the amplification of gel purified amplicons from the amplification of the selection products (5x Second Amp Cocktail): 32.6 μM MH14, 43.5 μM MH 6, 1.5 mM dNTPs, 5 mM $MgSO_4$, and 5x Thermopol Buffer. Formamide loading buffer: 10 ml formamide (Sigma), 0.2 ml 0.5 M EDTA, pH 8.0, 10 mg bromophenol blue (0.1 % w/v), 10 mg xylene cyanol (0.1% w/v).

In vitro selection

For round 1, 15 pmol of template **1** was annealed to 15 pmol of ribose-embedded primer **2**. The primer was extended using the following conditions: 1x Sequenase buffer, 5 mM dithiothreitol, Single Stranded Binding Protein (5 U), Super RNase In (5 U), 50 μM $dA^{imm}TP$, 10 μM $dU^{ga}TP$, 10 μM dGTP, 10 μM $dC^{aa}TP$, 5-15 μCi dGTP$\alpha^{32}P$ and, added last, Sequenase Version 2.0 (4.6 U) in a final volume of 20 μl. The reaction was overlaid with mineral oil and incubated at 37 °C for five hours. After the five hours, a 0.5 M solution of EDTA (0.5 μl) was added to quench the reaction. The reaction mixture was added to prewashed streptavidin magnetic particles and incubated at room temperature for 30 minutes. The streptavidin magnetic particles with bound modified DNA were washed with TEN (2 × 50 μl), (0.1M NaOH, 1 mM EDTA) (5 × 50 μl), 25 mM cacodylate, pH 6 buffer (1 × 100 μl), DEPC treated H_2O (1 × 100 μl). Cleavage buffer (50 μl) was added to the bound modified single stranded oligonucleotides and left to incubate for one hour at room temperature. After one hour of incubation, the beads were magnetized and the supernatant was removed. 5x First Amp cocktail (2 μl) and 1% $LiClO_4$ in acetone (1 ml) were added to the supernatant to precipitate cleaved product. The cleaved products were centrifuged for 15 minutes and the supernatant was separated from the pellet. The pellet was washed with of ethanol (500 μl), agitated briefly on a vortexer and spun in the centrifuge (13000 rpm) for 15 minutes. The ethanol was decanted and any residual ethanol was removed in the speedvac. Formamide loading buffer (20 μl) was added to the pellet. For size controls, water (10 μl) was added to the beads which were then split into two equal portions. To one set of beads, RNaseA (10 μg) was added and incubated at room temperature for 20 minutes. After twenty minutes of incubation, 99:1 formamide loading buffer:biotin (100 mM in DMF) (200 μl) was added to the beads. To the other set of bead, 99:1 formamide loading buffer:biotin (100

mM in DMF) (200 µl) was added directly. Cleavage products and size controls were resolved by 7 % denaturing PAGE. In the early rounds of selection, products are not detectable and the gel was excised at the height where the cleaved product control appears. Gel slices were crushed using a flame-sealed pipette tip and the DNA was eluted by freezing and thawing (2 × 500 µl elution buffer). Elutions were combined in a single eppendorf tube and dried in a speedvac. The dried sample was dissolved in water (100 µl) and 5x First Amp Cocktail (2 µl). Ethanol (1 ml) was added to precipitate the DNA. The sample was agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was removed and the pellet was dried using a speedvac to evaporate residual ethanol. After redissolving in water (30 µl), the DNA was desalted using a short spin column. PCR amplification of the purified products was carried out with the addition of 5x First Amp Cocktail (8 µl), dGTPα^{32}P (3.3-10 µCi) and Vent (exo-) (3 U) in a total volume of 40 µl. The reaction was thermocycled 30 × (95/15s, 54/15s, 75/40s). Amplicons were extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (40 µl) and precipitated with the addition of ethanol (400 µl). After agitating briefly on a vortexer and 15 minutes of centrifuging (13000 rpm), the ethanol was decanted and residual amounts of liquid were evaporated. The amplicons were dissolved in water (35 µl), 10x lambda exonuclease buffer (4 µl) and of 5 U lambda exonuclease (1 µl of a 5 U/µl stock solution). Degradation of the phosphorylated strand was carried out for 1.5 hours at room temperature. The reaction was extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (40 µl). To precipitate the single stranded DNA, 3% $LiClO_4$ in acetone (300 µL) was added. The sample was agitated briefly on a vortexer and centrifuged (13000 rpm) for 15 minutes. The supernatant was decanted and the pellet was washed using ethanol (400 µl). The sample was again agitated briefly on a vortexer and centrifuged for 15 minutes. The ethanol was decanted and any residual was evaporated by heating the sample to 65 °C. To load the sample onto a 10% denaturing polyacrylamide gel, it was first dissolved in water (15 µl) and formamide loading buffer (15 µl). The single stranded product was visualized using autoradiography and excised. The gel slices were crushed, eluted, precipitated, dissolved in water (30 µl) and desalted as before. A second amplification of the products involved combining 5x Second Amp Cocktail (40 µl), water (150 µl), Vent (exo-) (18 U) and DNA product (4 µl) obtained in the first amplification. Depending on the round, varying amounts of the purified single stranded products were used as templates for the second amplification to increase stringency on the selection. The reaction was thermocycled using the same program as in the first amplification. The reaction was extracted using phenol:chloroform:isoamyl alcohol 25:24:1 (200 µl). The amplicon product was precipitated with ethanol (2 ml), agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was decanted. Residual liquid was evaporated by heating the sample to 65°C and allowing it to air dry. The DNA was dissolved in water (90 µl) and 10x lambda exonuclease buffer (10 µl). The phosphorylated strand was degraded with the addition of lambda exonuclease (5 U). The degradation reaction was incubated at room temperature overnight. The reaction was extracted using phenol:chloroform:isoamyl alcohol 25:24:1 (100 µl). The single stranded DNA was precipitated with the addition of ethanol (1 ml), agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was decanted and residual liquid was evaporated by heating the sample to 65 °C and allowing it to air dry. The single stranded

DNA was dissolved in formamide loading buffer (40 μl) and 1 M NaOH (1 μl) prior to loading on a 10% denaturing polyacrylamide gel. The single stranded product was visualized by UV-shadowing and excised. The gel slice was crushed and eluted by freezing and thawing (3 × 500 μl elution buffer). The sample was dried using a speedvac and dissolved in water (100 μl) and precipitated with the addition of 3% $LiClO_4$ in acetone (1 ml). The sample was agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was decanted and the pellet was washed with ethanol (400 μl). The sample was agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was decanted and residual ethanol was evaporated. The single stranded template was dissolved in water (50 μl) and desalted using a long G-25 column. The template was quantified using UV absorption.

The remaining rounds of the selection were carried out as above with the following modifications. Synthesis of the modified pools for rounds 2-20 were done on a 30 pmol scale. Decants from the reaction mixture were taken at 60 minutes for rounds 1 to 3. From rounds 4 to 21, decants were taken at 1 minute, 5 minutes, and 60 minutes with fresh cleavage buffer being added after the 1 minute and 5 minute decants. From round 9 onwards, the water used to wash the beads before the addition of cleavage buffer was also collected and resolved on the selection gels. In order to increase the stringency, varying amounts of first amplification product corresponding to different decants were used to prepare the second amplification product. Rounds 1 to 3 used gel purified first amplification product (4 μl). Rounds 4 to 7 used the following amounts of first amplification product: 1 minute (1 μl), 5 minutes (1 μl), and 60 minutes (4 μl). Rounds 8 to 10 used the following amounts of first amplification product: water wash (1 μl), 1 minute (1 μl), of 5 minutes (1 μl), and 60 minutes (1 μl). Rounds 11 to 17 used the following amounts of first amplification product: water wash (1 μl), 1 minute (1 μl) and 5 minutes (1 μl). Round 18 to 20 used first amplification products from the water wash (1 μl) and 1 minute decant (1 μl). Generation 20 was the final generation and the 21st round was used only to assess the activity of generation 20. A generation 21 was not produced.

Cloning and sequencing of Generation 20

Using primers **5** and **6**, generation 20 was amplified with Taq polymerase 20 × (94/30 seconds, 55 °C/30 seconds, 72 °C/30 seconds) to produce double stranded DNA with 3′ A overhangs. The amplicon product was purified on 2 % agarose and extracted using Qiagen's QIAQuick Gel Extraction kit. The purified amplicon was TA cloned using Promega's TA cloning pGEM-T Easy cloning kit. The ligation was dialyzed on 1% agarose for at least one hour followed by electroporation using Tritech's Bactozapper and of Invitrogen's DH10B Electromax cells (20 μl). The cells were incubated for one hour and a portion of cells (20 μl) were used for blue/white screening. White colonies were chosen at random and used to inoculate TB media (1ml). Harvested plasmids were screened for single inserts of correct size by restriction digest. Plasmids bearing single inserts were sent to UBC's Nucleic Acids and Protein Services Unit for sequencing.

Screening of the clones

Single stranded templates for individual sequences were ordered from Integrated DNA Technologies. To screen the clones for self cleavage activity, 6 pmol of primer **2** were annealed to 5 pmol of template and extended using $dC^{aa}TP$, $dU^{ga}TP$, $dA^{imm}TP$, natural dGTP and $dGTP\alpha^{32}P$. Clones were tested using the selection conditions (cleavage buffer, 40µl, 24 °C). At time points 1, 5, 30, 60, 120 and 1320 minutes an aliquot (5 µl) was removed and quenched by adding 99:1 formamide loading buffer: biotin (100 mM in DMF) (15 µl). Cleaved and uncleaved oligonucleotides were resolved on a 7% denaturing polyacrylamide gel, visualized with a phosphorimager (Amersham Typhoon 9200) and polygons were drawn around the bands corresponding to cleaved and uncleaved products. The data was fitted to a first ordered exponential equation (1) using Sigmaplot 2001.

$$P_t = P_\infty \times (1 - e^{-kt}) \qquad 1$$

where:

P_t = amount of cleaved product
P_∞ = total amount of DNAzyme
k = observed rate constant
t = time

Preparation of modified templates for PCR amplification

Modified templates were synthesized on a 15 pmol scale. Nucleotides used for extensions were: 1) 10 µM natural dNTPs, 5-15 µCi $dGTP\alpha^{32}P$ 2) 10 µM dGTP, 10 µM $dC^{aa}TP$, 10 µM $dU^{ga}TP$, 50 µL $dA^{imm}TP$, and 5-15 µCi $dGTP\alpha^{32}P$ 3) 10 µM dGTP, 10 µM $dC^{aa}TP$, 10 µM $dU^{ga}TP$, 50 µL $dA^{ime}TP$ and 5-15 µCi $dGTP\alpha^{32}P$ and 4) 10 µM dGTP, 10 µM $dC^{aa}TP$, 10 µM $dU^{ga}TP$, 50 µM $dA^{imp}TP$ and 5-15 µCi $dGTP\alpha^{32}P$. Synthesized templates were bound on streptavidin, washed TEN (2 × 50 µL), (0.1M NaOH, 1 mM EDTA) (5 × 50 µL), 25 mM cacodylate, pH 6 buffer (1 × 100 µl), DEPC H_2O (1 × 100 µl), and resuspended in DEPC treated H_2O (20 µl). The beads were split into two equal portions. To one portion RNaseA (1 µl of a 10 mg/ml stock solution) was added and the sample was left to incubate at room temperature for 20 minutes. 99:1 loading buffer:biotin (100 mM in DMF, 10 µl) were added to each sample. The samples were heated at 95 °C for 5 minutes and resolved using 7 % PAGE. Bands corresponding to uncleaved and RNaseA-cleaved material were excised. The gels containing cleaved products were crushed and the DNA was eluted by freezing and thawing (elution buffer, 2 × 500 µl). Templates were dried in a speedvac. Pellets were dissolved in H_2O (100 µl), 5x First Amp buffer (1 µl) and ethanol (1 ml). Samples were agitated briefly on a vortexer and centrifuged for 15 minutes. The supernatant was decanted and the pellet was air dried on a 65 °C heat block.

The pellet was dissolved in DEPC H_2O (50 µl) and desalted by passing the sample through a G25 spin column.

Standardizing Modified Templates by Autoradiography

A calibration curve relating the radioactivity of a sample to the concentration of G in a solution was produced. The calibration curve uses a set of standards produced by graphing the relation between signal obtained by autoradiography (which is proportional to the amount of radioactivity in the sample and thus the amount of dG incorporated. In this case, both extension reactions and the stock standard solution contained the same ~2.5 µCi/20 µL concentration. The exact amount of radiation does not need to be known, but it is critical that both extension and standards are prepared using the same concentration of ∝-^{32}P-dGTP at the same specific activity. The constant ratio where radioactivity represents nucleoside concentration is established; in this case it is a constant ratio of ~2.5 µCi/10 µM dGTP. Since this is a constant ratio, the concentration of dG can be calculated once a dilution factor is established using equation (3).

$$\ln x = \frac{\ln y - b}{a} \qquad 3$$

where:

x = dilution factor
y = volume
a = slope
b = intercept

Determination of the concentration of dG using this dilution factor eliminates the need to know the exact amount of radioactivity used in the extension and standards. The relative intensity of the radiation is represented in volumes. Dilutions used were 30 ×, 90 ×, 900 ×, 1800 ×, 18000 × (Figure S5**A**). The natural log of the volumes were plotted against the natural log of the dilution and fitted to equation (1) to obtain values for a and b. The calibration curve is shown in Figure S5**B**. Purified DNA templates were spotted along with the calibration standards in 1 µl and 10 µl volumes (Figure S5**A**). The radioactive volumes for the 10 µl samples were used for determining the dilution factor of the sample. The dilution factor of G of the samples, x, can then used to determine the concentration of the DNA template based on the 19 dGs in the template according to the equation (4):

$$[\text{ template }] = \frac{[dGTP]\times 0.1}{19\times e^{x}}$$

4

where:

x = dilution factor
The 19 comes from the 19 G and the 0.1 corrects for the 10 µL sample volume.

Once the templates concentrations were determined, templates were standardized to 10 fM and used in 1 × Thermopol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1 % Triton X-100, 20 mM Tris-HCl pH 8.8), 200 µM dATP, dTTP, dGTP, and dCTP, 1 fM DNA template 10 µM of each primers **5** and **6** and Vent (exo-) (5 U) or Taq polymerase (5 U) in final volume of 50 µl.

The Vent (exo-) polymerase catalyzed reactions were thermocycled 45 times (94 °C/30 sec, 57 °C/30 sec, 72 °C/30 sec). Aliquots (10 µl) were removed after cycles 37, 39, 41, 43 and 45. The Taq polymerase catalyzed reactions were thermocycled 35 times (94 °C/30 sec, 57 °C/30 sec, 72 °C/30 sec). Aliquots (10 µl) were removed after cycles 27, 29, 31, 33 and 35. For comparison, the Vent (exo-)-produced amplicons (2 µl) from the 45th cycle were resolved on 2 % agarose gel (Figure 6). The same was done for the amplicons produced from the 33rd cycle of PCR using Taq polymerase (Figure S6).

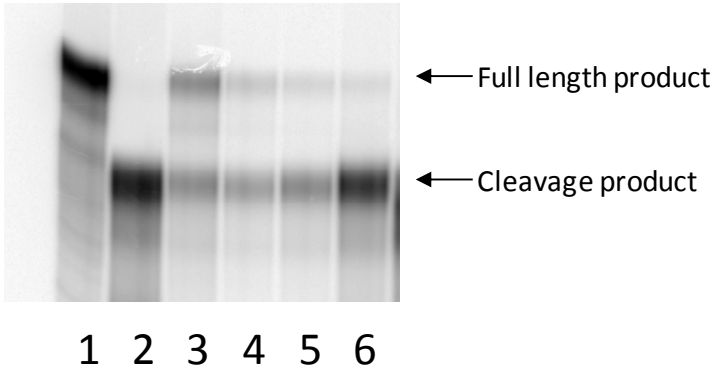

Figure S1. Polyacrylamide gel showing the self-cleavage products generated by generation 20. Lane 1 contains full length product from the synthesis using $dA^{imm}TP$ and Lane 2 is RNaseA-treated DNAzyme pool. Lane 3-6 are products collected from the supernatants which were removed at time 0 (water wash), 1, 5, and 60 minutes, respectively. Full length product appearing in the water wash (Lane 3) is from beads that failed to magnetize and were also decanted.

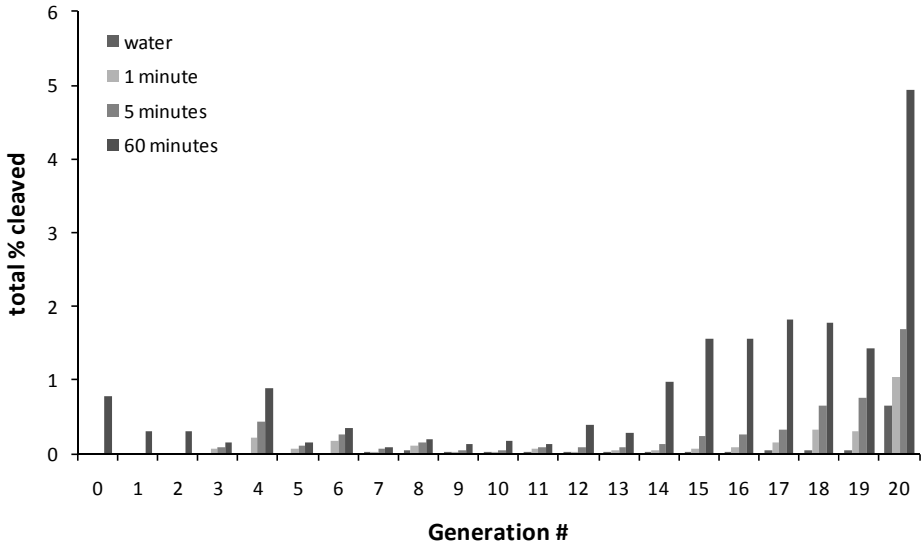

Figure S2. Progress of the selection: Self-cleavage activity is shown for each generation. Seemingly high total % cleaved in the early rounds could be due to truncates and low counts in the controls due to lack of full extension.

Table S3. Generation 20 sequences and self-cleavage activity

Clone #	Degenerate Region Sequence (N40)	# of A's	Total # of bases	% yield	Truncates	k_{obs} ($\cdot 10^{-3}$ min^{-1})
1	AUGCAUGGUUAUUGUAGCAUGUGCUGUGUAGCAGCAGCGUUU	8	42	<5	+++	n.d.
2	AUGCAUGGUUAUUGAGUCGAGGCAUGUUAGUGAGUGUGUGCUU	8	43	<5	++	n.d.
4	CUGCAUGGUUAUUGAGGCGAGGCAUGUGAGGGAUUGGCUG	7	40	<5	+	n.d.
5	UCAUAGUCUCGGUGGCACGUUCGUAGGUGUGAUUGUGUGU	5	40	<5	++	n.d.
6	AGUUAUGCUCUCCAGUGGCUCGCAUGAUGUGUAGUGUGUG	6	40	6	+	2.91
7*	GUAUGAGCAGUGUGGUGGGAGGCGCGCUUGUGCUUGCGUUAGU	5	43	64	+	4.73
8	AGUCAUGUAGUCAGUCUGCGGCACGCCGUGGUGAGGGAUGUGC	7	43	14	+	7.90
9	AUGCAUGCUUAUUGAGGCGAGGCAUGCGUCGAGUGUGUGUGCGGU	7	45	<5	+	n.d.
10	AGUCAUGUAUUCCGUUGCUAGCGCAGCAUGUGCUGUGUGUUG	6	42	<5	++	n.d.
11	GUGUUUGCUCGGCUGUGGUGCGCAGUGUGGUCGAAGUGUGU	3	41	<5	+	n.d.
13	GUAUGAGUGGAGUGGUGGGAGGCAUGCUUGUGGUGAGGUGGCUUU	6	45	80	+++	2.43
14	ACUGUUGAGCACUAGUGAGGUGUGCACGAGUGGUGUCGGUCU	7	42	<5	++	n.d.
18	AUGCAUGCUUAUUGAGGCGUGGCACAGUAUGUGUGUGAGU	8	40	<5	+++	n.d.
19	AUGCAUGGUUAUUGAGGCGUGGCCAGGGUGGCAGUAGUGUU	7	41	<5	+	n.d.
21	AUGCAUGGUUAUUGAGGCGUGGUACGCUUGUGCUUGUAGUGAGU	7	44	<5	++	n.d.
22	CUGCAUGGUUAUUGAGUCGAGGCAUGUGAGGGAUUGGCUG	7	40	<5	-	n.d.
24	AUGCAUGGUUAUUGAGUCUAGGCACGUGAUGAGUGUGAGUGCG	9	43	<5	-	n.d.
25	AUGCAUGGUUAUUGAGUCGAGGCUUGUGAGGGAUGGGCUG	7	40	<5	-	n.d.
31	UUGCAUGGUUAUUGAGGCGUGGUCGCAGUGGUAGUGAGU	6	39	<5	+++	n.d.
35	ACUACCAUGUGGUCUACAAUGGCGGAGCACCAGUUAUGUUU	10	41	<5	++++	n.d.
38	UGUCAUGUUCUCCGUGGCUCGUACGCCGUGUGUGUGUGUUA	3	41	<5	+	n.d.
41	UUCGCAUCGUGAGUGAGGCACGGUGGCGACGUGUUGUGGUGCA	6	43	<5	+	n.d.
43	AUGCAUGGUUAUUGAGGUGUGGCCAGGUGGCAGUAGUGUU	7	40	<5	++	n.d.
44	AGUCAUGCUCUCCAGUGGUUCGCAUGUAUGUGAGUGGAGUGU	7	42	20	+	3.49
45	AGCAGCUCGAGUCAGUUUGCGGCACGCAUGGUGGUUCGCGUGU	6	43	<5	+	n.d
46	AUGCAUGGUUAUUGAGGCGUGGCACAGUAUGGGUGUGAGU	8	40	<5	+	n.d.
48	AUGCAUGGUUAUUGAGUCGAGUGUAGUGUAGCAGCAGCGUUG	9	42	<5	+++	n.d.
49	AGUCAUGCUCUCUAGUGGUUCGCAGGUCGUGUGGGUCGUU	4	40	80	+	3.33
50	AUGCAUGGUUAUUGAGUCGAGUGUAGUGUAGCAGUAGCGUUG	9	42	<5	+++	n.d.
52	GCAUAGUCUCGGUGGCACACUCGUAGAGGUGGUAGUGUCA	8	40	<5	++++	n.d.
54	AGUCAUGUAGUCCGUUGCUAGCGCGCCAUGUGCUGUGUUG	5	40	<5	++	n.d.
55	AUGCAUGGUUAUUUAGUCGAGGCAUGUGAGGGAUUGGGUG	8	40	<5	-	n.d.
57	CUGUUUGCUCGACUAUGGCGCGCAGUGUGGUCUUAGUGUUU	4	41	<5	++	n.d.
	For reference: Dz10-66 constructed using $dA^{imm}TP$					
10-66	CUAGCAGCGCAAGUGAGGCGCGCUAUGAGUGUGGUGCGUGUAU	8	43	<5	-	n.d.

n.d. = no detectable cleavage
- = no significant truncates
+ = slight truncates
++ = truncates
+++ = significant truncates
++++ = almost no fully extended products
* = slight smearing

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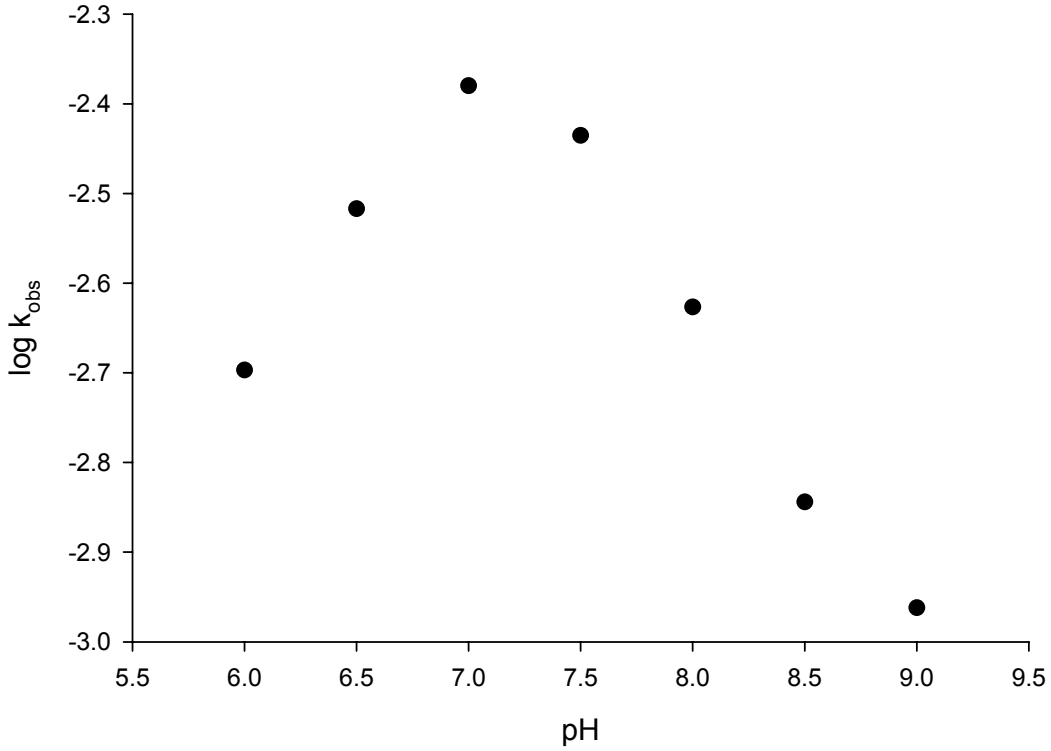
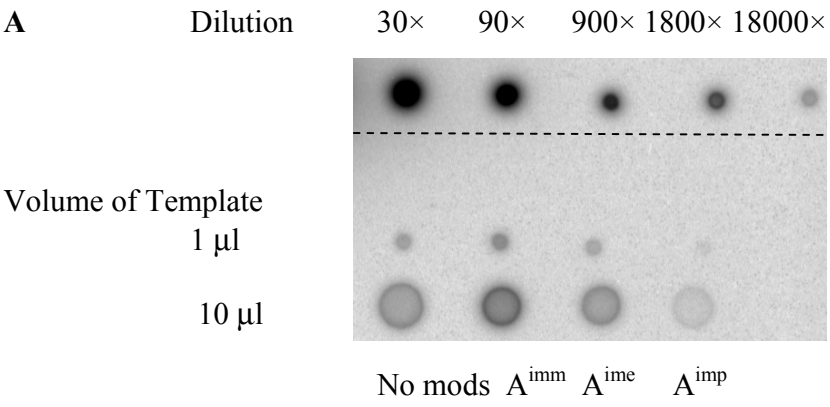


Figure S4. pH rate profile showing the effect of pH on log $k_{obs.}$

A



B

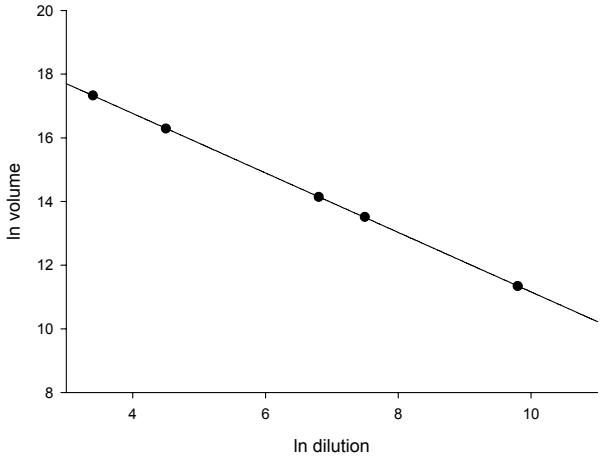


Figure S5. **A** Exposed screen showing the radioactive intensity of calibration standards at various dilutions and 1 µl and 10 µl of synthetic template. **B** Calibration curve produced by autoradiography for determining modified template concentration. Graph fitted to equation $y = -0.9344x + 20.5035$, $R^2 > 0.99$.

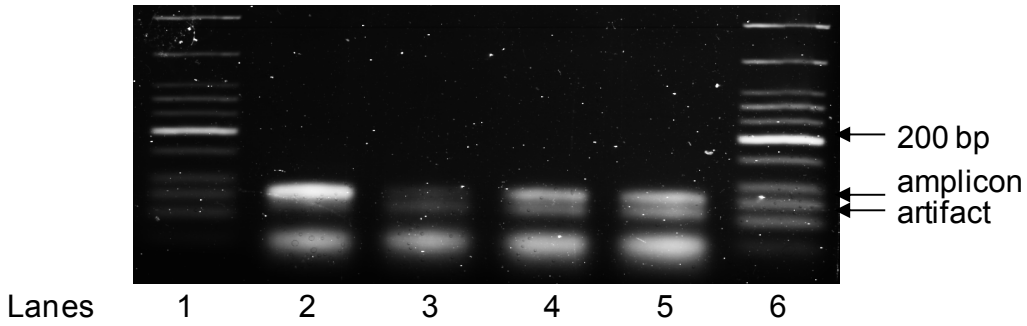


Figure S6. PCR amplification of modified templates by Taq (33 cycles). Lanes 1 and 6 contain NEB Low Molecular Weight Ladder. For a control, a completely unmodified ribophosphodiester bond cleaved version of Dz20-49 was used as the control template (Lane 2). For modified templates, Dz20-49 was constructed with dGTP, **6**, **7** and one of: **2** (Lanes 3), **3** (Lane 4) or **4** (Lane 5).