SUPPORTING INFORMATION

In Vivo Structure-Activity Relationships and Optimization of an Unnatural Base Pair For Replication in a Semi-Synthetic Organism

Aaron W. Feldman and Floyd E. Romesberg*
Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA

Table of Contents ..... **Page**

Methods

In vivo UBP replication

Assembly of UBP-containing plasmids

Inserts for Golden Gate assembly bearing the UBP were generated by PCR of chemically synthesized oligonucleotides bearing d**NaM** (**O1** for context 1, **O2** for context 2, **O3** for context 3, **O4** for context 4; 0.025 ng per 50 μL reaction) using the primers **P1**/**P2** for context 1 or **P3**/**P4** for contexts 2–4 and conditions described previously,[1] under the following thermocycling protocol (times denoted as mm:ss): [96 °C 0:30 | 25 × [96 °C 0:30 | 47 °C 0:30 | 68 °C 4:00]].

Golden Gate assembly reactions were prepared by combining destination plasmid pUCX2 (1 μg),[2] PCR insert (3:1 insert:plasmid molar ratio), T4 DNA ligase (533 U), BsaI-HF (53.3 U), and ATP (1 mM) in 1× NEB CutSmart buffer (final volume 80 μL). The reactions were thermocycled under the following conditions: [37 °C 20:00 | 40× [37 °C 5:00 | 16 °C 10:00 | 22 °C 5:00] | 37 °C 20:00 | 50 °C 15:00 | 70 °C 30:00]. Following thermocycling, T5 exonuclease (13.3 U) and additional BsaI-HF (26.6 U) were added, and the reactions were incubated at 37 °C for an additional hour. Assembled plasmids were purified on a Zymo-Spin I column, and quantified fluorometrically using a Qubit fluorometer (Thermo-Fisher). Refer to Table S1 for list of sequences.

Transformation and replication of UBP-containing plasmids

The *E. coli* SSO was made electrocompetent as described previously,[2] and an aliquot (50 μL) was transformed with 2 ng pINF plasmid. Upon electroporation, cells were immediately diluted with media supplemented with chloramphenicol (950 μL). An aliquot of diluted cells (10 μL) was immediately diluted 10-fold with the same media, and further supplemented with d**NaM**TP (125 μM) and d**TPT3**TP (25 μM) for plasmid recovery. Transformed cells were incubated at 37 °C for 1 h before pelleting each sample by centrifugation. Recovery media was removed, and cells were resuspended in an equivalent volume of fresh media lacking unnatural triphosphate and supplemented with ampicillin for pINF selection. An aliquot of these cells (10 μL) was used to inoculate cultures of fresh media supplemented with ampicillin for pINF selection, as well as fresh unnatural triphosphates at specified concentrations (final volume of 100 μL). Samples were then further incubated at 37 °C, monitoring for growth, and harvesting by pelleting (9,000 r.c.f., 10 min) at an OD_{600} of ~0.7.

Analysis of pINF plasmids by biotin shift PCR

Plasmids were isolated for analysis using commercial miniprep kits (ZR Plasmid Miniprep Classic, Zymo Research), and analyzed for UBP retention by biotin shift PCR, as described previously.[1,2] PCR conditions were as follows: plasmid miniprep (0.5 μL, diluted 5-fold for samples grown to saturation), dNTPs (400 μM), 1× SYBR Green, $MgSO_4$ (2.2 mM), primers (**P5**/**P6** for context 1, **P7**/**P8** for contexts 2–4; 1 μM each), d**5SICS**TP and d**MMO2**$^{\mathbf{BIO}}$TP (65 μM each), OneTaq DNA polymerase (0.018 U/μL), and DeepVent DNA polymerase (0.007 U/μL) in 1× OneTaq standard reaction buffer (final volume 15 μL). Thermocycling conditions were as follows: [20 × [95 °C 0:15 | 52 °C 0:15 | 68 °C 4:00]]. An aliquot (1 μL) of each reaction was mixed with a solution of streptavidin (2.5 μL, 2 μg/μL, Promega), and incubated at room temperature for 10 min. Streptavidin-bound amplicon was then resolved from unbound amplicon on a 6% polyacrylamide (29:1 acrylamide:bis-acrylamide) TBE gel, at 120 V for 25 min. Gels were then stained with 1× SYBR Gold (Thermo Fisher) and imaged using a Molecular Imager Gel Doc XR+ equipped with a 520DF30 filter (Bio-Rad).

UBP retention was measured by densitometric analysis of the gels (Quantity One 1-D Analysis Software, Bio-Rad) from the biotin shift assay and calculation of a percent raw shift, which equals the intensity of the streptavidin-shifted band divided by the sum of the intensities of the shifted and unshifted bands. Reported UBP retentions are percent raw shift for a given sample normalized to the percent raw shift of the input pINF plasmid used.

Table S1. Oligonucleotides and plasmids used in this study.

	Sequence (5'→3')
O1	CTGTTTCCTGTGTGAAATTGTTATCCGCTCACA-d**NaM**-TTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCC
O2	CTCGAGTACAACTTTAACTCACACAATGTA-d**NaM**-AGATCACGGCAGACAAACAAAAGAATGGAATC
O3	CTCGAGTACAACTTTAACTCACACAATGTAC-d**NaM**-CATCACGGCAGACAAACAAAAGAATGGAATC
O4	CTCGAGTACAACTTTAACTCACACAATGTAC-d**NaM**-GATCACGGCAGACAAACAAAAGAATGGAATC
P1	ATGGGTCTCCAGTGGCTGTTTCCTGTGTGAAATTGTTATCCGC
P2	ATGGGTCTCTTCGTTGGCTTTACACTTTATGCTTCCGGC
P3	ATGGGTCTCCAGTGGCTCGAGTACAACTTTAACTCACAC
P4	ATGGGTCTCTTCGTTGATTCCATTCTTTTGTTTGTCTGC
P5	CTGTTTCCTGTGTGAAATTGTTATCC
P6	GGCTTTACACTTTATGCTTCCG
P7	CTCGAGTACAACTTTAACTCACAC
P8	GATTCCATTCTTTTGTTTGTCTGC

pUCX2 (3174 bp)

AACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCC
CTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGA
GTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGC
TGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTAAGTTAGCTCACTCATTAGGCAC
CCCACTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATT
CACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCC
TTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGG
CGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCT
CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGC
ATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCG
AGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCA
CTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACA
ATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC
TTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGG
GTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCC
AATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGC
CGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAA
GAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAA
GGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCC
ATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTAC
TTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCT
TCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGCTCTCGCGGTATCATTGCAGCACTGGGGCCA
GATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCG
CTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAA
ACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTT
TCGTTGAGACCATTCTCACCAATAAAAAACGCCCGGCGGCAACCGAGCGTTCTGAACAAATCCAGATGGAGTTCTGAGG
TCATTACTGGATCTATCAACAGGAGTCCAAGCGAGCTCTCAGTCCTGCTCCTCGGCCACGAAGTGCACGCAGTTGCCGG
CCGGGTCGCGCAGGGCGAACTCCCGCCCCCACGGCTGCTCGCCGATCTCGGTCATGGCCGGCCCGGAGGCGTCCCGGAA
GTTCGTGGACACGACCTCCGACCACTCGGCGTACAGCTCGTCCAGGCCGCGCACCCACACCCAGGCCAGGGTGTTGTCC
GGCACCACCTGGTCCTGGACCGCGCTGATGAACAGGGTCACGTCGTCCCGGACCACACCGGCGAAGTCGTCCTCCACGA
AGTCCCGGGAGAACCCGAGCCGGTCGGTCCAGAACTCGACCGCTCCGGCGACGTCGCGCGCGGTGAGCACCGGAACGGC
ACTGGTCAACTTGGCCATGGTTTAGTTCCTCACCTTGTCGTATTATACTATGCCGATATACTATGCCGATGATTAATTG
TCAACGACGGGGTCTCCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGC
GTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTT
TTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTT
CAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG
TGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACAC
AGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGA
AGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAAC
GCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGTGCTCGTCAGGGGGGCG
GAGCCTATGGAAA

Table S2. Nucleotides used in this study.

d**X**TPs						d**Y**TPs		
		Ref.			Ref.			Ref.
1	d**DMN**	[3]	39	d**TOK587**	[4]	1	d**ICS**	[3]
2	d**4OMe**	[5]	40	d**TOK576**	[4]	2	d**4MICS**	[6]
3	d**IQ**	[7]	41	d**TOK581**	[4]	3	d**5MICS**	[6]
4	d**2MN**	[3]	42	d**TOK580**	[4]	4	d**NICS**	[8]
5	d**3OMe**	[5]	43	d**PhMO**	[9]	5	d**SICS**	[8]
6	d**QL**	[7]	44	d**PyMO1**	[9]	6	d**4SICS**	[10]
7	d**2Np**	[3]	45	d**PyMO2**	[9]	7	d**5SICS**	[10]
8	d**DM4**	[11]	46	d**PMO3**	[9]	8	d**SNICS**	[8]
9	d**BEN**	[11]	47	d**PMO2**	[9]	9	d**ONICS**	[8]
10	d**MM1**	[11]	48	d**FuMO1**	[9]	10	d**7OFP**	[12]
11	d**DM2**	[11]	49	d**FuMO2**	[9]	11	d**7OTP**	[12]
12	d**DM**	[3]	50	d**TpMO1**	[9]	12	d**4OTP**	[12]
13	d**3FB**	[14]	51	d**TpMO2**	[9]	13	d**TPT3**	[13]
14	d**MMO1**	[5]	52	d**FIMO**	[9]	14	d**FTPT1**	[13]
15	d**DM5**	[11]	53	d**MIMO**	[9]	15	d**TPT1**	[13]
16	d**2Py**	[7]	54	d**FEMO**	[9]	16	d**TPT3^{PA}**	[13]
17	d**3MPy**	[7]	55	d**IMO**	[9]			
18	d**4MPy**	[7]	56	d**MEMO**	[9]			
19	d**5MPy**	[7]	57	d**EMO**	[9]			
20	d**34DMPy**	[7]	58	d**MMO2**	[5]			
21	d**35DMPy**	[7]	59	d**DMO**	[5]			
22	d**EPy**	[7]	60	d**5FM**	[15]			
23	d**45DMPy**	[7]	61	d**2OMe**	[5]			
24	d**BP**	[16]	62	d**NaM**	[15]			
25	d**BTp**	[16]	63	d**APy**	[7]			
26	d**IN**	[16]	64	d**MAPy**	[7]			
27	d**BzT**	[16]	65	d**DMAPy**	[7]			
28	d**BF**	[16]	66	d**FDMO**	[9]			
29	d**Tp**	[17]	67	d**ZMO**	[9]			
30	d**MTp**	[17]	68	d**TfMO**	[9]			
31	d**AM**	[18]	69	d**VMO**	[9]			
32	d**ADM**	[18]	70	d**ClMO**	[9]			
33	d**MAN**	[18]	71	d**CNMO**	[9]			
34	d**MMAN**	[18]	72	d**MMO2^{A}**	[19]			
35	d**DMMAN**	[18]	73	d**MMO2^{PA}**	[19]			
36	d**TOK588**	[4]	74	d**MMO2^{BIO}**	[2]			
37	d**TOK582**	[4]	75	d**MMO2^{SSBIO}**	[19]			
38	d**TOK586**	[4]						

Table S3. UBP retention data for Figure 4a. An asterisk indicates no cell growth was observed.

	125 μM				10 μM			
name	**Trial 1**	**Trial 2**	**Trial 3**	**avg ± sd**	**Trial 1**	**Trial 2**	**Trial 3**	**avg ± sd**
dMMO2	103	110	107	107 ± 4	86	80	82	83 ± 3
dDMO	100	104	102	102 ± 2	4	12	12	10 ± 5
dClMO	103	95	99	99 ± 4	88	75	81	81 ± 7
dCNMO	99	96	98	98 ± 1	78	95	85	86 ± 9
d5FM	100	95	98	98 ± 3	89	107	73	90 ± 17
dNaM	105	90	97	97 ± 7	44	44	36	41 ± 5
dFDMO	96	94	95	95 ± 1	22	40	27	30 ± 9
dFIMO	100	88	94	94 ± 6	72	43	62	59 ± 15
dN3MO	93	95	94	94 ± 1	6	31	26	21 ± 13
dIMO	98	89	94	94 ± 5	58	67	44	56 ± 11
dMIMO	103	84	93	93 ± 9	6	5	1	4 ± 3
dFEMO	96	83	90	90 ± 6	66	49	26	47 ± 20
dMMO2^{A}	93	86	89	89 ± 4	33	58	44	45 ± 13
d2OMe	99	68	84	84 ± 16	13	30	24	22 ± 8
dCF3MO	85	70	77	77 ± 7	1	12	3	5 ± 6
dMEMO	*	86	75	54 ± 47	7	2	1	3 ± 3
dVMO	*	79	69	50 ± 43	17	31	11	20 ± 10
dDM5	85	9	23	39 ± 40	*	13	2	5 ± 7
d2MN	77	6	21	34 ± 38	4	10	2	5 ± 4
d45DMPy	82	5	15	34 ± 42	2	0	8	3 ± 4
dEMO	55	9	32	32 ± 23	5	6	5	6 ± 1
dDM	62	4	17	28 ± 30	1	12	4	6 ± 5
dTOK581	65	*	16	27 ± 34	5	15	5	8 ± 6
dTOK587	45	5	13	21 ± 21	5	2	3	3 ± 1
dPyMO2	45	5	13	21 ± 21	4	9	0	5 ± 5
d35DMPy	48	*	12	20 ± 25	1	8	3	4 ± 4
d5MPy	45	*	11	19 ± 24	2	6	0	3 ± 3
dPyMO1	27	9	18	18 ± 9	3	0	14	6 ± 7
dPMO2	36	5	10	17 ± 16	4	5	1	4 ± 2
dMMO1	29	6	9	15 ± 13	0	3	5	3 ± 2
dBTp	21	8	15	15 ± 6	2	2	0	1 ± 1
dDM2	29	6	9	14 ± 12	4	7	4	5 ± 2
dMMO2^{SSBIO}	25	3	14	14 ± 11	1	10	4	5 ± 5
dMMO2^{PA}	23	5	14	14 ± 9	8	5	6	6 ± 1
dDMMAN	26	4	7	12 ± 11	4	0	2	2 ± 2
dQL	19	6	12	12 ± 6	1	4	0	2 ± 2
dMM1	24	5	7	12 ± 10	1	3	0	1 ± 2
d4MPy	26	0	7	11 ± 14	3	0	0	1 ± 2
dBzT	19	6	6	11 ± 7	4	6	1	4 ± 2

d3OMe	20	4	6	10 ± 9	0	5	5	3 ± 3
dPhMO	6	14	10	10 ± 4	4	13	10	9 ± 5
d2Py	13	7	10	10 ± 3	1	3	5	3 ± 2
d3MPy	23	0	6	10 ± 12	1	6	0	2 ± 3
d4OMe	14	5	10	10 ± 5	0	7	5	4 ± 4
dPMO3	16	3	10	10 ± 6	1	10	0	4 ± 5
dBPy	20	3	6	10 ± 9	3	5	7	5 ± 2
dMAN	11	7	9	9 ± 2	2	1	7	3 ± 4
dDMN	9	10	9	9 ± 1	0	7	10	6 ± 5
dIQ	15	3	9	9 ± 6	1	3	5	3 ± 2
d2NP	14	4	9	9 ± 5	3	14	2	6 ± 7
dFuMO2	19	1	5	9 ± 9	5	1	2	3 ± 2
dEPy	11	5	8	8 ± 3	3	5	4	4 ± 1
dDM4	11	4	8	8 ± 4	1	9	3	4 ± 4
dMMAN	11	4	8	8 ± 3	2	9	12	8 ± 5
dMTp	15	0	8	8 ± 8	1	7	0	2 ± 4
d3FB	10	4	7	7 ± 3	1	9	2	4 ± 4
dIN	14	0	7	7 ± 7	3	2	4	3 ± 1
dBEN	10	4	7	7 ± 3	4	6	0	3 ± 3
dMAP	6	8	7	7 ± 1	2	1	0	1 ± 1
dTp	13	0	6	6 ± 6	0	6	2	3 ± 3
dDMAP	8	5	6	6 ± 1	0	6	7	4 ± 4
d34DMPy	12	0	6	6 ± 6	1	9	2	4 ± 4
dBF	12	0	6	6 ± 6	3	2	0	2 ± 2
dTOK588	6	5	6	6 ± 1	4	3	4	3 ± 1
dAM	8	4	6	6 ± 2	5	16	4	8 ± 7
dTOK586	7	4	6	6 ± 1	3	9	1	4 ± 4
dTOK576	6	5	6	6 ± 1	2	3	5	3 ± 1
dAP	6	4	5	5 ± 1	5	1	2	3 ± 2
dTOK580	4	7	5	5 ± 2	1	28	0	10 ± 16
dFuMO1	11	0	5	5 ± 5	4	1	2	3 ± 1
dTpMO1	7	3	5	5 ± 2	3	0	3	2 ± 2
dMMO2^{BIO}	7	2	4	4 ± 2	4	10	2	5 ± 5
dTpMO2	7	2	4	4 ± 3	1	1	0	1 ± 1
dTOK582	4	4	4	4 ± 0	3	0	5	3 ± 2
dADM	0	6	3	3 ± 3	2	8	1	4 ± 4

Table S4. UBP retention data for Figure 4b. An asterisk indicates no cell growth was observed.

	125 µM				25 µM			
name	**Trial 1**	**Trial 2**	**Trial 3**	**avg ± sd**	**Trial 1**	**Trial 2**	**Trial 3**	**avg ± sd**
d**TPT3**^{PA}	98	69	94	87 ± 16	70	93	93	85 ± 13
d**TPT3**	92	71	89	84 ± 12	87	97	87	90 ± 6
d**SICS**	96	54	96	82 ± 24	91	97	98	95 ± 4
d**FPT1**	80	88	78	82 ± 5	73	82	85	80 ± 6
d**4SICS**	59	90	90	80 ± 18	84	90	92	89 ± 4
d**TPT1**	79	69	79	76 ± 6	80	96	90	89 ± 8
d**5SICS**	59	82	86	76 ± 14	68	89	93	83 ± 13
d**NICS**	81	62	79	74 ± 10	76	88	85	83 ± 6
d**SNICS**	65	71	41	59 ± 16	71	86	89	82 ± 10
d**ICS**	*	*	*	*	71	86	86	81 ± 8
d**4MICS**	*	*	*	*	66	80	94	80 ± 14
d**5MICS**	*	*	*	*	59	74	80	71 ± 11
d**ONICS**	*	*	*	*	*	*	*	*
d**7OFP**	*	*	*	*	*	*	*	*
d**7OTP**	*	*	*	*	*	*	*	*
d**4OTP**	*	*	*	*	*	*	*	*

Table S5. UBP retention data for Figure 4c.

dTPT3 analog	dNaM analog	Trial 1	Trial 2	Trial 3	avg ± sd
d**SICS**	d**5FM**	88	52	63	68 ± 18
	d**MMO2**	39	9	9	19 ± 18
	d**CNMO**	65	82	89	79 ± 12
	d**ClMO**	66	50	66	61 ± 10
d**TPT3**	d**5FM**	100	78	83	87 ± 12
	d**MMO2**	90	93	80	88 ± 7
	d**CNMO**	97	94	94	95 ± 2
	d**ClMO**	98	95	96	96 ± 2
d**TPT1**	d**5FM**	0	0	0	0 ± 0
	d**MMO2**	0	0	0	0 ± 0
	d**CNMO**	0	0	0	0 ± 0
	d**ClMO**	0	0	0	0 ± 0
d**4SICS**	d**5FM**	0	0	0	0 ± 0
	d**MMO2**	2	3	0	2 ± 1
	d**CNMO**	9	5	4	6 ± 3
	d**ClMO**	0	5	6	4 ± 3
d**TPT3**^{PA}	d**5FM**	0	0	0	0 ± 0
	d**MMO2**	0	4	3	3 ± 2
	d**CNMO**	5	2	0	2 ± 2
	d**ClMO**	2	5	6	4 ± 2
d**5SICS**	d**5FM**	0	0	0	0 ± 0
	d**MMO2**	0	0	0	0 ± 0
	d**CNMO**	6	5	5	5 ± 1
	d**ClMO**	12	0	9	7 ± 6
d**NICS**	d**5FM**	0	0	0	0 ± 0
	d**MMO2**	2	1	4	3 ± 2
	d**CNMO**	0	7	2	3 ± 4
	d**ClMO**	3	0	4	2 ± 2
d**SNICS**	d**5FM**	0	0	5	2 ± 3
	d**MMO2**	0	0	0	0 ± 0
	d**CNMO**	1	5	0	2 ± 3
	d**ClMO**	6	11	2	6 ± 4
d**ICS**	d**5FM**	4	3	5	4 ± 1
	d**MMO2**	5	2	0	2 ± 2
	d**CNMO**	1	0	0	0 ± 1
	d**ClMO**	6	3	4	4 ± 1
d**FPT1**	d**5FM**	0	4	0	0 ± 0
	d**MMO2**	1	4	4	3 ± 2
	d**CNMO**	4	0	4	3 ± 2
	d**ClMO**	0	5	0	0 ± 0
d**4MICS**	d**5FM**	1	4	6	4 ± 3

	d**MMO2**	4	4	1	3 ± 2
	d**CNMO**	0	0	0	0 ± 0
	d**ClMO**	3	5	3	4 ± 1
d**5MICS**	d**5FM**	1	3	6	4 ± 3
	d**MMO2**	2	2	3	2 ± 0
	d**CNMO**	0	0	0	0 ± 0
	d**ClMO**	1	2	2	2 ± 1

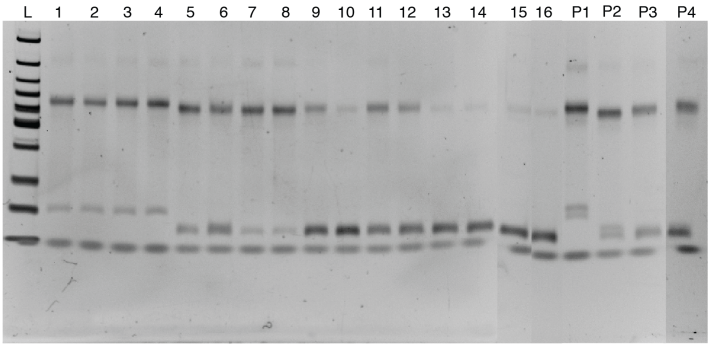

Figure S1. Representative 6% native PAGE gel of a single trial from Figure 6. Lane "L" is low molecular weight DNA ladder. Each grouping of four lanes from lane 1 to 16 represents, from left to right, d**NaM**TP at 125 µM, d**NaM**TP at 25 µM, d**CNMO**TP at 125 µM, and d**CNMO**TP at 25 µM. Lanes 1–4 show replication under these conditions in context 1. Lanes 5–8 show replication under these conditions in context 2. Lanes 9–12 show replication under these conditions in context 3. Lanes 13–16 show replication under these conditions in context 4. Lane P1 shows PCR products directly from *in vitro* constructed plasmids of context 1. Lane P2 shows PCR products directly from *in vitro* constructed plasmids of context 2. Lane P3 shows PCR products directly from *in vitro* constructed plasmids of context 3. Lane P4 shows PCR products directly from *in vitro* constructed plasmids of context 4.

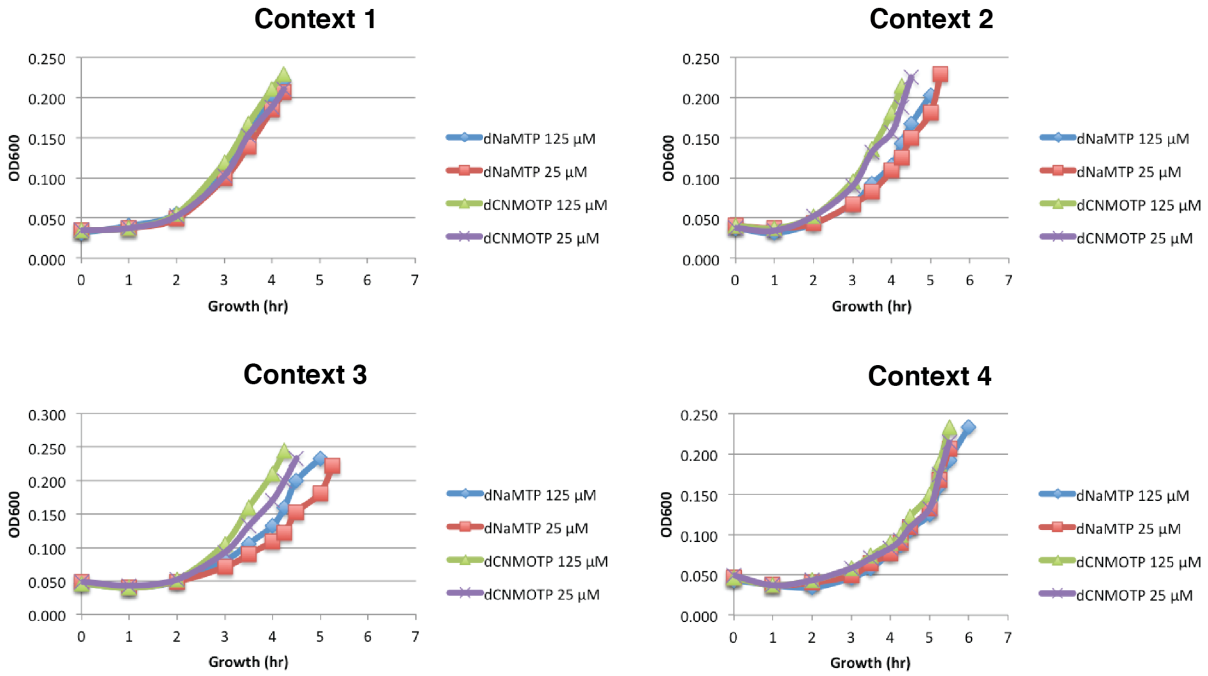

Figure S2. Growth curves of a single trial for the data presented in Figure 6.

References

(1) Malyshev, D. A.; Dhami, K.; Lavergne, T.; Chen, T.; Dai, N.; Foster, J. M.; Correa, I. R., Jr.; Romesberg, F. E. *Nature* **2014**, *509*, 385−388.

(2) Zhang, Y.; Lamb, B.; Feldman, A. W.; Zhou, A. X.; Lavergne, T.; Li, L.; Romesberg, F. E. *Proc. Natl. Acad. Sci. USA* **2017**, *114,* 1317-1322..

(3) Ogawa, A. K.; Wu, Y.; Berger, M.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.* **2000**, *122*, 8803−8804.

(4) Dhami, K.; Malyshev, D. A.; Ordoukhanian, P.; Kubelka, T.; Hocek, M.; Romesberg, F. E. *Nucleic Acids Res.* **2014**, *42*, 10235−10244.

(5) Matsuda, S.; Leconte, A. M.; Romesberg, F. E. *J. Am. Chem. Soc.* **2007**, *129*, 5551−5557.

(6) Wu, Y.; Ogawa, A. K.; Berger, M.; McMinn, D. L.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.* **2000**, *122*, 7621-7632.

(7) Hari, Y.; Hwang, G. T.; Leconte, A. M.; Joubert, N.; Hocek, M.; Romesberg, F. E. *Chembiochem* **2008**, *9*, 2796−2799.

(8) Yu, C.; Henry, A. A.; Romesberg, F. E.; Schultz, P. G. *Angew. Chem. Int. Ed.* **2002**, *41*, 3841−3844.

(9) Lavergne, T.; Degardin, M.; Malyshev, D. A.; Quach, H. T.; Dhami, K.; Ordoukhanian, P.; Romesberg, F. E. *J. Am. Chem. Soc.* **2013**, *135*, 5408−5419.

(10) Leconte, A. M.; Hwang, G. T.; Matsuda, S.; Capek, P.; Hari, Y.; Romesberg, F. E. *J. Am. Chem. Soc.* **2008**, *130*, 2336−2343.

(11) Matsuda, S.; Romesberg, F. E. *J. Am. Chem. Soc.* **2004**, *126*, 14419−14427.

(12) Henry, A. A.; Yu, C.; Romesberg, F. E. *J. Am. Chem. Soc.* **2003**, *125*, 9638−9646.

(13) Li, L.; Degardin, M.; Lavergne, T.; Malyshev, D. A.; Dhami, K.; Ordoukhanian, P.; Romesberg, F. E. *J. Am. Chem. Soc.* **2014**, *136*, 826−829.

(14) Henry, A. A.; Olsen, A. G.; Matsuda, S.; Yu, C.; Geierstanger, B. H.; Romesberg, F. E. *J. Am. Chem. Soc.* **2004**, *126*, 6923−6931.

(15) Seo, Y. J.; Hwang, G. T.; Ordoukhanian, P.; Romesberg, F. E. *J. Am. Chem. Soc.* **2009**, *131*, 3246−3252.

(16) Matsuda, S.; Henry, A. A.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.* **2003**, *125*, 6134−6139.

(17) Berger, M.; Luzzi, S. D.; Henry, A. A.; Romesberg, F. E. *J. Am. Chem. Soc.* **2002**, *124*, 1222−1226.

(18) Heo, J.-Y.; Hwang, G. T. *Bull. Korean Chem. Soc.* **2010**, *31*, 3794−3796.

(19) Seo, Y. J.; Malyshev, D. A.; Lavergne, T.; Ordoukhanian, P.; Romesberg, F. E. *J. Am. Chem. Soc.* **2011**, *133*, 19878−19888.