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Amplification, Mutation, and Sequencing of a Six-Letter Synthetic Genetic System

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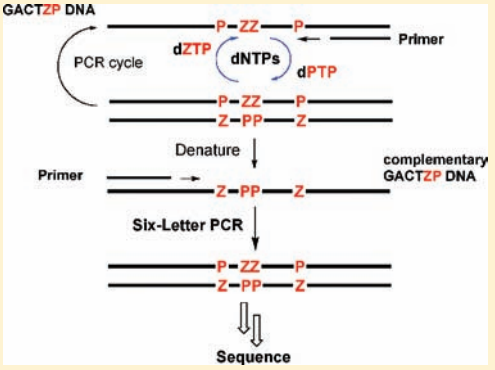
Supporting Information

ABSTRACT: The next goals in the development of a synthetic biology that uses artificial genetic systems will require chemistry−biology combinations that allow the amplification of DNA containing any number of sequential and nonsequential nonstandard nucleotides. This amplification must ensure that the nonstandard nucleotides are not unidirectionally lost during PCR amplification (unidirectional loss would cause the artificial system to revert to an all-natural genetic system). Further, technology is needed to sequence artificial genetic DNA molecules. The work reported here meets all three of these goals for a six-letter artificially expanded genetic information system (AEGIS) that comprises four standard nucleotides (G, A, C, and T) and two additional nonstandard nucleotides (Z and P). We report polymerases and PCR conditions that amplify a wide range of GACTZP DNA sequences having multiple consecutive unnatural synthetic genetic components with low (0.2% per theoretical cycle) levels of mutation. We demonstrate that residual mutation processes both introduce *and* remove unnatural nucleotides, allowing the artificial genetic system to evolve as such, rather than revert to a wholly natural system. We then show that mechanisms for these residual mutation processes can be exploited in a strategy to sequence "six-letter" GACTZP DNA. These are all not yet reported for any other synthetic genetic system.



INTRODUCTION

Efforts to develop analogues of nucleic acids to support various synthetic biologies[1,2] are characterized by chemical successes that now must confront biological realities. Over a dozen nonstandard genetic molecules have been prepared,[3] including many with nucleotide modifications and expansions. Proposed additions to DNA alphabets include pairing based on different hydrogen bonding patterns,[4,5] steric complementarity,[6,7] and even no hydrogen bonds at all.[8,9]

The ability of these analogues to bind specifically and orthogonally to natural DNA makes several of them important in clinical diagnostics. For example, an alternative genetic system that expands the number of nucleotides in DNA by rearranging hydrogen bonding patterns to give a six-letter artificially expanded genetic information systems (AEGIS)[10] today personalizes the care of 400 000 patients infected with viral diseases such as HIV and hepatitis.[11]

However, these chemical successes have forced synthetic biologists to encounter the realities of natural biochemistry, which has evolved for billions of years to accept G, A, C, and T. Although many natural polymerases can accept some components of unnatural genetic systems, all synthetic genetic systems examined to date suffer unidirectional *losses* when amplifying unnatural components.[12] In addition, their performance is generally limited to the acceptance of single unnatural nucleotides or nonadjacent unnatural pairs;[13] template-based synthesis of DNA containing runs of consecutive nonstandard base pairs is largely unknown.

Clinical diagnostics can use architectures where polymerases add single nonstandard nucleotides. For example, such single AEGIS addition supports architectures that diagnose respiratory diseases[14] and detect cystic fibrosis mutations.[15] However, a fully functioning synthetic genetic system also needs to support PCR amplification of *any* oligonucleotide sequence built from six or more different building blocks.

Further, the tools that we take for granted in standard molecular biology are unavailable to most synthetic genetic systems. In particular, rapid sequencing methods are not available for any artificial genetic alphabet. Instead, most artificial genetic sequences are analyzed on individual exemplars using difficult methods.[16]

To overcome these barriers for synthetic genetics, we focused on what is known about the chemical details of molecular interactions between DNA polymerases and DNA. For example, some time ago, Joyce, Steitz, and others noted that all four standard nucleotides (G, A. C, and T, or GACT) present electron density to the minor groove,[17] either from N3 of the purines or from the exocyclic oxygen of the pyrimidines (green lobes, Figure 1).

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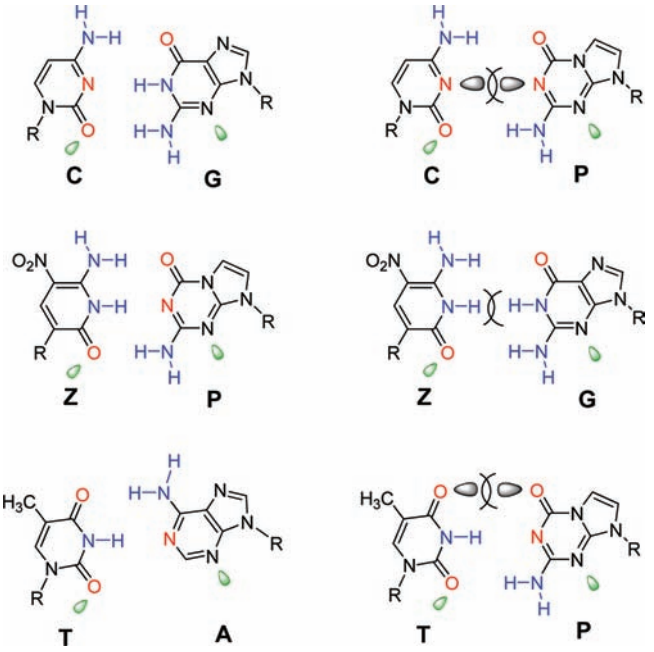

Figure 1. Expanded GACTZP genetic system. Left column: Matched C:G, Z:P, and T:A pairs all fit the Watson−Crick geometry (a small pyrimidine analogue with one ring complements in size a large purine analogue with two rings, and all but A:T are joined by three hydrogen bonds). Electron density presented to the minor groove is represented as shaded green lobes. Right column: Mismatched C:P, Z:G, and T:P pairs. Note clashes between electrons (gray lobes) or hydrogens, which can be mitigated by protonation/deprotonation, respectively.

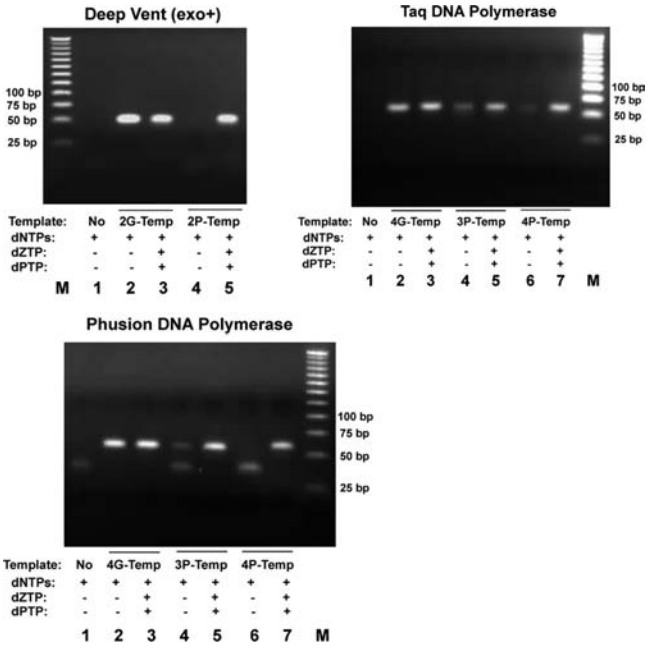

Figure 2. Agarose gel (3%) resolving amplicons from "six-letter" GACTZP PCR with standard templates and synthetic templates containing multiple consecutive dPs. Lane 1: Control without template. Lane 2: Amplification of standard template, without dZTP and dPTP. Lane 3: Amplification of standard template, with dZTP and dPTP. Lanes 4 and 6: Amplification of synthetic template, without dZTP and dPTP. Lanes 5 and 7: Amplification of synthetic template, with dZTP and dPTP. dNTPs (0.1 mM for each), dZTP (0.05 mM), and dPTP (0.6 mM). M: 25 bp marker. See Methods and Materials for PCR conditions.

Little else in common is presented by G, A, C, and T to either groove. Therefore, Joyce, Steitz, and co-workers suggested their "minor groove scanning hypothesis", which holds that polymerases seek this electron density as a way of achieving uniform acceptance of their four substrates. Although we and others have successfully copied single, nonconsecutive unnatural nucleotides lacking this electron density, often with mutated polymerases,[18] we reasoned that if we were to move forward in our development of synthetic genetic systems, the easiest path would be to accommodate this need of natural polymerases arising from billions of years of evolution.

Two matched components of the AEGIS synthetic genetic system present this electron density to the minor groove (Figure 1), at the same time having rearranged hydrogen bonding, permitting them to pair exclusively with each other, and not with GACT nucleobases. These are 6-amino-5-nitro-3-(1′-β-D-2′-deoxyribofuranosyl)-2(1H)-pyridone and 2-amino-8-(1′-β-D-2′-deoxyribofuranosyl)-imidazo[1,2-*a*]-1,3,5-triazin-4(8H)-one), respectively, implementing hydrogen bond *donor*−*donor*−*acceptor* and *acceptor*−*acceptor*−*donor* patterns on small and large nucleobases. We trivially named these **Z** and **P**. These particular implementations were also chosen as the fifth and sixth nucleobase[19,20] because, in addition to their presenting the scanned electron density, they are also insensitive to both oxidation[21] and epimerization.[22]

We report here polymerases that support PCR amplification of six-letter GACTZP DNA having essentially any sequence, including sequences containing as many as four consecutive nonstandard nucleotides. Error under optimized conditions was shown to be ca. 0.002 per theoretical cycle for both gain and loss of **Z**:**P** pairs. Furthermore, the chemical mechanism for the residual mutation was studied and was found to resemble mechanisms for mutation in standard DNA. The resulting understanding of mechanisms of mutation allowed us to manipulate mutation rates to maximize the *loss* of Z and P during copying and PCR amplification. This loss allowed us to develop procedures to exploit standard sequencing methods to sequence GACTZP DNA. This provides a critical analytical tool to advance this particular synthetic biology.

METHODS AND MATERIALS

Phosphoramidites, Triphosphates, and Polymerases. Protected phosphoramidites of the nonstandard nucleosides d**Z** (protected as the O-NPE ether) and d**P**, and the triphosphates d**Z**TP and d**P**TP were obtained from the Foundation for Applied Molecular Evolution (www.ffame.org, phosphoramidite of "d**Z**" (cat. # DZPhosphor-101), Phosphoramidite of "d**P**" (cat. # DPPhosphor-102), d**Z**TP (cat. # DZTP-ZY101), 117 d**P**TP (cat. # DPTP-ZY102)). Polymerases were obtained from New England Biolabs. GACT DNA was obtained from IDT (Coralville, IA). Other reagents were obtained from Promega and Sigma-Aldrich, and used as received.

GACTZP Oligonucleotide Synthesis. Oligonucleotides containing d**Z** and d**P** were synthesized using standard phosphoramidite chemistry on an ABI 394 DNA synthesizer on controlled pore glass supports. Following synthesis, oligonucleotides containing d**Z** were first treated with 1 M DBU in anhydrous acetonitrile to remove the O-protection group (O-NPE either), following the standard deprotection procedure in aqueous concentrated ammonia overnight at 55 °C. The deprotection procedure for oligonucleotides containing d**P** is the same as the standard procedure.[19]

Table 1. Oligonucleotides Used in "Six-Letter" GACTZP PCR and Sequencing[a]

2P-Temp:	5'-GCGTAATACGACTCACTATAGACGAPPCTACTTTAGTGAGGGTTAATTCGC-3'
2Z-Temp:	3'-CGCATTATGCTGAGTGATATCTGCTZZGATGAAATCACTCCCAATTAAGCG-5'
3P-Temp:	5'-GCGTAATACGACTCACTATAGACACTPPPTACTCACTTTAGTGAGGGTTAATTCGC-3'
3Z-Temp:	3'-CGCATTATGCTGAGTGATATCTGTGAZZZATGAGTGAAATCACTCCCAATTAAGCG-5'
4P-Temp:	5'-GCGTAATACGACTCACTATAGACACTPPPPTACTCACTTTAGTGAGGGTTAATTCGC-3'
4Z-Temp:	3'-CGCATTATGCTGAGTGATATCTGTGAZZZZATGAGTGAAATCACTCCCAATTAAGCG-5'
2G-Temp:	5'-GCGTAATACGACTCACTATAGACGAGGCTACTTTAGTGAGGGTTAATTCGC-3'
4G-Temp:	5'-GCGTAATACGACTCACTATAGACACTGGGGTACTCACTTTAGTGAGGGTTAATTCGC-3'
ZZ-2P:	5'-GACACTAGTAGCACTCACTATACGTGACTCPTCACZZAGTGCPACTACGGTCATAGCTGTTTCCTGTGTGCGA-3'
PP-2Z:	3'-CTGTGATCATCGTGAGTGATATGCACTGAGZAGTGPPTCACGZTGATGCCAGTATCGACAAAGGACACACGCT-5'
Bsp-Z:	5'-CTAGGACGACGGACTGCCTATGAGAGACATGA*GGGCCZ*GGTACCATCGATACGTTGCGATCGCTCCTTCCTG-3'
Bsp-P:	3'-GATCCTGCTGCCTGACGGATACTCTCTGTACT*CCCGGP*CCATGGTAGCTATGCAACGCTAGCGAGGAAGGAC-5'
Bsp-C:	5'-CTAGGACGACGGACTGCCTATGAGAGACATGA*GGGCCC*GGTACCATCGATACGTTGCGATCGCTCCTTCCTG-3'
Bsp-G:	3'-GATCCTGCTGCCTGACGGATACTCTCTGTACT*CCCGGG*CCATGGTAGCTATGCAACGCTAGCGAGGAAGGAC-5'

[a] Underlined sequences are either primers or primer binding sequences. The recognition sequences of restriction endonuclease Bsp120I is shown in underlined italic letters.

Polymerase Extension Reading through Multiple Consecutive Nonstandard Nucleotides. 5′-^{32}P-Labeled primer (Primer-F1 or Primer-R1, 0.2 pmol of radio-labeled primer plus 4 pmol of non-radio-labeled primer, final concentration 70 nM) was annealed to a template containing multiple consecutive nonstandard nucleobases (d**P** or d**Z**, 6 pmol, final concentration 100 nM) in 1× ThermoPol polymerase reaction buffer (pH = 8.0 at room temperature) or 1× HF Phusion buffer (pH = 8.3 at room temperature) by heating at 96 °C for 5 min and then slow cooling (0.5 h) to room temperature. dNTPs (final 0.1 mM for each) or dNTPs, d**Z**TP, and d**P**TP (final 0.1 mM for each) were added at room temperature. The mixture was preheated at 72 °C for 30 s. Extension was initiated by adding *Taq* (2.5 units), Deep Vent (exo$^+$, 2 units), or Phusion (1 unit) DNA polymerase to give a final volume of 60 μL. The primer was extended at 72 °C and aliquots (7 μL) were taken from each reaction at time intervals (1, 2, 4, 8, and 16 min), quenched by PAGE loading/quench buffer (10 μL, 10 mM EDTA in formamide). Samples were resolved by electrophoresis using a 16% PAGE (7 M urea). The gel was analyzed using MolecularImager software. See Supporting Information Table S1 for the sequences of primer and template and Figure S1 for gel images.

PCR Amplification of the Synthetic GACTZP DNA (Figure 2). Six-letter PCR amplification of GACTZP DNA containing multiple consecutive nonstandard nucleobases (2P-Temp, 3P-Temp, and 4P-Temp, final 0.5 nM for each, Table 1) was carried out in 1× ThermoPol reaction buffer (pH = 8.0 at room temperature, for Deep Vent (exo$^+$) and *Taq* DNA polymerase, respectively), or 1× HF Phusion buffer (pH = 7.0 at room temperature, for Phusion DNA polymerase), 0.5 μM of each Primer-F1 and Primer-R1, four standard dNTPs (each 0.1 mM), dZTP (0.05 mM), dPTP (0.6 mM), and 0.05 unit/μL DNA polymerase (*Taq*) or 0.02 unit/μL (Deep Vent (exo$^+$) and Phusion, respectively) on the DNAEngine Peltier Thermal Cycler (Bio-Rad) in a total volume of 50 μL. The following PCR conditions were used: one cycle of 95 °C for 2 min; followed by 21 cycles of (95 °C for 20 s, 58 °C for 25 s, 72 °C for 3 min); and finally 72 °C for 10 min. Upon completion of PCR, samples (10 μL) were taken from each PCR mixture, mixed with 6× agarose loading dye (2 μL, Promega), and analyzed on a 3% agarose gel. See Figure 2 for gel images.

Mutation Interconverting Z:P, C:G, and T:A Pairs (Figure 3). *a. The "Forward" Mutation Converting C:G pairs into **Z**:**P** Pairs Using Digestion with the Restriction Endonuclease (Bsp120I).* Eight parallel PCRs were performed in 1× ThermoPol buffer at two different pHs (8.8 and 8.0 at 25 °C). The PCR mixture contained identical amounts of primers, (Primer-F3 (5 pmol) and Primer-R3 (1 pmol of 5′-^{32}P-labeled primer and 4 pmol of non-^{32}P-labeled primer, each 250 nM final, template (Bsp-G, 0.25 nM final), four standard dNTPs (0.2 mM each), and JumpStart *Taq* DNA polymerse (0.075 unit/μL, Sigma). Two nonstandard nucleotide triphosphates, d**Z**TP and d**P**TP (each 0.2 mM), were absence or present in each PCR mixture (see Figure 3a for details). The PCR mixtures (20 μL of total volume) were cycled using the following conditions: one cycle of 95 °C for 1 min; followed by 26 cycles of (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min); and finally 72 °C for 10 min. After PCR amplification, samples (5 μL) were taken from each PCR mixture, mixed with PAGE loading/quench buffer (7 μL, 10 mM EDTA in formamide), and resolved by electrophoresis using 10% PAGE (7 M urea). The gel was analyzed using MolecularImager software. The results shown all primers were consumed and PCR amplicon was produced with the expected length. Then, another 1 μL of the PCR mixture was digested with Bsp120I (0.5 μL, final 0.5 units/μL) in 1× Buffer B (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA, pH 7.5) at 37 °C for 20 h (10 μL of reaction volume). An additional 0.5 μL of Bsp120I was added to the digestion mixture and incubated for another 20 h. The digestion products were resolved on 10% PAGE gel (7 M urea) and visualized by autoradiography (see Figure 3a for results).

*b. Measuring the "Reverse" Mutation of **Z**:**P** Pair to Give C:G and T:A Pair Using Restriction Endonuclease (Bsp120I).* Six parallel PCRs were performed in 1× ThermoPol buffer at two different pHs (8.8 and 8.0, measured at 25 °C). The PCR mixture contained identical amounts of primers (Primer-F3 (5 pmol) and Primer-R3 (1 pmol of 5′-^{32}P-labeled primer and 4 pmol of non-^{32}P-labeled primer), each 250 nM final, synthetic templates (Bsp-Z and Bsp-P, Table 1, each 0.25 nM final), four standard dNTPs (200 μM each), various amounts of d**Z**TP and d**P**TP (20 μM (lane 1), 10 μM (lane 2), and 5 μM (lane 3), respectively, Figure 3b), and JumpStart *Taq* DNA polymerse (0.075 unit/μL, Sigma). The PCR mixture (20 μL of total volume) was cycled (26 rounds of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min). Upon the consumption of all of the primers, the PCR mixture (1 μL) was digested with BsP120I (0.5 μL, final 0.5 units/μL) in 1× Buffer B (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA, pH 7.5) at 37 °C for 20 h (10 μL of reaction volume). An additional 0.5 μL of Bsp120I was added to the digestion mixture and incubated for another 20 h. The digestion products were

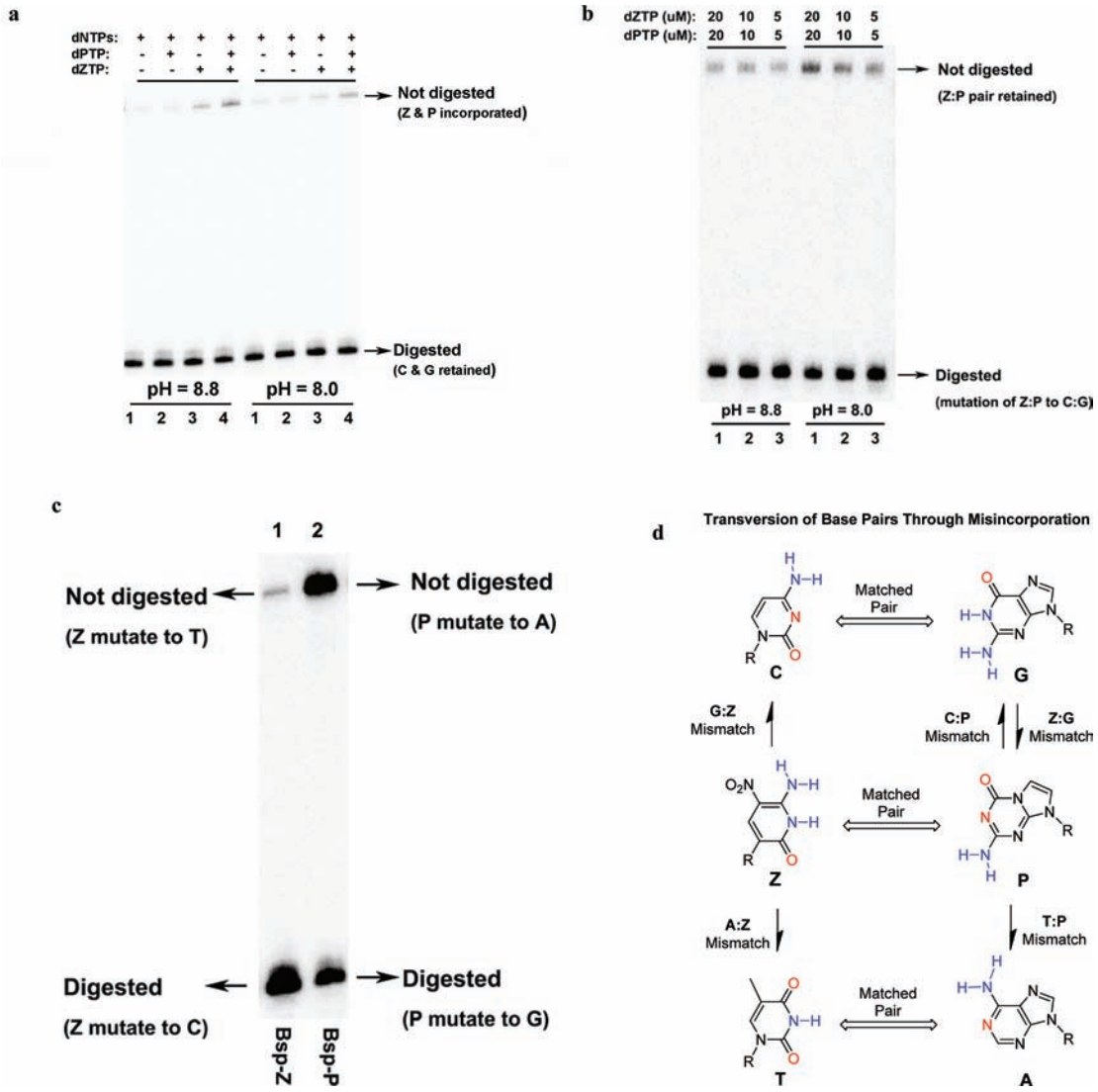

Figure 3. Mutation interconverting Z:P, C:G and T:A Pairs. (a) Measuring the "forward" mutation converting C:G pairs into **Z**:**P** pairs using digestion with the Bsp120I restriction endonuclease. Standard oligonucleotide (Bsp-G, Table 1) containing the Bsp120I recognition sequence (5′-GGGCCC-3′) were 1000-fold amplified using *Taq* DNA polymerase at pH = 8.8 or 8.0 with standard dNTPs (0.2 mM of each) with or without d**Z**TP and d**P**TP. Then, PCR amplicon was digested by endonuclease (Bsp120I). Lane 1: In the absence of both d**Z**TP and d**P**TP. Lane 2: With d**P**TP (0.2 mM). Lane 3: With d**Z**TP (0.2 mM). Lane 4: With both d**Z**TP and d**P**TP (0.2 mM for each). Not digested: indicates the fraction of PCR product resisted endonuclease digestion. Digested: indicates the fraction of PCR product was digested. See Methods and Materials for PCR conditions. (b) Measure of the "reverse" mutation of **Z**:**P** to give C:G and T:A using restriction endonuclease. Two complementary synthetic templates (Bsp-Z and Bsp-P, Table 1) containing 5′-GGGCC**Z**-3′ and 3′-CCCGG**P**-5′, were 1000-fold amplified using *Taq* with standard dNTPs (200 μM), d**Z**TP and d**P**TP (with various concentration, lane 1 (20 μM), lane 2 (10 μM), lane 3 (5 μM)), then, PCR amplicon were digested by endonuclease (Bsp120I). See Methods and Materials for details. (c) Measuring the mutation of **Z** into C and T (left) and **P** into A and G (right) using restriction endonuclease digestion. Single-stranded synthetic oligonucleotide containing either 5′-GGGCC**Z**-3′ (left, lane 1, Bsp-Z) or 3′-CCCGG**P**-5′ (right, lane 2, Bsp-P) was 1000-fold amplified using *Taq* with only four standard dNTPs (0.2 mM) in 1× ThermoPol reaction buffer (pH 8.8 at room temperature). Then, PCR amplicon was digested by endonuclease (Bsp120I). See Methods and Materials for details. (d) Observed pathways of mutation between nonstandard nucleotides and standard nucleotides. Conversion of C:G pair to Z:P pair (forward mutation) involves mis-incorporation of d**Z**TP opposite template-G to form Z:G mismatch at high pH (8.8) (panel a). Conversion of Z:P pair to C:G pair (reverse mutation) involves two pathways: (1) the most likely pathway, mis-incorporation of dGTP opposite template-Z to form G:Z mismatch at high pH (8.8) (panel b); (2) mis-incorporation of dCTP opposite template-P to form C:P mismatch (panel c, lane 2). Conversion of Z:P pair to T:A pair (reverse mutation) involves two pathways: (1) the most likely pathway, mis-incorporation of dTTP opposite template-P to form T:P mismatch; (2) mis-incorporation of dATP opposite template-Z to form A:Z mismatch (panel c). See Figure 1 for corresponding matched and mismatched base pairs.

resolved on 10% PAGE gel (7 M urea) and visualized by autoradiography (see Figure 3b for results).

*c. Measuring the Conversion of **Z** into C and T and **P** into A and G Using the Restriction Endonuclease Bsp120I.* In 1× ThermoPol reaction buffer (pH 8.8 at 25 °C) and 0.2 mM of each four standard dNTPs (without d**Z**TP and d**P**TP), single-stranded synthetic template Bsp-Z (Figure 3c, lane 1) or Bsp-P (Figure 3c, lane 2) was 1000-fold amplified with primers (Primer-F3 and Primer-R3) using JumpStart *Taq* DNA polymerse (0.08 unit/μL, Sigma). Upon the completion of PCR amplification, 1 μL of PCR mixture was digested with BsP120I (1 μL,

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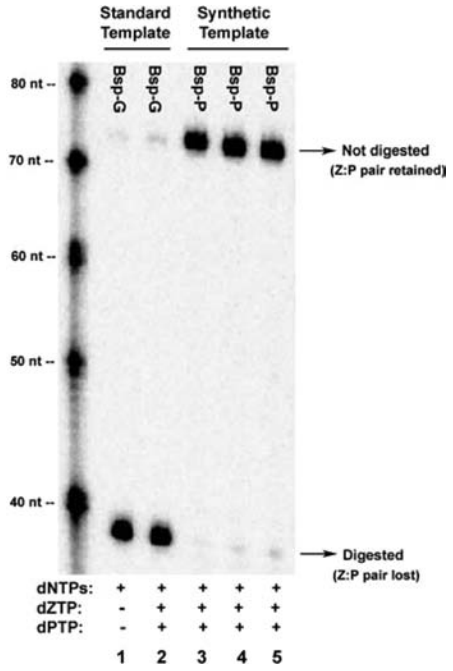

Figure 4. Measuring the retention and mutation of Z:P pair in optimized six-letter PCR. Standard template (Bsp-G, Table 1) and synthetic template (Bsp-P, Table 1) were amplified using *Taq* DNA polymerase under 1× Thermopol buffer (pH 8.0), followed by endonuclease digestion (Bsp120I). dA,T,G/TPs = 0.1 mM, dCTP = 0.4 mM, d**Z**TP = 0.05 mM, and d**P**TP = 0.6 mM. Lanes 1 and 2: Standard template was amplified 10^4-fold using *Taq*, without (lane 1) and with (lane 2) d**Z**TP and d**P**TP. Lanes 3−5: Synthetic template, 10^3- (lane 3), 10^4- (lane 4), and 10^5- (lane 5) fold amplification, with both d**Z**TP and d**P**TP. Not digested: indicates the fraction of PCR product retained the **Z**:**P** pair and, therefore, resisted endonuclease digestion. Digested: indicates the fraction of PCR product was digested. See Methods and Materials for details.

final 1 units/μL) in 1× Buffer B at 37 °C for 20 h (10 μL of reaction volume). Then, an additional 0.5 μL of Bsp120I was added to the digestion mixture and incubated for another 20 h. The digestion products were resolved on 10% PAGE gel (7 M urea) and visualized by autoradiography (see Figure 3c for results).

Measuring the Retention and Mutation of Z:P Pair in Optimized Six-Letter PCR (Figure 4). In 1× ThermoPol reaction buffer (pH 8.0 measured at 25 °C), synthetic template (Bsp-P, Table 1) or standard template (Bsp-G, Table 1) was amplified (1000- to 100 000-fold, respectively) with primers (250 nM final concentration of Primer-F3 and Primer-R3) and dA,T,G/TPs = 0.1 mM, dCTP = 0.4 mM, d**Z**TP = 0.05 mM, and d**P**TP = 0.6 mM using JumpStart *Taq* DNA polymerse (0.08 unit/μL, Sigma). The PCR mixtures were cycled using the following conditions: one cycle of 95 °C for 1 min; followed by 31 cycles of (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min); and finally 72 °C for 10 min. Upon the consumption of primers, 1 μL of PCR mixture was digested with BsP120I (0.5 μL, final 0.5 units/μL) in 1× Buffer B at 37 °C for 20 h (10 μL of reaction volume). An additional 0.5 μL of Bsp120I was added to the digestion mixture and incubated for another 20 h. The digestion products were resolved on 10% PAGE gel (7 M urea) and visualized by autoradiography (see Figure 4 for results).

PCR Amplification of the GACTZP DNA and Sanger Sequencing of the PCR Products (Figure 5 and Supporting Information Table S2). Synthetic GACTZP DNA containing various numbers of **Z** and **P** nucleotides incorporated at various positions, adjacent and spaced apart (final 0.04 nM of each, Supporting Information Table S1), were amplified in 1× ThermoPol reaction buffer (pH = 8.0, measured at room temperature) containing primers (0.4 μM each of Primer-F1 and Primer-R1, or Primer-F2 and Primer-R2, or Primer-F3 and Primer-R3), dA,T,G/TPs (each 0.1 mM), dCTP (0.2 mM to 0.4 mM), dZTP (0.05 mM), dPTP (0.6 mM), and 0.05 unit/μL of JumpStart *Taq* DNA polymerase in a total volume of 50 μL. The following PCR conditions were used: one cycle of 95 °C for 1 min; followed by 21 cycles of (95 °C for 20 s, 58 °C for 25 s, 72 °C for 3 min); and finally 72 °C for 10 min. Upon the completion of the PCR, samples (10 μL) were taken from each PCR mixture, mixed with 6× agarose loading dye (2 μL, Promega), and analyzed on agarose gel.

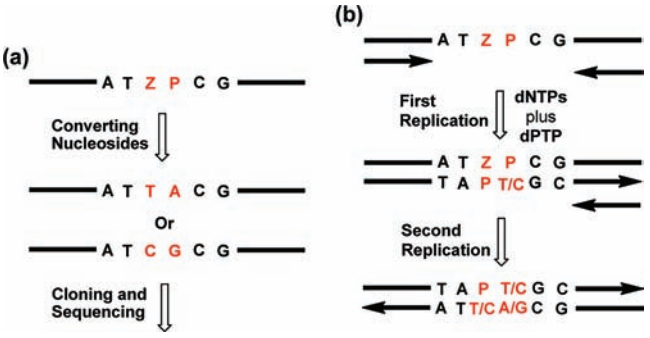

Figure 5. Strategy for sequencing GACTZP DNA. (a) Positions of **Z** and **P** in an amplicon are inferred by a process that converts **Z**:**P** pairs into a mixture of T:A pairs and C:G pairs, followed by standard Sanger sequencing. Comparison of the resulting sequences shows only T:A or C:G pairs at sites where T:A or C:G pairs were present in the initial amplicon, but mixtures of T:A and C:G pairs at sites where **Z**:**P** pairs were present in the initial amplicon. (b) Manipulation of the concentrations of d**P**TP without d**Z**TP allows stepwise conversion of **Z**:**P** pairs into C:G pairs or into T:A pairs.

As a first step toward sequencing, the remaining single stranded primers and excess triphosphates were degraded in the amplicon mixture by incubating aliquots (20 μL) of the PCR mixture with ExoSAP-IT (8 μL, USB, Cleveland, OH) at 37 °C for 30 min and then at 80 °C for 15 min. Double stranded amplicons were then recovered by using the Qiaquick Nucleotide Remove Kit (Qiagen, Valencia, CA). The GACTZP DNA was eluted from the spin column using EB buffer (200 μL, 10 mM Tris.Cl, pH 8.5) and sequenced following the strategies and protocols described below.

The purified GACTZP DNA was further amplified using JumpStart *Taq* DNA polymerse (0.05 unit/μL) in 1× ThermoPol reaction buffer (pH = 8.8 at room temperature), 0.25 μM of each Primer (Primer-F1 and Primer-R1, or Primer-F2 and Primer-R2, or Primer-F3 and Primer-R3, Supporting Information Table S1), four standard dNTPs (final 0.2 mM of each), and d**P**TP (final 0.2 mM, Figure 5b). The following PCR conditions were used: one cycle of 95 °C for 1 min; followed by 25 cycles of (95 °C for 20 s, 58 °C for 25 s, 72 °C for 1.5 min); and finally 72 °C for 15 min. Upon completion of PCR, PCR products were analyzed by agarose gel electrophoresis.

Fresh PCR products were cloned into the pCR2.1-TOPO vector and transformed into the recombinant vector into One Shot DH5α-T1^R chemically competent cells using the TOPO TA Cloning Kits (Invitrogen, Carlsbad, CA). Blue-white screening gave 24−40 colonies that were submitted for Sanger sequencing (BioBasic, Canada). The sequence results are shown in Supporting Information Table S2.

RESULTS

Polymerases to amplify GACTZP six-letter DNA were screened using templates containing various numbers of **Z** and **P** nucleotides adjacent and apart (Table 1). Consistent with the

minor groove scanning hypothesis, and different from experiments with AEGIS components that do not present electron density to the minor groove,[18] no polymerase rejected the **Z**:**P** pairs entirely. Further, polymerases accepting **Z**:**P** pairs at single site also accepted **Z**:**P** pairs when spaced apart (data not shown).

However, some polymerases had difficulty accepting multiple consecutive **Z** and **P** nucleotides. For example, Deep Vent (exo$^+$) accepted two consecutive template-**P**'s and **Z**'s but not three. *Taq* and Phusion, in contrast, incorporated d**Z**TP opposite three and four consecutive **P**'s, and d**P**TP opposite three and four consecutive **Z**'s (Supporting Information Figure S1). Phusion and *Taq* DNA polymerases also PCR-amplified templates containing three and four consecutive **Z** and **P** nucleotides with efficiencies only slightly lower than that with standard DNA (Figure 2, lanes 2, 3, 5, 7). Here, the retention of artificial bases in the PCR products obtained from *Taq* was verified by sequencing (see below, Supporting Information Tables S1 and S2). Performance at this level has not been seen with any other artificial genetic system, including those developed in this laboratory.[12,18] The relative facility with which this performance is obtained might be viewed as being consistent with the Steitz−Joyce scanning hypothesis.

But what about fidelity? To follow the misincorporation of **Z** and **P** into standard sequences and the loss of **Z**:**P** pairs from GACTZP DNA, we exploited our observation that the Bsp120I restriction endonuclease does not cleave its recognition sequence (5′-GGGCCC-3′) if any C or G is replaced by **Z** or **P**.[23] A DNA molecule containing a 5′-GGGCCC-3′ sequence was amplified by *Taq* PCR (at pH 8.8 or 8.0) with and without d**Z**TP and d**P**TP. The amplicons were then treated with Bsp120I. In the presence of 0.2 mM of both d**Z**TP and d**P**TP (Figure 3a, lane 4), after 1000-fold PCR amplification, 16% and 6% of the amplicon obtained at pH 8.8 and 8.0 (respectively) resisted digestion (see also Supporting Information Figure S2). These results indicate *Taq* only slowly replaces C:G by **Z**:**P** pairs, with the error at high "error-generating" pH 8.8 (ca. 0.25% per theoretical cycle per site; the pH is measured at room temperature) dropping to less than 0.1% per theoretical cycle per site at the lower pH of 8.0. The observed pH dependency suggested that mismatching arises predominantly as a result of deprotonated d**Z**TP pairing with G, which becomes significant at high pH (Figure 3a, left lane 3). In contrast, dPTP pairing with C is negligible at both pHs (Figure 3a, lane 2).

These fidelity results also demonstrated the existence of small amounts of "forward" mutation, where *nonstandard* components *enter* a sequence during copying, rather than being lost (Figure 3a). In artificial genetic systems, in general, polymerases show only a natural propensity to *lose* unnatural components. This is, we believe, the first example of forward mutation in any synthetic genetic system.

Mutation of T:A pairs to **Z**:**P** pairs was even rarer. To identify rare substitutions of this kind, we exploited our observation (unpublished) that DraI does not cut at its recognition sequence (5′-TTTAAA-3′) if any site contains **Z** or **P**. Here, DNA containing a 5′-TTTAAA-3′ sequence was amplified 1000-fold with and without d**Z**TP and d**P**TP at pH 8.8 and 8.0. Here, no detectable fraction of the amplicon became resistant to cleavage (data not shown). This showed that any T:A to **Z**:**P** forward mutation was less facile than mutation of C:G to **Z**:**P** and did not occur to less than one part in ca. 50 000.

To measure the rates of "reverse" mutation that convert **Z**:**P** pairs into C:G or T:A pairs, PCR amplification was performed on a template that contained the Bsp120I recognition sequences disrupted by **Z** and **P** nucleotides under forcing conditions. Here, double-strand GACTZP DNA (Bsp-Z and Bsp-P, Table 1), containing 5′-GGGCC**Z**-3′ and 3′-CCCGG**P**-5′, was 1000-fold amplified with low concentrations of d**Z**TP and d**P**TP (5 μM to 20 μM each) and the four standard dNTPs (200 μM each). Followed by endonuclease digestion, the fraction of cleavable amplicons was used as a metric to quantitate **Z**:**P** loss. As shown in Figure 3b, most amplicon was digested by Bsp120I, indicating reverse mutation of the **Z**:**P** pair to a C:G pair, recreating the recognition sequence. At pH 8.0, loss was less than 7% per theoretical cycle at 20 μM of d**P**TP and d**Z**TP (Figure 3b, right lane 1).

To drive the loss of **Z**:**P** pairs more forcibly, DNA containing 5′-GGGCC**Z**-3′ (Bsp-Z, Table 1) or 3′-CCCGG**P**-5′ (Bsp-P, Table 1) sequences were amplified without *any* d**Z**TP or d**P**TP. Here, 95% of PCR product was digested (Figure 3c, lane 1), indicating that *Taq* incorporates both dGTP (95%) and dATP (5%) opposite template **Z** in the absence of d**P**TP, mutating **Z** into C or T. In contrast, 70% of PCR product resisted digestion (Figure 3c, lane 2), indicating that *Taq* incorporates both dTTP (70%) and dCTP (30%) opposite template **P** in the absence of d**Z**TP, mutating **P** into A or G.

The observed mutation under forcing conditions and pH dependency suggested mechanisms for mutation (Figure 3d) and conditions that might maximize the fidelity of copying GACTZP DNA. At pH 8.0 (measured at room temperature), decreasing the concentration of d**Z**TP from 0.2 to 0.05 mM significantly reduced the "forward mutation" (converting C:G pairs into Z:P pairs). This d**Z**TP concentration (0.05 mM) is also sufficient to faithfully incorporate d**Z**TP opposite template-P, and also prevents mispairing of dCTP and dTTP with template-P. This was also verified by the subsequent sequencing results in Supporting Information Figure S4.

Next, increasing the concentration of dCTP (from 0.2 to 0.6 mM) essentially eliminates mispairing of d**Z**TP with G (Supporting Information Figure S2b). Likewise, increasing the concentration of d**P**TP to 0.6 mM ensures d**P**TP pairing with template-**Z** in competition with dGTP. Last, decreasing the concentration of dA,T,G/TPs to 0.1 mM and adjusting the ratio of standard to nonstandard triphosphates finishes the optimization process. Under these optimized conditions, retention of **Z**:**P** pairs averaged 99.8% per theoretical PCR cycle, while the loss and gain of **Z**:**P** pairs is ca. 0.2% per theoretical PCR cycle (Figure 4 and Supporting Information Figure 2b). In contrast, under normal triphosphate concentrations (0.2 mM, without optimizing the concentrations), the retention of one Z−P pair is 99.2% per theoretical PCR cycle, and about 0.6% per theoretical cycle from natural to artificial base pair (for all Z/Ps in the recognition sequence) (Supporting Information Figure S2a).

Any biotechnology based on an evolvable genetic molecule built from six nucleotide letters needs analytical tools to determine its sequence. Accordingly, we developed such a tool for GACTZP DNA, which we describe here and use to show that amplicons arising from known initial sequences retain **Z** and **P** at their proper positions.

This sequencing tool exploited both the power of high throughput DNA sequencing technologies[24] and the understanding of how **Z**:**P** pairs in duplex DNA might evolve to give C:G and/or T:A pairs during PCR amplification (Figure 3d). *Opposite* of our goal while developing high fidelity six-letter PCR, which sought to *minimize* the loss of **Z** and **P**, our goal in developing sequencing tools was to *maximize* the loss of **Z** and **P**.

The sequencing procedure developed had these steps (Figure 5 and Supporting Information Figure S3):

(a) A sample of duplex GACTZP DNA is PCR-amplified using dNTPs (0.2 mM each) and *just* d**P**TP (0.2 mM), *without* d**Z**TP. Lacking d**Z**TP, any **P** in the template directs addition of either dTTP or dCTP to the primer. The presence of some d**P**TP allows any **Z** in any template to direct the incorporation of **P** in a product strand; the derived **P** subsequently directs incorporation of either T or C in the next copying step (Supporting Information Figure S3b).
(b) The products of the "conversion" PCR reaction are then shotgun cloned.
(c) Individual DNA molecules from the clones are sequenced.
(d) The resulting sequences are aligned and compared.
(e) Sites in the alignment that hold both C and T in various aligned sequences are inferred to have arisen from sites in the parent sequence that held **Z**; sites in the alignment that hold both G and A are inferred to have arisen from sites in the parent sequence that held **P**. Sites in the alignment that consistently hold G, A, C, and T in *all* of the aligned sequences are inferred to have arisen from sites in the parent sequence that held G, A, C, and T, respectively.

In more detail, sites that originally held **P** in the precursor would hold either G or A in the converted sequence as a result of steps that involved **P**:C and **P**:T mispairing (respectively) in the absence of d**Z**TP. If the mismatching is balanced, the result will generate a "G" call in half of the sequences and an "A" call in the other half. Similarly, sites that originally held **Z** will generate either a "C" call or a "T" call, through a first step involving **Z**:**P** pairing and a second involving **P**:C and **P**:T mispairing (respectively). Sites that originally held G, A, C, and T will give uniform calls in all of the sequences returned though consistent G:C and T:A pairing (*pace* an occasional PCR error). Thus, the sequence of the precursor and the positions of **Z** and **P** in that sequence can be inferred (Figure 5 and Supporting Information Figure S3).

We found that *Taq* DNA polymerase supports the needed level of mismatching of template **P** against T or C at these concentrations in the total absence of d**Z**TP (Figure 3c). In contrast, in conditions that we examined, template-**Z** in the total absence of d**P**TP directed overwhelmingly the incorporation of G (leading to amplicons where **Z** is replaced by C), not the balanced mixture of G and A that would be most useful to infer a sequence.

To demonstrate the use of "conversion PCR" to sequence GACTZP DNA, DNA molecules containing various consecutive and nonconsecutive **Z**′s and **P**′s (Supporting Information Table S1) were first amplified under optimized six-letter PCR conditions (Supporting Information Figure S3a). To convert **Z**:**P** pairs in PCR amplicon to a mixture of T:A and C:G pairs (Supporting Information Figure S3b), a second PCR was performed in 1× ThermoPol reaction buffer (pH = 8.8, measured at room temperature) with *Taq*, standard dNTPs (0.2 mM each), no d**Z**TP, and d**P**TP (0.2 mM) to further amplify the **Z**:**P** containing PCR amplicon. The second PCR products were then cloned into the pCR-2.1-TOPO plasmid and transformed into *E. coli* (DH5α), colonies were picked, plasmids were isolated and Sanger sequenced, and the separate sequencing results compared (Supporting Information Table S2).

As expected, at sites in the amplicon that originated as A:T or G:C pairs, all Sanger sequences concurred (Supporting Information Table S2). However, at sites in the amplicon that originated as **Z**:**P** pairs, the sequences differed and showed a mixture of T:A and C:G pairs at those sites. Thus, the positions of the **Z**:**P** pairs in the parent amplicons could be inferred, and were found to be where they were placed in the original template that was PCR amplified. Control experiments amplifying targets that contained no **Z**:**P** pairs in a GACT sequence (Bsp-C, Table 1) showed negligible false calls of T:A and C:G pairs.

DISCUSSION

These results show that both *Taq* and Phusion polymerases support six-letter GACTZP PCR for sequences containing up to (and including) four consecutive nonstandard synthetic nucleotides. This represents a considerable advance over the current "art", where only single or nonadjacent nonstandard nucleotides can be part of a PCR amplification. This confirms the value of the "minor groove scanning" hypothesis to guide the design of at least one synthetic genetic system.

When constructing random sequences from a six letter alphabet, with each nucleotide being present in equal amounts (16.7%), a longer run of five (or more) consecutive d**Z** or d**P** nucleotides is expected to occur only twice in ca. every 7800 sites. As a typical plasmid contains ca. 3000 nucleotides, the level of performance demonstrated here should be sufficient to ensure the replication of a plasmid-sized DNA molecule containing entirely random sequences. Work is now in progress to engineer strains of *E. coli* that accept plasmids containing **Z**:**P** pairs.

With *Taq*, the minimized error after optimization is ca. 0.002 per theoretical cycle in both directions, the "forward" direction that gains **Z**:**P** pairs and the "reverse" direction that loses **Z**:**P** pairs. Absent selection pressure, this implies that GACTZP DNA would "evolve" to randomize their sequence with respect to G, C, **Z**, and **P**, rather than lose **Z** and **P** and gradually revert to natural DNA. To date, all other artificial genetic systems evolve to lose their unnatural components.[12]

The mechanism for mutation resembles mechanisms for mutation in standard DNA, as evidenced by the dependence on pH of the mutation rate. Before deprotonation, **Z** presents to its partner a hydrogen bond donor−donor−acceptor pattern, proceeding from the major to the minor groove, a pattern that is complementary to the acceptor−acceptor−donor pattern of **P**. After deprotonation, with a $pK_a \approx 7.8$ free in solution, **Z** presents a donor−acceptor−acceptor hydrogen bonding pattern, as does standard C, and is complementary to standard G. The predominant mutation processes can be explained by **Z**:G mismatching from deprotonated **Z**.

This allows mutation to meets two goals necessary to support Darwinian evolution. The level of mutation is sufficient to allow mutation, at the same time as not being so high as to cause an "error catastrophe" in a small genome. Further, the mutation is bidirectional; because it allows **Z** to be introduced as well as lost, the unnatural system does not trivially evolve to return to an entirely natural DNA molecule.

Of special importance in this work are the procedures that allow us to sequence GACTZP DNA. Sequencing strategies were developed using various hypothetical mechanisms for mutation. These allowed us to manipulate mutation rates to controllably convert d**Z** and d**P** nucleotides to standard nucleotides that could be cloned or directly sequenced using any next generation sequencing technologies. These should allow GACTZP DNA to support SELEX experiments. Work to develop these is also underway.

■ ASSOCIATED CONTENT

Supporting Information. Polymerase extension reading through multiple consecutive nonstandard nucleobases, PCR amplification of the GACTZP DNA, and sequencing results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Author Contributions

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Notes

The authors declare that they are inventors on various patent applications covering various of the compounds and methods reported here. Correspondence and requests for materials should be addressed to S.A.B. (sbenner@ffame.org).

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