

# The use of thymidine analogs to improve the replication of an extra DNA base pair: a synthetic biological system

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## ABSTRACT

**Synthetic biology based on a six-letter genetic alphabet that includes the two non-standard nucleobases isoguanine (isoG) and isocytosine (isoC), as well as the standard A, T, G and C, is known to suffer as a consequence of a minor tautomeric form of isoguanine that pairs with thymine, and therefore leads to infidelity during repeated cycles of the PCR. Reported here is a solution to this problem. The solution replaces thymidine triphosphate by 2-thiothymidine triphosphate (2-thioTTP). Because of the bulk and hydrogen bonding properties of the thione unit in 2-thioT, 2-thioT does not mispair effectively with the minor tautomer of isoG. To test whether this might allow PCR amplification of a six-letter artificially expanded genetic information system, we examined the relative rates of misincorporation of 2-thioTTP and TTP opposite isoG using affinity electrophoresis. The concentrations of isoCTP and 2-thioTTP were optimal to best support PCR amplification using thermostable polymerases of a six-letter alphabet that includes the isoC–isoG pair. The fidelity-per-round of amplification was found to be ~98% in trial PCRs with this six-letter DNA alphabet. The analogous PCR employing TTP had a fidelity-per-round of only ~93%. Thus, the A, 2-thioT, G, C, isoC, isoG alphabet is an artificial genetic system capable of Darwinian evolution.**

## INTRODUCTION

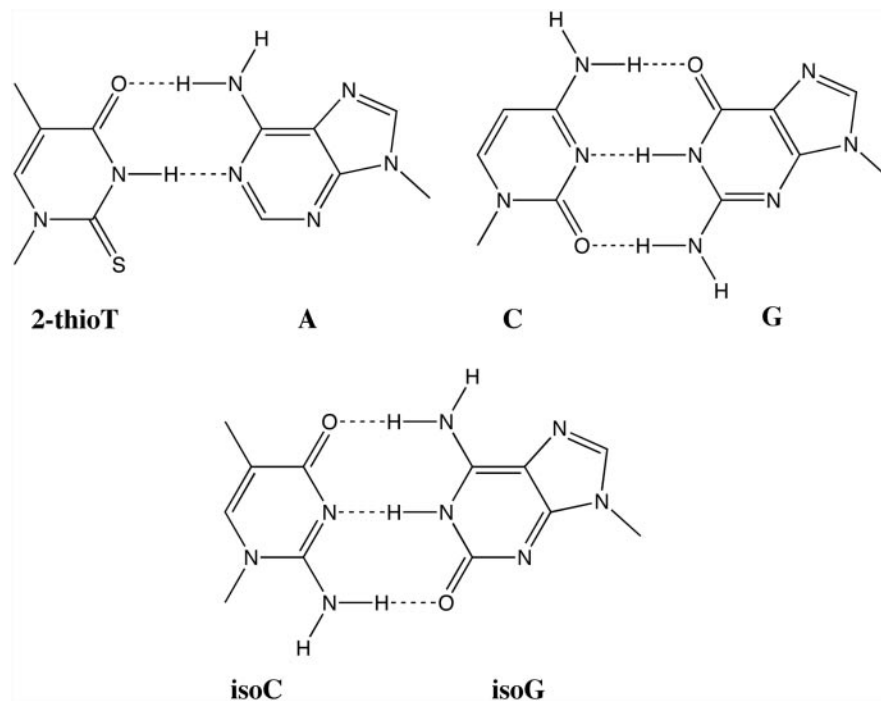
The creation of an artificially expanded genetic information system, or AEGIS, has attracted much attention in the literature

(1–6) and scientific press (7,8) in recent years. Toward this goal, we recently reported an artificial genetic system built from 6 nucleotide analogs that selectively bind to form three nucleobase pairs that bind orthogonally (Figure 1). Other laboratories are working toward similar synthetic biological systems (9–11).

A six-letter expanded genetic alphabet today supports clinical assays to quantitate through simple Watson–Crick binding the levels of human immunodeficiency virus, hepatitis B and hepatitis C viruses in patients; approximately 400 000 individuals annually have their health care improved using this synthetic biological system (12,13). The enzymatic synthesis of DNA containing AEGIS components, however, remains problematic (14–16). This is due, in part, to the highly evolved specificity of natural DNA polymerases, which have evolved for billions of years to handle the standard nucleotides (A, G, T, C). Polymerases must ensure, with high accuracy, that the correct deoxynucleotide triphosphate is paired with its complementary partner in the template, and must do so for four distinct reacting partners (17). This means that natural polymerases, especially those that operate at high temperature, have binding interactions to their substrates that cause them to discriminate against non-standard substrate structures (18–20).

Nevertheless, we recently reported the PCR amplification of DNA containing a pair between 2,4-diaminopyrimidine and xanthine (called the pyDAD:puADA base pair, because the pyrimidine presents a hydrogen bond Donor–Acceptor–Donor hydrogen bond pattern, from the major groove to the minor groove, complementary to the Acceptor–Donor–Acceptor pattern presented by the purine xanthine) (1). This was achieved using a double mutant of the reverse transcriptase from HIV I that was obtained by a combination of *in clinico* selection and rational design. This mutant amplified an oligonucleotide containing a single pyDAD over five rounds of PCR with an overall fidelity (per round) of >99% for the pyDAD:puADA base pair. A similar PCR amplification of a duplex containing

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**Figure 1.** Artificially expanded genetic information system used in this study.

an isoC:isoG (pyAAD:puDDA) base pair was achieved for several dozen rounds of amplification (2) using a fragment of DNA polymerase I from *Thermus aquaticus* that lacked the 5'→3' exonuclease domain, with a fidelity of only ~96% per round.

While >98% is a useful fidelity, the HIV RT is not thermally stable with respect to denaturation, making it inconvenient to use in a practical PCR assay. The *T.aquaticus* polymerase is, of course, thermally stable, but a fidelity of <98% per round is not adequate for most practical applications. We reasoned that we might gain both acceptable fidelity and the practicality of a thermostable polymerase if we exploited the tactic of sterically complementarity used, e.g. in the pairs proposed by Kool, Schultz, Hirao and Yokoyama. (21–23) It was assumed that the insufficient fidelity in the reported pyAAD:puDDA PCR was due to a mispairing of TTP opposite isoG. This mispairing was well documented in earlier work by Switzer *et al.* (15). As this is the most common mispair formed during replication of oligonucleotides containing isoG, inhibiting formation of this mispair could significantly enhance the fidelity of replication.

It has long been known that thiones (as in thioT) do not serve effectively as hydrogen bond acceptors in Watson–Crick pairing (24–27). For example, while a nucleobase pair between 2-thioT and adenine contributes to duplex stability (as measured by  $\Delta G$ ), as well as a pair between T and A, each pair between 2-thioT and 2-aminoadenine destabilizes the duplex by 0.8 kcal/mol (corresponding to a 2.4°C decrease in  $T_m$  in a 20 nt duplex) (28). This effect was attributed to the increased steric bulk of the C=S H–NH– unit in the minor groove of the DNA helix. As the minor tautomer of isoG, responsible for its mispairing with thymidine, displays a hydroxy group into the minor groove (2-position), we reasoned that the 2-thiothymidine-isoG (minor tautomer) base pair may also

be disfavored relative to the thymidine-isoG (minor tautomer) pair as well in the polymerase active site (Figure 2).

Here, we report experiments to show that the 'Klenow' fragment of *Taq* polymerase, which does not discriminate well against the T-isoG pair, discriminates better against the 2-thioT:isoG pair. We then optimized conditions for the discrimination and used those conditions in a PCR process.

## MATERIALS AND METHODS

### Oligonucleotides and enzymes

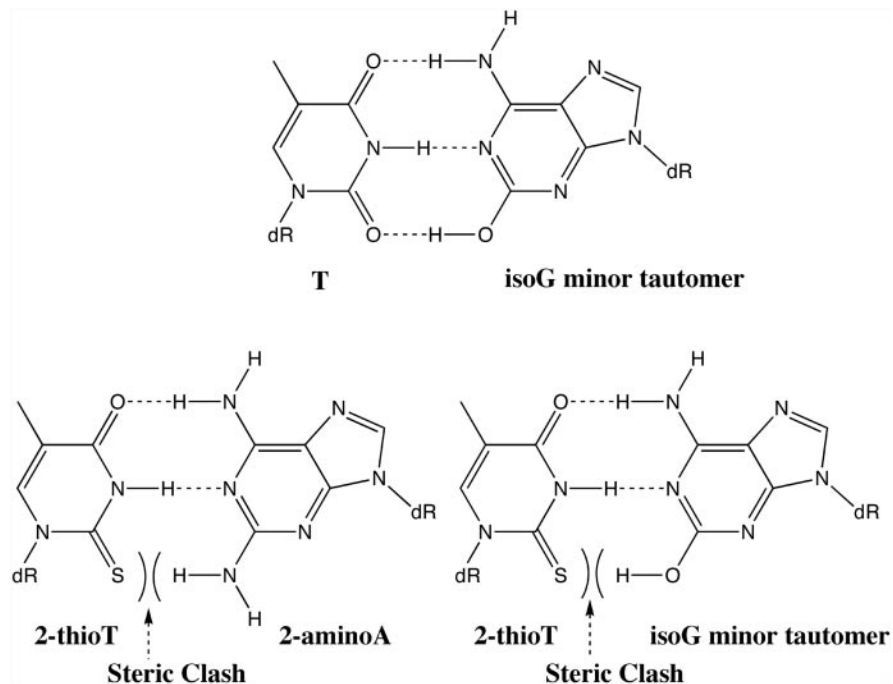
Oligonucleotides (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). All oligodeoxynucleotides used in this study were purified by PAGE (10–20%). The purity of all oligonucleotides used was >99%.

The triphosphate of isoG was purchased from ChemGenes (Wilmington, MA.) The triphosphate of isoC was synthesized by the method of Jurczyk *et al.* (29). The natural deoxynucleoside triphosphates were purchased from Promega (Madison, WI). 2-Thiothymidine triphosphate was purchased from Trilink Biotechnologies (San Diego, CA).

The 'Klenow' fragment of *Taq* polymerase (Titanium™ *Taq*) used in this study was purchased from BD Biosciences (Mountain View, CA). As Titanium™ *Taq* is a 'hot start' enzyme, the enzyme was heated to 95°C for 2 min, followed by rapid cooling to ambient temperature prior to any primer extension reactions. Similarly, all PCRs included an initial 2 min 95°C denaturation cycle.

### Primer extension reactions

In a typical primer extension reaction (25  $\mu$ l total volume), 5'-<sup>32</sup>P labeled primer (P-1, 25 pmol) and template (T-1, 30 pmol) were mixed with buffer (10 mM bis-trispropane-HCl, pH 9.1,



**Figure 2.** Steric exclusion of 2-thioT-isoG base pair. Shown is the mispair between thymidine (T) and the minor tautomer of isoG, resulting in replication infidelity. Also shown is the pair between 2-thiothymidine (2-thioT) and 2-aminoadenine, known to destabilize DNA duplexes due to the steric clash in the minor groove.

**Table 1.** Oligonucleotides used in this study

T-1 d(GTC TTC GTG TCA CG(isoG) CCA TAG TGA GTC GTA TTA CGC)
T-2 d(GCG AAT TAA CCC TCA CTA AAG TAC G(isoG)T CGT CTA TAG TGA GTC GTA TTA CGC)
T-3 d(GCG AAT TAA CCC TCA CTA AAG TAC GAT CGT CTA TAG TGA GTC GTA TTA CGC)
P-1 d(GCG TAA TAC GAC TCA CTA T)
P-2f d(GCG TAA TAC GAC TCA CTA TAG)
P-2r d(GCG AAT TAA CCC TCA CTA AAG)

40 mM potassium acetate, 5 mM magnesium chloride, 0.1 mg/ml BSA), heated (95°C, 5 min) and allowed to cool to ambient temperature over 1 h. Polymerase (1 unit) was added, and the mixture was again heated (72°C, 10 s). Each reaction was initiated by adding the appropriate dNTPs (final concentration 100 μM). Aliquots (2 μl) were taken from each reaction at the appropriate time, and the reaction quenched by dilution into PAGE loading/quench buffer (2 μl, 20 mM EDTA in formamide). Samples were then heated (95°C, 5 min) and resolved by electrophoresis using a 20% PAGE (7 M urea) gel. The gel was analyzed using MolecularImager<sup>®</sup> software.

To improve reproducibility, master mixes of the primer/template in buffer were prepared in large scale (100 μl).

### Nucleotide competition reactions

Single turnover primer extension reactions were performed by annealing 5'-<sup>32</sup>P labeled primer (P-2f, 1 pmol) and template (T-2 or T-3, 1 pmol) in the appropriate buffer as described above. Polymerase (1 pmol) was added, and the mixture was heated (72°C, 10 s). The reaction was initiated with the addition of isoCTP (100 μM) and either 2-thioTTP (100 μM) or

TTP (100 μM) in the presence of unlabeled trap DNA (P-2f, 100 pmol, T-2, 100 pmol) and the reaction was quenched (20 mM EDTA in formamide) after 20 s. The samples were resolved by electrophoresis using a 20% PAGE (7 M urea) gel containing *p*-acrylamidophenylmercury chloride (APM, 1 μg/ml). This permitted the separation of oligonucleotides containing thiothymidine (which ran slower) from those that did not. APM was synthesized as described previously (30).

### PCR amplification and fidelity assay

For each six-letter nucleotide system investigated, seven parallel PCR mixtures containing natural dNTPs (100 μM each; dATP, dCTP, dGTP and TTP or 2-thioTTP) and noncanonical nucleotide triphosphates (200 μM each; isoCTP and isoGTP) were cycled (30 rounds, 95°C:45 s/45°C:45 s/72°C:1.5 min) with identical amounts of primers P-2f (<sup>32</sup>P labeled) and P-2r (1 pmol; 6 × 10<sup>11</sup> molecules) and various concentrations of templates T-2. These were obtained by 10-fold serial dilutions (from 6 × 10<sup>4</sup> to 6 × 10<sup>10</sup> molecules per reaction). As each 10-fold dilution in template was equivalent to ~3.32 rounds of amplification, the fidelity of the isoC:isoG replication could be monitored on a round-by-round basis, with each amplicon requiring a different number of exponential amplifications to consume the primers (Table 2).

Following PCR amplification, the reaction mixtures were treated with an equal volume of acetic acid (0.1 mM) and incubated (95°C, 30 min), a procedure that depyrimidinylates the iso-cytidines that have been incorporated. The tubes were then opened, and the volatiles removed by evaporation at atmospheric pressure. Two volumes of ammonium hydroxide (0.1 mM) were then added, and incubation was continued (95°C, 5 min). This step cleaves the product DNA strands at

**Table 2.** PCR amplicons

Template molecules	$6 \times 10^{10}$	$6 \times 10^9$	$6 \times 10^8$	$6 \times 10^7$	$6 \times 10^6$	$6 \times 10^5$	$6 \times 10^4$
Primer molecules	$6 \times 10^{11}$	$6 \times 10^{11}$	$6 \times 10^{11}$	$6 \times 10^{11}$	$6 \times 10^{11}$	$6 \times 10^{11}$	$6 \times 10^{11}$
Amplification	10	$10^2$	$10^3$	$10^4$	$10^5$	$10^6$	$10^7$
Doublings (PCR rounds)	3.32	6.64	9.97	13.29	16.61	19.93	23.25

To trace the fidelity of each six-letter amplification, each amplicon shown was cycled through 30 rounds of PCR. The number of perfect doublings required to convert all primer to product is dependent on the primer to template ratio (amplification) and is calculated as described. The number of doublings is equal to the number of rounds of PCR (under ideal conditions, i.e. exponential phase) needed to consume the primer. Doublings =  $\log_2$  (number of primer molecules/number of template molecules).

the site of where isoC had been located. The ammonium hydroxide was allowed to evaporate, and the mixtures were dissolved 2-fold with gel loading buffer (98% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol FF) and analyzed by denaturing PAGE (17%). Quantitation of the cleaved (isoC containing) versus full-length (not containing isoC) product indicated the fidelity of isoC and isoG replication.

Amplicons testing the substitution of 2-thioTTP for TTP in a PCR were generated as above, using primers P-2f and P-2r (1 pmol each), template T-3 ( $6 \times 10^7$  molecules) and either all four natural dNTPs (100  $\mu$ M each) or by substituting 2-thioTTP for TTP.

### Data analysis

To estimate the fidelity-per-round of the PCRs, the percent of product containing isoC, as determined by the cleavage assay, was graphed against the number of doublings required to consume all of the primer added. The number of product molecules generated in a PCR is equal to

$$N = n2^r, \quad 1$$

where  $n$  equals the number of template molecules,  $N$  equals the number of product molecules, and  $r$  equals the number of rounds of perfect doubling required to use all primer molecules. Similarly, the number of product molecules containing isoC is equal to

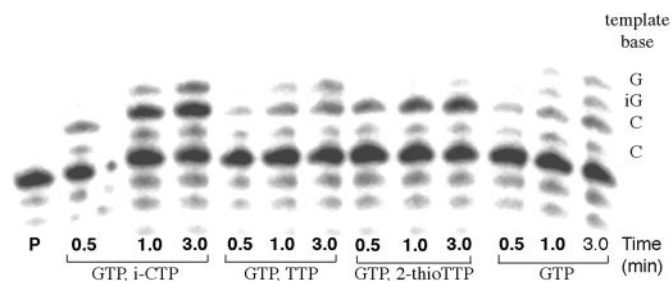
$$N_{iC} = n(1 + f)^r, \quad 2$$

where  $f$  is the fidelity per round. The percentage of the PCR product containing the isoC:isoG base pair is equal to  $N_{iC}/N$ , which simplifies to  $(1/2 + f/2)^r$ . Data from the PCR amplifications were graphed and fit to the equation  $y = 100 \times (1/2 + f/2)^x$  using the program Kaleidagraph Version 3.5; Synergy Software, Reading, PA), where  $x$  is the number of doublings (i.e. PCR rounds) as calculated in Table 2 and  $y$  is the percent cleaved product from each reaction.

## RESULTS

### Primer extension studies

Running start primer extension reactions were performed with KlenTaq polymerase to examine its ability to incorporate either TTP or 2-thioTTP opposite isoG. For each reaction, the polymerase was challenged to misincorporate the respective triphosphate opposite the isoG residue at position 22 in the template (T-1), 3 nt downstream of the primer (P-1) terminus. Reactions were run in parallel, one containing dGTP and TTP,



**Figure 3.** Primer extension reactions comparing 2-thioTTP and TTP incorporation opposite isoG. *Taq* polymerase was incubated with template (T-1), primer (P-1), dGTP and either isoCTP, TTP or 2-thioTTP at 72°C. Time points taken at 0.5, 1.0 and 3.0 min.

one containing dGTP and 2-thioTTP, one containing dGTP and isoCTP (positive control), and one containing only dGTP (negative control). Aliquots of each reaction were quenched at various times and analyzed by PAGE on a 20% polyacrylamide gel.

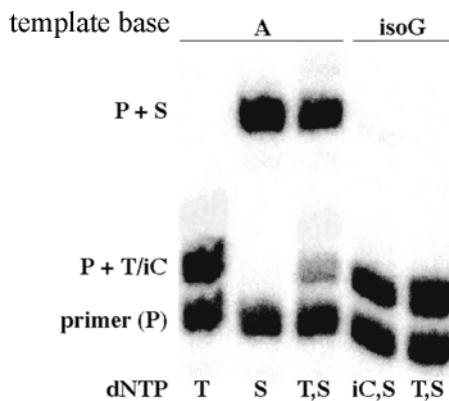
In running start primer extension reactions (Figure 3), KlenTaq polymerase incorporated all three dNTPs tested (isoCTP, TTP, 2-thioTTP) opposite an isoG in the template, with isoCTP incorporated most efficiently, followed by TTP and 2-thioTTP. It is noteworthy that after 1 and 3 min of incubation, the polymerase incorporated  $\sim$ 2-fold more isoCTP than TTP opposite isoG.

This result illustrates the well-known nonspecificity of polymerases challenged with a template containing isoG. Most polymerases also incorporate T as well as isoC opposite isoG, either via a wobble base pair or, more likely, opposite the minor tautomer of isoG that is complementary (in the Watson–Crick hydrogen bonding sense) to T. (31)

These data also show that 2-thioTTP was minimally misincorporated opposite isoG in the 0.5 and 1 min incubations. After 3 min, misincorporation gave rise to a more obvious band (Figure 3). This establishes that 2-thioTTP is misincorporated opposite isoG to a much lesser extent than is TTP. This is especially so at incubation times relevant for a typical PCR elongation step (for DNA  $\leq$  2 kb) of between 45 and 90 s.

Also observed in these data is the misincorporation of GTP opposite isoG. As it is well known that polymerases will attempt to incorporate a mismatch if the correct nucleotide is not available, such ‘minus’ experiments, wherein the correct nucleotide is not available, are not good metrics for replication fidelity in the presence of a complete set of triphosphates.

In two parallel reactions, one containing equal concentrations of 2-thioTTP and TTP, and one containing equal concentrations of 2-thioTTP and isoCTP, KlenTaq polymerase was



**Figure 4.** Direct competition studies opposite isoG. Primer extension reactions examined the incorporation of 2-thioTTP and either TTP or isoCTP opposite isoG (lanes 4 and 5, primer P-2f, template T-2). Control reactions challenged the polymerase incorporate TTP or isoCTP opposite A (lanes 1–3, primer P-2f, template T-3). S indicates 2-thioTTP; iC indicates isoCTP; P + T/iC is the product from the polymerase adding a single TTP or isoCTP to the primer (P); the product resulting from the addition of a single 2-thioTTP to the primer is annotated as P + S.

challenged to choose a nucleotide to incorporate opposite isoG. Affinity electrophoresis on a polyacrylamide gel (20%) containing *p*-acrylamidophenylmercury chloride (APM, 10  $\mu$ g/ml) was used to separate those products extended with a 2-thioT from those extended with a non-sulfur-containing dNTP (isoCTP or TTP). (30) The gel was analyzed via radioimaging.

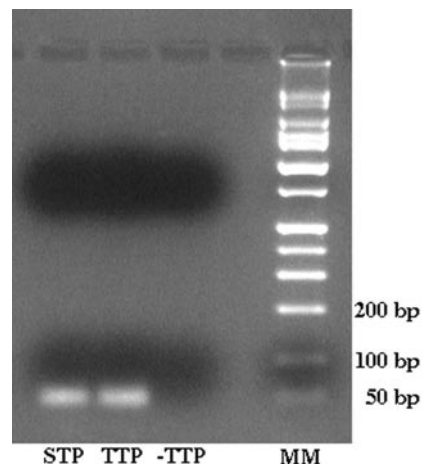
Figure 4 shows the results of the direct competition experiments, wherein the DNA containing 2-thioT migrates at a slower rate than a typical oligonucleotide due to the interaction of its thione group with the mercury in the APM. It is observed that when placed in direct competition for incorporation, the polymerase incorporates either isoCTP or TTP opposite isoG, with <1% of the extended product resulting from incorporation of 2-thioTTP. Also notable is the observation that *Taq* polymerase prefers 2-thioTTP over TTP as a substrate for incorporation opposite adenosine. This unexpected result was not observed for Family B polymerases (data not shown).

### PCR amplification

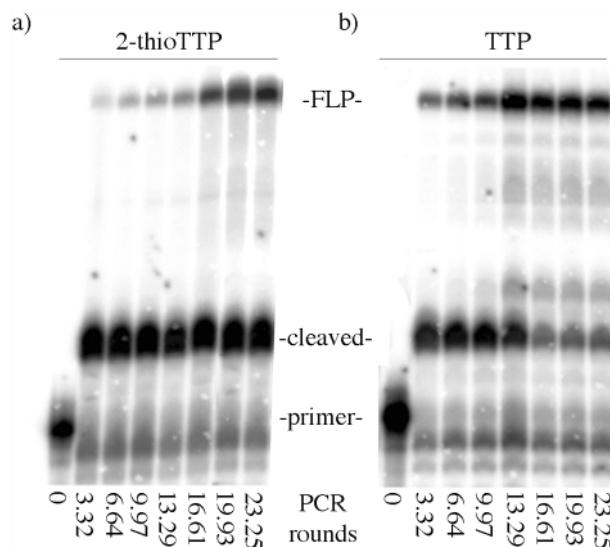
After showing that 2-thioTTP is misincorporated less frequently than TTP opposite isoG residues, we then established that 2-thioTTP works in a PCR amplification. For this purpose, replicate PCRs with KlenTaq polymerase were performed using the PCR replicon consisting of primers P-2f and P-2r and template T-3. Three reactions were run in parallel, one containing the four natural dNTPs (positive control), one containing dCTP, dGTP, dATP and 2-thioTTP, and one without TTP (negative control). Each amplification was cycled for 30 rounds, and the products were analyzed by electrophoresis on a 2% agarose gel.

As shown in Figure 5, the PCRs with TTP and 2-thioTTP generated comparable amounts of product. This result shows that 2-thioTTP is not only a satisfactory substrate for a polymerase, but can, in fact, be used as a substitute for TTP with little affect on the yield of products.

To analyze the products of a PCR with isoC, isoG, thioT, A, G and C, amplification of the non-standard base pair, we used

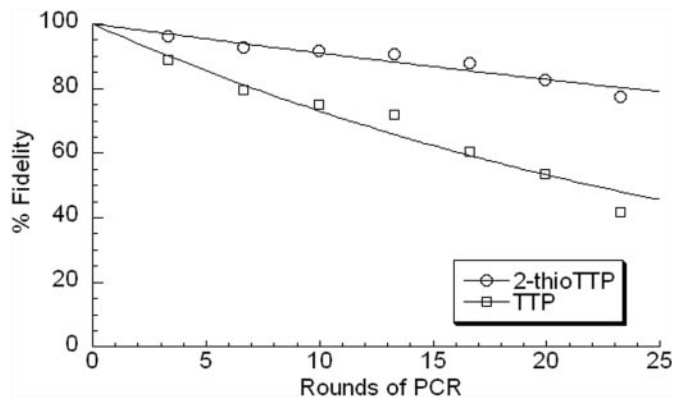


**Figure 5.** PCR amplification using 2-thioTTP. The products generated from a 30 round PCR amplifying template (T-3) in a reaction mixture containing *Taq* polymerase, primers (P-2f and P-2r), ATP, GTP, CTP, and either no thymidine analog (-TTP), TTP or 2-thioTTP (STP). MM indicates a molecular weight marker.



**Figure 6.** Fidelity assay of isoC:isoG PCR. The PCR amplification of DNA containing a single isoG nucleoside (template T-2, and primers P-2f and P-2r) and subsequent chemical cleavage of product DNA at isoC nucleosides. (a) Amplification of amplicons (Table 2) in the presence of dATP, dGTP, dCTP, 2-thioTTP, isoCTP and isoGTP. (b) Amplification of amplicons in the presence of dATP, dGTP, dCTP, TTP, isoCTP and isoGTP. The amount of full-length product (FLP) and cleaved product remaining after chemical cleavage indicate the retention of the isoC:isoG base pair after the indicated number of PCR rounds (i.e. theoretical doublings).

the acid cleavage method of Johnson *et al.* (2) This method exploits the facile depyrimidinylation of isocytidine upon incubation in acid under conditions where the cleavage of the glycosyl bonds of the standard nucleotides is slow. The resulting abasic site is then cleaved with base, and the products are analyzed by PAGE. The relative amount of isoC that was remaining in a full-length PCR product is estimated by the intensity of the cleavage band at the position where the isoC is expected and normalized by the amount of full-length product (Figure 6). These are crude estimates, as some cleavage occurs at other sites as well.



**Figure 7.** Comparison of PCR fidelity using 2-thioTTP or TTP. Shown is the percentage of PCR product containing isoC (% fidelity) versus rounds of amplification when TTP (squares) or 2-thioTTP (circles) was the thymidine analog present in the reaction mixture amplifying template (T-2) with primers (P-2f and P2-r) with *Taq* polymerase. The data were fit to the equation  $y = 100 \times (1/2 + f/2)^x$ . Displayed are the data from each reaction set and the fitted curves. The series of amplicons containing 2-thioTTP displayed a fidelity-per-round ( $f$ ) of 98%, whereas the TTP series yielded a value of 93%.

Figure 7 shows the disappearance of isoC in the PCR product as a function of rounds of PCR for both reactions containing TTP and those substituting 2-thioTTP. This diagram shows that isoC is lost from the PCR product much more rapidly when TTP is used than when 2-thioTTP is used. The fidelity-per-round was obtained by fitting the data to the theoretical curve  $y = (1/2 + f/2)^x$  (see Data Analysis section for details), where  $f$  is the fidelity per round. A PCR containing 2-thioTTP displays a fidelity-per-round of 98%. In contrast, the fidelity-per-round for PCR containing TTP is only 93%, under the conditions reported by Johnson *et al.* (2).

## DISCUSSION

This work shows that substituting 2-thioTTP for TTP in a PCR sequence significantly increases the fidelity in a PCR amplification of an oligonucleotide containing the isoC–isoG base pair. This represents the first chemistry–enzymology combination that has both sufficient fidelity and thermostability for practical application as a six-letter thermocycling PCR.

Direct competition experiments coupled with mercuric gel separations, as exploited here, should be generally useful in the future to assess the fidelity of incorporation of different non-standard nucleotides, when sulfur-containing nucleosides are involved. These experiments allow rapid estimation of the relative kinetic properties for competing dNTPs (17,32,33). This technique is superior to the standard single nucleotide addition (primer extension) reactions or the Scintillation Proximity Assay (34) often used to distinguish those nucleotide triphosphates incorporated opposite a particular non-natural nucleoside from those that are not. This technique can also be used to optimize reaction parameters, such as relative dNTP concentrations, buffers and elongation time.

The combination of the two different approaches used to engineer artificial genetic systems, manipulating hydrogen bonding and steric complementarity base pairs, has also been shown to be useful here (23,35). Here, the large sulfur of 2-thioTTP was used to ‘sterically steer’ the fidelity of replicating

a noncanonical base pair in a more favorable direction. This steric steering approach to fidelity may find application in the next generation of noncanonical DNA alphabets as 2-thioCTP, 4-thioTTP and 6-thioGTP are all known to be readily incorporated into DNA by polymerases.

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*Conflict of interest statement.* None declared.

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