

## **SUPPORTING INFORMATION**

### **Enhanced Base Pairing and Replication Efficiency of Thiothymidines, Expanded-size Variants of Thymidine**

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### Oligonucleotides containing modified nucleotides

The phosphoramidite derivatives of 4S-T (cat. no. 10-1034) and 2S-T (cat. no. 10-1036) were purchased from Glen Research. DNA oligonucleotides were synthesized on an Applied Biosystems 394 synthesizer using standard  $\beta$ -cyanoethylphosphoramidite chemistry. Oligomers were synthesized in DMT-off mode. Oligomers containing 2S-T were deprotected in ammonium hydroxide (55°C, 8 hr). For the synthesis of oligonucleotides containing 4S-T, protecting groups of the natural bases were phenoxyacetyl (PAC) for A and C and tert-butylphenoxyacetyl (tBPAC) for G and the solid support used was 2'-oxalyl lcaa CPG (from ChemGenes corporation). Oligonucleotide containing 4S-T was treated with 1.0M DBU in anhydrous acetonitrile at room temperature for 2hrs to remove the cyanoethyl protection, followed by further deprotection using 50mM NaSH in ammonium hydroxide (room temperature, 2 hr). The oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis, isolated by the crush and soak method, and quantitated by absorbance at 260 nm. 4S-T was also quantitated by absorbance at 335 nm. The molar extinction coefficient for 2S-T at 277 nm is 13,280 and that for 4S-T at 335 nm is 16,254. The shorter oligomers (12 mer) were characterized by mass spectrometry.

### MS MALDI

5'-AAGAA(4S-T)GAAAAG; expected 3751.4 for [oligomer + 11H], found 3752.6.

5'-AAGAA(2S-T)GAAAAG; expected 3751.4 for [oligomer + 11H], found 3752.9.

The longer oligomers (28 nt) were characterized by comparing mobility on an analytical electrophoresis gel with a standard (oligomer containing natural thymine at the substitution site). Incorporation of both 4S-T and 2S-T were also verified spectroscopically by their characteristic absorption bands at 335 nm and 277 nm respectively.

### Thermal Denaturation Studies

After the samples were prepared in melting buffer (100 mM NaCl, 10 mM MgCl<sub>2</sub> and phosphate buffer (pH 7.0)), they were transferred to a Teflon-stoppered 1 cm path length quartz cells on a Varian Cary 1 UV-vis spectrophotometer equipped with thermoprogrammer. The samples were heated to 80 °C, allowed to slowly cool at a rate of 1 °C/min to 4 °C and warmed at a rate of 1 °C/min to 70 °C. Absorbance was monitored at 260 nm for base pairing. In all cases the complexes displayed sharp, apparently two-state transitions. The data were analyzed by the melt

curve processing program, MeltWin v. 3.0. Melting temperatures ( $T_m$ ) were determined by computer-fit of the first derivative of absorbance with respect to  $1/T$ .

### Steady-state kinetics

*<sup>32</sup>P Labeling of Primer.* Primer (final concentration, ~100 nM) was labeled with 5'-[ $\gamma$ -<sup>32</sup>P]ATP (Cat. No. PB10218 from Amersham Bioscience) and T4 polynucleotide kinase (Cat. No. 18004-010 from Invitrogen) and purified using ethanol precipitation.<sup>i</sup>

*Steady-State Kinetics.* Steady-state kinetics for single-nucleotide insertions were carried out as described.<sup>ii</sup> Briefly, insertion reactions were carried out in 10  $\mu$ L volumes in the reaction buffer: 50 mM Tris·HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 50  $\mu$ g/mL BSA for exonuclease-free Klenow enzyme DNA polymerase I (Cat. No. E70057Z from Amersham Bioscience).

Labeled primer (sequence 5'-dTAA TAC GAC TCA CTA TAG GGA GA; final concentration, ~20 nM), template (sequence 5'-dACT GXT CTC CCT ATA GTG AGT CGT ATT A, where X is a variable base), and unlabeled primer were mixed in 2x reaction buffer and gave a final total concentration of primer–template of approximately 20  $\mu$ M. The primer–template duplexes were annealed by heating to 90°C, and slow-cooling to 4°C over 1 hour.

The commercial polymerase showed evidence of contaminating pyrophosphate in control reactions. By pretreating the polymerase enzyme with approximately 0.0002 units of inorganic pyrophosphatase per unit of KF for 10 min at room temperature before adding the primer–template duplex, interfering pyrophosphorolysis reactions were prevented.

A 2x concentrated stock solution of duplex was mixed with 2x KF exo- polymerase for 2 min at 37°C, and the reaction was initiated by adding a 2x solution of the appropriate dNTP in buffer A (200 mM Tris·HCl, 20 mM MgCl<sub>2</sub>·H<sub>2</sub>O, 6 mM 2-mercaptoethanol, pH 8.0). Enzyme concentration and reaction time were adjusted in different dNTP reactions to give 1–20% incorporation in time periods  $\leq$  90 min. Ranges of enzyme concentrations (units/ $\mu$ L) maximum triphosphate concentrations and times (min) that were used are as follows; 4S-TTP + X; (X = A, 0.0025, 1min 15s, 75. X = G, 0.1, 45s, 125. X = C, 0.1, 5 min, 125. X = T, 0.1, 2.5 min, 125). 2S-T + X;

(X = A, 0.0025, 1min 15s, 75. X = G, 0.1, 3 min, 125. X = C, 0.1, 9 min, 125. X = T, 0.01, 1.5 min, 125).  
 T + X; (X = A, 0.0025, 1min 15s, 75. X = G, 0.1, 2 min, 125. X = C, 0.1, 18 min, 125. X = T, 0.1, 30 min,  
 125). ATP + X; (X = 4S-T, 2S-T or T, 0.0025, 4 min, 75). GTP + X; (X = 2S-T or T, 0.1, 45 s, 125. X =  
 4S-T, 0.01, 5 min, 125). CTP + X; (X = 4S-T or T, 0.1, 18 min, 125. X = 2S-T, 0.1, 9 min, 125). TTP +  
 X; (X = 4S-T, 0.1, 5 min, 125. X = 2S-T, 0.1, 2.5 min, 125).

Example:

0.1, 18 min, 125 = (enzyme concentration (unit/uL), reaction time, maximum [dNTP] ( $\mu$ M)

X = template containing indicated base at insertion site.

Reactions were quenched with 15 $\mu$ l of loading buffer I [80% formamide, 1x TBE (89 mM Tris•borate [pH 8.3], 2 mM EDTA), 0.05% xylene cyanol, and bromophenol blue]. Extents of reaction were determined by running quenched reaction samples on a 20% denaturing polyacrylamide gel to separate unreacted primer from insertion products.

*Kinetics data analysis.* Radioactivity was quantified using a PhosphorImager (Molecular Dynamics) and the ImageQuant program. Relative velocity  $v$  was measured as the ratio of the extended product ( $I_{\text{ext}}$ ) to remaining primer ( $I_{\text{prim}}$ ) as follows  $v = I_{\text{ext}}/I_{\text{prim}} t$ , where  $t$  represents the reaction time, and normalized for the lowest enzyme concentration used. The apparent  $K_M$  and  $V_{\text{max}}$  values were obtained from Hanes-Woolf plots.

**Table S1.** Base pairing properties of 2-thio- and 4-thiothymidine in the context of the 12 base pair DNA duplex 5'CTTTTCXTTCTT • 5'AAGAAYGAAAAG<sup>a</sup>

X	Y	T <sub>m</sub> (°C)	ΔT <sub>m</sub> (°C) <sup>b</sup>	−ΔG <sub>37</sub> <sup>°c</sup> (kcal/mol)
A	2S	41.2	+1.4	9.5 ± 0.5
A	4S	38.1	-1.7	9.2 ± 0.1
A	T	39.8	--	9.6 ± 0.1
T	2S	29.2	+3.5	7.5 ± 0.1
T	4S	26.0	+0.3	6.2 ± 0.1
T	T	25.7	--	6.4 ± 0.1
C	2S	24.1	+2.7	7.6 ± 0.1
C	4S	21.6	+0.2	5.3 ± 0.4
C	T	21.4	--	5.3 ± 0.1
G	2S	29.2	-3.1	7.8 ± 0.1
G	4S	32.0	-0.3	--
G	T	32.3	--	7.9 ± 0.1

<sup>a</sup>Conditions: 2 μM DNA, 10 mM phosphate buffer (pH 7.0), 10 mM MgCl<sub>2</sub> and 100 mM NaCl. Errors in T<sub>m</sub> are less than ±0.5 °C.

<sup>b</sup>ΔT<sub>m</sub> values are relative to pairing of natural T with each natural base.

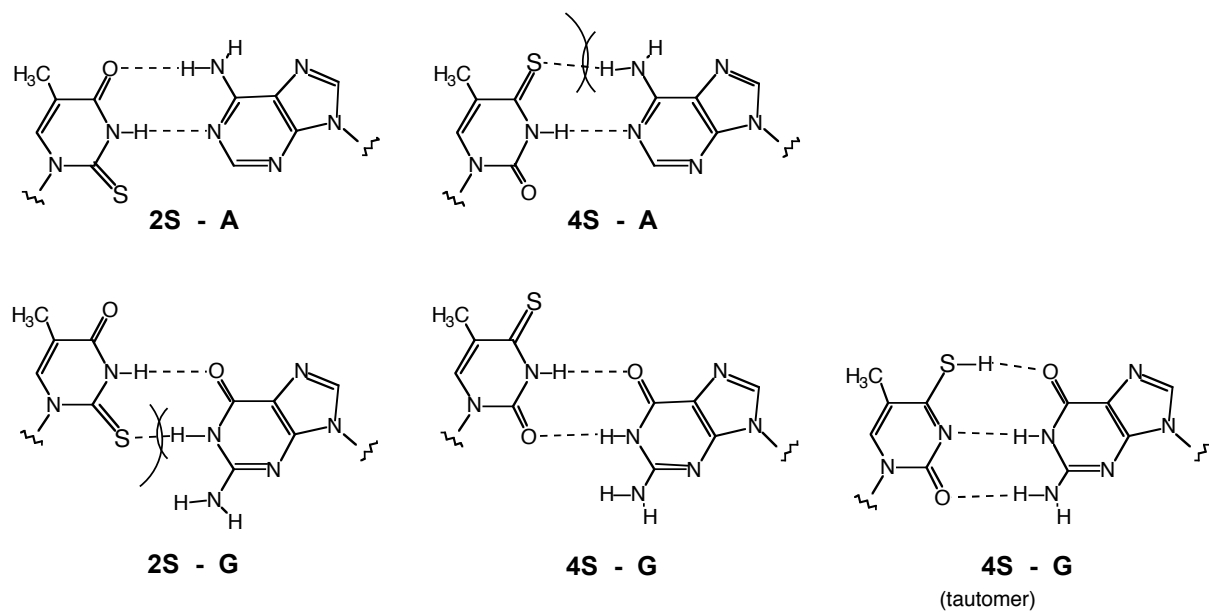
<sup>c</sup>Obtained from curve fitting using 2-state assumption.

**Table S2.** Steady-state kinetics for processing of base pairs and mismatches involving thiothymidine nucleotides by the Klenow fragment of DNA Pol I (exo-).<sup>a</sup>  
See Table 1 (main text) for data with **2S** and **4S** as incoming dNTP analogs.

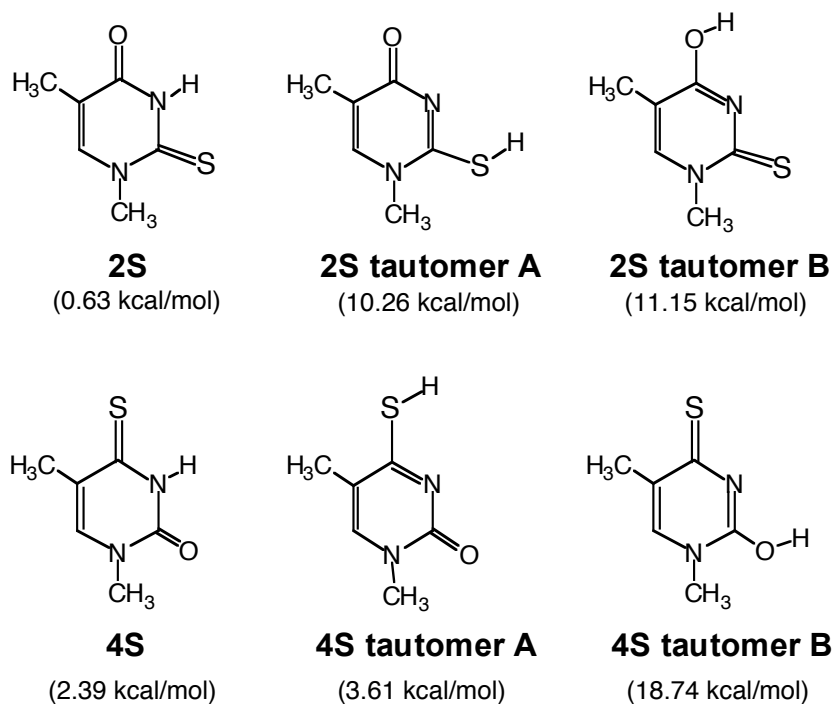
nucleoside triphosphate	template base (X)	$K_m$ ( $\mu$ M)	$V_{max}$ (% min <sup>-1</sup> ) <sup>b</sup>	efficiency ( $V_{max} / K_m$ )	relative efficiency
A	2S	5.4 (1.1)	6.0 (0.8)	$1.2 \times 10^6$	1
A	4S	2.8 (0.5)	3.2 (0.4)	$1.1 \times 10^6$	$9.2 \times 10^{-1}$
A	T	5.0 (1.7)	5.8 (0.8)	$1.2 \times 10^6$	1
T	2S	120 (31)	0.094 (0.011)	$8.2 \times 10^2$	$6.8 \times 10^{-4}$
T	4S	104 (46)	0.027 (0.008)	$2.7 \times 10^2$	$2.3 \times 10^{-4}$
T	T	80 (13)	0.015 (0.005)	$1.8 \times 10^2$	$1.5 \times 10^{-4}$
C	2S	263 (150)	0.096 (0.013)	$4.2 \times 10^2$	$3.5 \times 10^{-4}$
C	4S	80 (37)	0.014 (0.005)	$1.8 \times 10^2$	$1.5 \times 10^{-4}$
C	T	47 (5)	0.006 (0.001)	$1.3 \times 10^2$	$1.1 \times 10^{-4}$
G	2S	134 (21)	0.248 (0.028)	$1.9 \times 10^3$	$1.6 \times 10^{-3}$
G	4S	12 (1.2)	0.129 (0.015)	$1.1 \times 10^4$	$9.2 \times 10^{-3}$
G	T	179 (34)	0.596 (0.086)	$3.3 \times 10^3$	$2.8 \times 10^{-3}$

<sup>a</sup>Conditions: 200 nM 23mer/28mer primer-template duplex and varied polymerase concentrations in a buffer containing 50 mM Tris•HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 50  $\mu$ g/mL BSA and 1 mM dithiothreitol, incubated at 37°C in a reaction volume of 10  $\mu$ L. Standard deviations are given in parentheses.

<sup>b</sup>Normalized for the lowest enzyme concentration used.

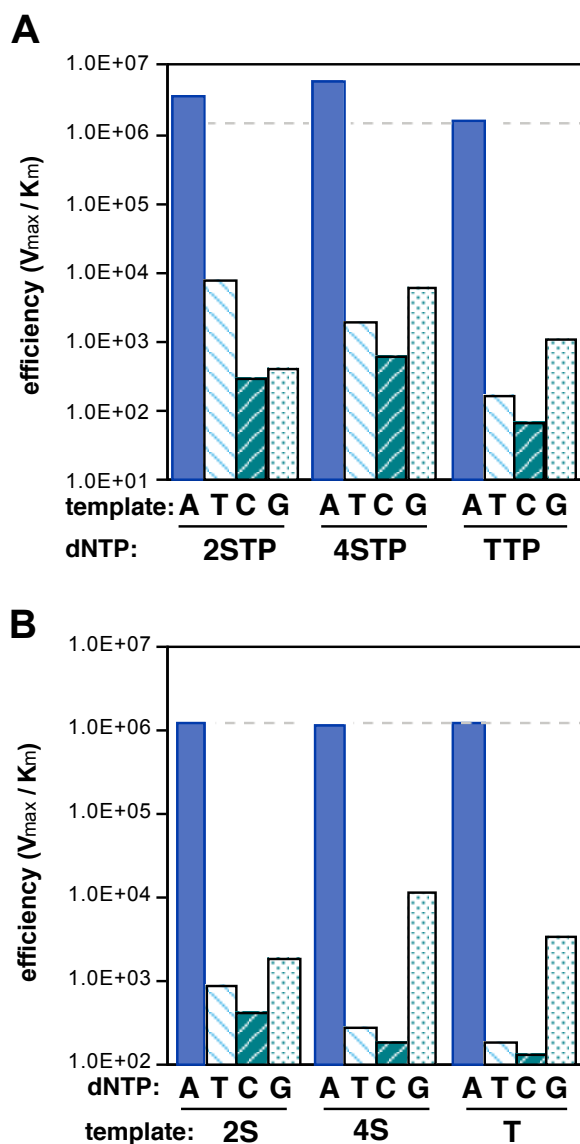


**Figure S1.** Proposed structures of thioT pairs with adenine and of mispairs with guanine, with possible steric clashes as indicated. The added steric size of the thiocarbonyl group may explain the relatively higher stability of 2S-A, and the lowered stability of 4S-A pairs and 2S-G mispairs.



**Figure S2.** AM1-calculated heats of formation for **2S**, **4S**, and their enethiol and enol tautomers. Calculations were performed in the gas phase; appropriate caution should be used in conclusions, since solvation can have significant effects on relative tautomer stability.





**Figure S3.** Histograms showing efficiency and selectivity of replication of **2S** and **4S** paired opposite natural DNA bases, using Kf DNA polymerase (exo-). Note log scale of efficiency (as  $V_{\max} / K_M$ ). (A) Data for modified dNTP analogs being inserted opposite natural bases; (B) Efficiencies for natural dNTP's being inserted opposite thioT analogs in a DNA template. Dashed lines mark the efficiency of the natural A-T base pair.

## References

- <sup>1</sup> Sambrook, J.; Russell, D.W. *Molecular Cloning* 3<sup>rd</sup> ed.; CSHL Press, 2001, vol. 2, 10.17, 10.20.
- <sup>2</sup> (a) Goodman, M. F.; Creighton, S.; Bloom, L. B.; Petruska, J. *Crit. Rev. Biochem. Mol. Biol.* **1993**, 28, 83. (b) Moran, S.; Ren, R. X.-F.; Kool, E. T. *Proc. Natl. Acad. Sci. USA* **1997**, 94, 10506.

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<sup>i</sup> Sambrook, J; Russell, D.W. *Molecular Cloning* 3<sup>rd</sup> ed.; CSHL Press, 2001, vol. 2, 10.17, 10.20.

<sup>ii</sup> (a) Goodman, M. F., Creighton, S., Bloom, L. B. & Petruska, J. *Crit. Rev. Biochem. Mol. Biol.* **1993**, 28, 83. (b)

Moran, S.; Ren, R. X.-F.; Kool, E. T. *Proc. Natl. Acad. Sci. USA* **1997**, 94, 10506.