Supplementary Information

Supplemental Methods

Synthesis and purification of oligodeoxynucleotides (ODNs)

6-thio-2'-deoxyguanosine (^{6S}G) substituted template and 4-thio-2-deoxyuridine (^{4S}U) substituted 5'-FAM-labelled flap ODNs were synthesised on a one µmol scale by AtdBio using the corresponding *S*-cyanoethyl phosphoramidites (Glen Research) and other nucleoside phopshoramidites with acetyl and phenoxyacetyl protecting groups. Solid supports were treated with 1M 1,8-diazabicyclo[5.4.0] undec-7-ene in anhydrous CH_3CN for three hours at room temperature to remove cyanoethyl groups. The ODN was then liberated form the support and other protecting groups removed by treatment with concentrated NH_4OH containing 50 mM NaSH for eight hours at room temperature. All other unmodified ODNs were prepared using standard methods by ATD bio ltd. ODNs were purified at 60ºC by reversed phase HPLC (ACE C18 column, HiChrom) using buffers A = 100 mM triethylammonium acetate pH 6.5, 5% CH_3CN; buffer B = 100 mM triethylammonium acetate pH 6.5 , 65% CH_3CN and desalted using NAP 10 columns (GE LifeSciences).

Formation of disulfide crosslinks

^{6S}G template (100-200 µM) and ^{4S}U flap strands (50-100 µM) were annealed at a 2:1 ratio by heating to 80°C for three mins and cooling slowly to 25°C in 100 µL of 100 mM TRIS pH 8.4, 100 mM KCl, 10 mM $MgCl_2$. After addition of 5-10 µl of 10 mM I_2 in methanol (final I_2 concentration 0.5-1 mM) the sample was incubated for up to 48 hours at 4°C, after which the iodine was removed using Et_2O (3 x 500 µL). The crosslinked products were isolated by HPLC at 60°C (gradient 5-30% buffer B over 30 mins, buffers as above) and desalted with a NAP5 column (GE Lifesciences).

Examination of the effects of exposure of ODNs to I_2 and formation of iodinated FAM substrates

ODNs were treated identically to above to yield iodinated species that were then used to assemble substrates as required. Mono- and di-iodination of FAM was confirmed by mass spec. Furthermore, fluorescence spectra of I_2 treated FAM-oligos showed characteristic red-shifts in the emission spectra consistent with previous reports[1].

Comparison of products formed from crosslinked and non-crosslinked hFEN1 substrates

Non-crosslinked substrates were prepared by annealing relevant FAM-flap and template strands in a 1:1.1 ratio by heating to 60°C for two minutes in 250 mM KCl, 50 mM HEPES pH 7.5, followed by cooling to room temperature. Crosslinked substrates were treated identically. Substrates were pre-incubated in reaction buffer at 37°C, and reaction was initiated by addition of hFEN1 (previously purified[2]) to give final concentrations of 100 nM substrate, 50 mM HEPES pH 7.5, 100 mM KCl, 8 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM DTT and hFEN1 (10 pM-1 nM, dependent on substrate and crosslinking). Reactions were monitored by removal of aliquots at appropriate time intervals and quenched with ten volumes of 8 M urea, 80 mM EDTA, 1 mM DTT to reduce disulfides prior to analysis. Rates of reaction were determined by analysis of samples using denaturing HPLC equipped with a fluorescence detector using the FAM or di-iodoFAM fluorescent tag for detection, (excitation wavelength 494 nm and emission 525 nm, and excitation 508 nm and emission 531 nm[1], respectively). Starting material and product(s) were separated using buffers A = 2.5 mM tetrabutylammonium bromide (TBAB), 0.1% acetonitrile, 1 mM EDTA, B = 2.5 mM TBAB, 70% acetonitrile, 1 mM EDTA, on a DNAsep® column (Transgenomic, Glasgow) at 50ºC, t = 0 min 5% B, t = 5 mins 30% B, t = 9 mins 50% B, t = 12 mins 70% B, t = 13.5 mins 100% B, t = 14.5 mins 100% B, t = 14.6 mins 5% B. The site(s) of reaction were determined by comparison to authentic samples.

The traces displayed in figure 3b (main text) used 100 nM substrate and the amounts of hFEN1 and incubation times indicated in brakets: [**ii**] Mismatches left, DF T-1•G17 (10 pM hFEN1, 20 mins); right, DF T+1•G18 20 (10 nM hFEN1, 20 mins). [**iii**] Mismatches left, DF T-1•G17 (10 pM hFEN1, 4 mins); right, DF T+1•G18 (10 nM hFEN1, 2 mins). [**iv**] **Oxidised** thio-substrate left, DF ^{4S}U-1•^{6S}G17 (10 nM hFEN1, 4 mins); right, DF ^{4S}U+1•^{6S}G18 (10 nM hFEN1, 4 mins). [**v**] Thio-substrates left, DF ^{4S}U-1•^{6S}G17 (100 pM hFEN1, 60 mins); right, DF ^{4S}U+1•^{6S}G18 (1 nM hFEN1, 70 mins). [**vi**] **Oxidised then reduced** thio-substrate, left, DF ^{4S}U-1•^{6S}G17 (1 nM hFEN1, 60 mins); right, DF ^{4S}U+1•^{6S}G18 (10 nM hFEN1, 120 mins).

Reductions of disulfide containing substrates and subsequent enzyme reactions

DF ^{4S}U+1•^{6S}G18

1 µl of 54 µM crosslinked DF ^{4S}U+1•^{6S}G18, 1 µl of 1 M DTT and 9 µl of 8 M urea were first incubated at room temperature for 30 minutes. To this mixture, 90 µl reaction buffer containing 500 mM HEPES pH 7.5, 1 M KCl, 80 mM $MgCl_2$ and 868 µl water were added for a final concentration of 54 nM substrate, 0.07 M urea, 50 mM HEPES pH 7.5, 100 mM KCl and 8 mM $MgCl_2$. The mixture was heated to 60°C for 2 minutes, and then, was allowed to cool to room temperature for 30-60 minutes, after which 1 µl of 1 M DTT and 20 µl of 5 mg/ml BSA was added to final concentrations of 2 mM DTT and 0.1 mg/ml BSA. Reaction was initiated by the addition of 10 µl of 1 µM hFEN1 in a buffer containing 500 mM HEPES pH 7.5, 1 M KCl and 80 mM $MgCl_2$. Aliquots (100 µl) were removed and quenched in 100 µl 8 M urea and 80 mM EDTA at appropriate time points. The extent of reaction was assessed by dHPLC equipped with a fluorimeter as described above.

DF ^{4S}U-1•^{6S}G17

2 µl of 2.8 µM DF ^{4S}U-1•^{6S}G17 , 1 µl of 0.1 M DTT and 10 µl of 8 M urea were first incubated at room temperature for 30 minutes. To this mixture, 10 µl reaction buffer containing 250 mM HEPES pH 7.5, 500 mM KCl, 40 mM $MgCl_2$ and 64 µl water were added for a final concentration of 56 nM substrate, 0.7 M urea, 50 mM HEPES pH 7.5, 100 mM KCl and 8 mM $MgCl_2$. The mixture was heated to 60°C for 2 minutes, and then, was allowed to cool to room temperature for 30-60 minutes after which 1 µl of 0.1 M DTT and 2 µl of 5 mg/ml BSA was added for final concentrations of 2 mM DTT and 0.1 mg/ml BSA. Reaction was initiated by the addition of 10 µl of 10 nM hFEN1 in a buffer containing 250 mM HEPES pH 7.5, 500 mM KCl and 40 mM $MgCl_2$. Aliquots (10 µl) were removed and quenched in 100 µl 8 M urea and 80 mM EDTA at appropriate time points. The extent of reaction was assessed using a dHPLC equipped with a fluorimeter as described above.

T-1•G17 and T+1•G18

Control reactions were performed as using T-1•G17 and T+1•G18 to determine whether the presence of 0.7 to 0.07 M urea, respectively, altered scissile phosphate selectivity. No change in retention times of products was observed.

Figure S1 Schematics of double flap substrates used in this study.

A) DF (perfect duplex), B) T+1•G18 (GT mismatch in the +1 position), C) T-1•G17 (GT mismatch in the -1 position), D) ^{4S}U+1•^{6S}G18 (thio GU basepair in the +1 position), E) ^{4S}U-1•^{6S}G17 (thio GU basepair in the -1 position). See also Table S2.

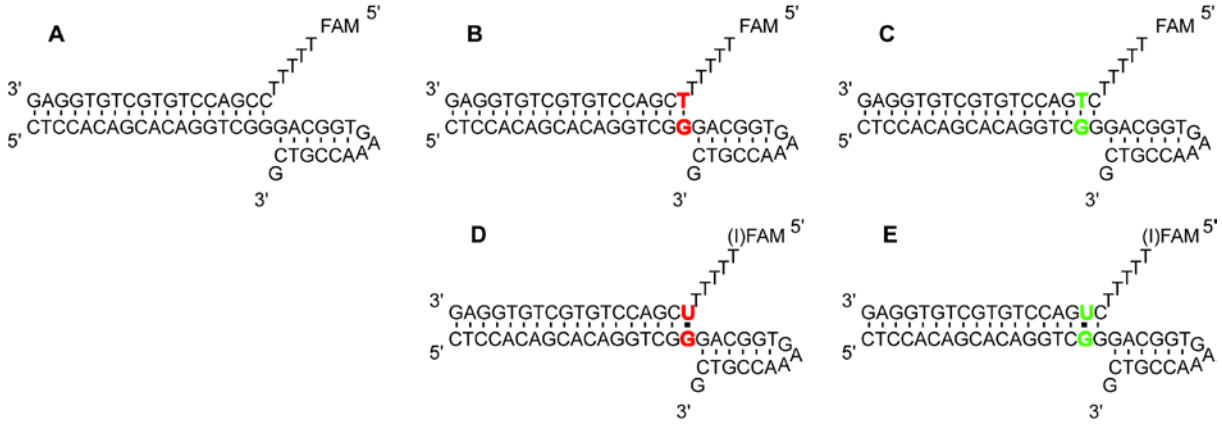
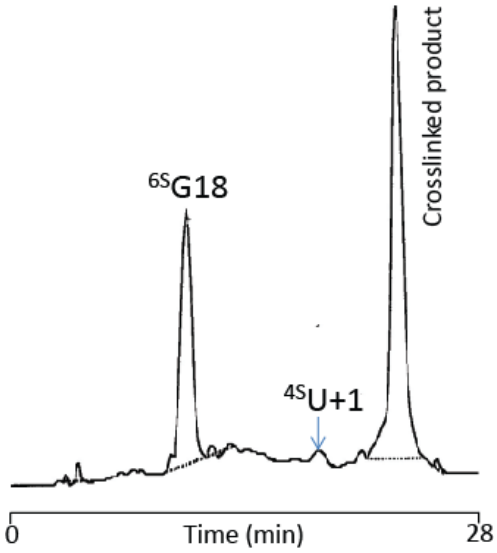


Figure S2 Denaturing HPLC Trace of Disulfide formation in a DF Substrate obtained at 260nm.



Denaturing HPLC trace of crosslinking reaction monitored at 260 nm between ^{6S}G18 and ^{4S}U+1 strands (^{6S}G18 strand present in 2:1 excess) after treated with 10-fold excess I_2 for 48 hours at 4° C. The crosslinked product (RT = 22.1 min) was isolated from unreacted ^{6S}G18 template strand (RT = 10.1 min) and ^{4S}U+1 flap strand (RT 17.5 min).

Table S1 Sequences and characterization of ODNs

Code	Sequence	MW calculated	MW found
^{6S}G18	d(CTCCACAGCACAGGTCG(^{6}SdG)GACGGTGAAACCGTCG)	10473.8	10476.8
^{6S}G18/I_2	d(CTCCACAGCACAGGTCG(^{6}SdG)GACGGTGAAACCGTCG)	10478.85	10482.4
^{4S}U+1	FAM-d(TTTTT(^{4}SdU)CGACCTGTGCTGTGGAG)	7603.7	7608.6
^{6S}G17	d(CTCCACAGCACAGGTC(^{6}SdG)GGACGGTGAAACCGTCG)	10473.8	10477.0
^{4S}U-1	FAM-d(TTTTTC(^{4}SdU)GACCTCTGCTGTGGAG)	7603.7	7610.6
^{4S}U-1/I_2	iodoFAM-d(TTTTTC(^{4}SdU)GACCTGTGCTGTGGAG)	7729.6 (+I) 7855.5 (+2I) 7839.5 (+2I, desulfurisation of ^{4S}U)	7842.0 (100 %, +2I, desulfurised)
T+1	FAM-d(TTTTTTCGACCTGTGCTGTGGAG)	7601.2	7606.0
T-1	FAM-d(TTTTTC(T)GACCTGTGCTGTGGAG)	7601.2	7605.0
T-1/I_2	iodoFAM-d(TTTTTC(T)GACCTGTGCTGTGGAG)	7727.5 (+1I) 7853.4 (+2I)	7723.0 (60% +I) 7856.0 (100% +2I)
G17 and G18	d(CTCCACAGCACAGGTCGGGACGGTGAAACCGTCG)	10457.8	10463.0
C+1 and C-1	FAM-d(TTTTTCCGACCTGTGCTGTGGAG)	7586.6	7590

The sequences of ODNs used to construct substrates used in this study. Molecular weights of thio-substituted oligomers were determined using electrospray and non-thio-substituted oligomer masses were determined using MALDI mass spectrometry. ODNs were exposed to I_2 as described above.

Table S2 ODNs used to construct double flap substrates

Double Flap Substrate	**ODNs**
DF (fully base paired)	G17 and G18 with C+1 and C-1
Mismatch DF T-1 • G17	T-1 with G17
Mismatch CF T+1 • G18	T+1 with G18
DF ^{4S}U-1•^{6S}G17	^{4S}U-1 with ^{6S}G17
DF ^{4S}U+1•^{6S}G18	^{4S}U+1 with ^{6S}G18

(1) Gabel, C. A.,Shapiro, B. M. *Anal Biochem* **1978**, *86*, 396-406.
(2) Tsutakawa, S. E.,Classen, S.,Chapados, B. R.,Arvai, A. S.,Finger, L. D.,Guenther, G.,Tomlinson, C. G.,Thompson, P.,Sarker, A. H.,Shen, B.,Cooper, P. K.,Grasby, J. A.,Tainer, J. A. *Cell* **2011**, *145*, 198-211.