Supporting Information

Heteropair Screen Protocol. Primers, varying at position **X** of the sequence 5'-dTAA TAC GAC TCA CTA TAG GGA GA**X**-3', were grouped into ten groups based loosely on their functional groups and general shape. Primer pools consisting of (n) nucleobases were constructed by equally representing all primers (final concentration of each individual primer = 1 mM/n) into a pool with a total DNA concentration of 1 mM. Primer pools were 5' radiolabeled with [γ^{33}P]-ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs). Primer pool was annealed with two-fold excess template of the complementary sequence, 3'-dATT ATG CTG AGT GAT ATC CCT CT**Y** GCT AGG TTA CGG CAG GAT CGC-5', in the reaction buffer by heating to 90°C and slow cooling to room temperature. Assay conditions include: 40 nM total primer-template duplex, 0.3 nM enzyme, 50 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 μg/mL BSA, 400 μM dCTP. Prior to reaction, dCTP was mixed with enzyme, and the reactions were initiated by adding the dCTP-enzyme mixture to an equal volume (5 μL) of a 2× DNA stock solution, incubated at 25°C for 5 min, and quenched with 20 μL of loading buffer (95% formamide, 20 mM EDTA). The reaction mixture, (3 μL) was then analyzed by 15% polyacrylamide gel electrophoresis. Radioactivity was quantified using a Phosphorimager (Molecular Dynamics) with overnight exposures and the ImageQuant program. Percent conversion was defined as the ratio of

$$\frac{\text{(singly extended product)}}{\text{(unextended product + singly extended product)}}$$

General Steady-State Kinetic Assay Protocol. Primer was 5' radiolabeled with [γ^{33}P]-ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs). Primer-template duplexes were annealed in the reaction buffer by heating to 90°C and slow cooling to room temperature. Assay conditions include: 40 nM template-primer duplex, 0.10-1.2 nM enzyme, 50 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL BSA. The reactions were initiated by adding the DNA-enzyme mixture to an equal volume (5 μL) of a 2× dNTP stock solution, incubated at 25°C for 2-12 min, and quenched with 20 μL of loading buffer (95% formamide, 20 mM EDTA). The reaction mixture, (8 μL) was then analyzed by 15% polyacrylamide gel electrophoresis. Radioactivity was quantified using a Phosphorimager (Molecular Dynamics) with overnight exposures and the ImageQuant program. The Michaelis-Menten equation was fit to the data using the program Kaleidagraph (Synergy software). The data presented are averages of triplicates.

General Full-Length Assay Protocol. Primer was 5' radiolabeled as described above. Primer-template duplexes were annealed in the reaction buffer by heating to 90°C and slow cooling to room temperature. Assay conditions include: 40 nM template-primer duplex, 3.7 nM enzyme, 50 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL BSA. The reactions were initiated by adding the DNA-enzyme mixture to an equal volume (5 μL) of a 2× dNTP stock solution containing either 50 μM of dATP, dCTP, dGTP, dTTP or 50 μM of dATP, dGTP, dTTP. Reactions were incubated at 25°C for 5 min, and quenched with 20 μL of loading buffer (95% formamide, 20 mM EDTA). The reaction mixture, (8 μL) was then analyzed by 15% polyacrylamide gel electrophoresis. Radioactivity was quantified using a Phosphorimager (Molecular Dynamics) with overnight exposures and the ImageQuant program.

Table S1. Steady State Rate Constants of Unnatural Self-Pair Extension by Insertion of dCTP[a]

5' - dTAATACGACTCACTATAGGGAGA(**X**)
3' - dATTATGCTGAGTGATATCCCTCT(**X**)GCTAGGTTACGGCAGGATCGC

X	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}min^{-1}$)
BFr[b]	0.81 ± 0.05	294 ± 9	2.8×10^3
BTp[b]	0.17 ± 0.04	230 ± 49	7.4×10^2
BTz[b]	14 ± 3	85 ± 20	1.7×10^5
IN[b]	0.4 ± 0.1	207 ± 30	1.8×10^3
4MP	2.9 ± 0.9	202 ± 56	1.5×10^4

Standard deviation in parentheses [a]See general steady-state kinetic protocol, above, for experimental details [b]Ref. 1

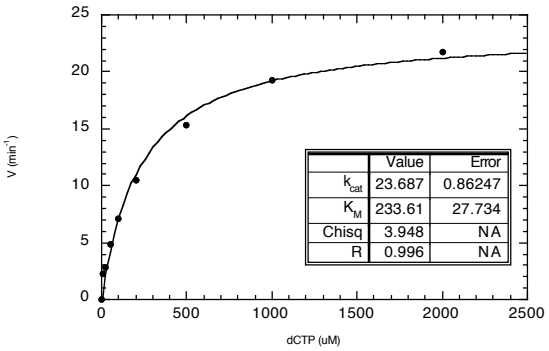

Figure S1. Sample Michaelis-Menten plot of Kf mediated extension of d**4MP**:d**BFr** terminus by incorporation of dCTP. Velocity is plotted as a function of triphosphate concentration and fit to the Michaelis-Menten equation using the non-linear graphing program Kaleidagraph (Synergy software).

REFERENCES

1. Matsuda, S.; Henry, A.A.; Schultz, P.G.; Romesberg, F.E., *J. Am. Chem. Soc.* **2003**, 125, 6134-6139.