Supporting Information. *Mechanistic Insight Through Irreversible Inhibition: DNA Polymerase θ Uses a Common Active Site for Polymerase and Lyase Activities*

*Daniel J. Laverty, Ifor P. Mortimer, and Marc M. Greenberg**

Department of Chemistry
Johns Hopkins University
3400 N. Charles St.
Baltimore, MD 2128

mgreenberg@jhu.edu

Contents:

Experimental

General Methods. Oligonucleotides were synthesized on an Applied Biosystems Inc. 394 DNA synthesizer using reagents from Glen Research (Sterling, VA) and deprotected according to the manufacturer's instructions. γ-^{32}P-dATP and α-^{32}P-ATP (cordycepin) were obtained from PerkinElmer. Protein purification was conducted using an AKTA FPLC and columns were from GE Healthcare. C_{18} Sep Pak cartridges were from Millipore. T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, Dpn1, Phusion polymerase, and dNTPs were obtained from New England Biolabs. Sephadex G-25 was from GE Healthcare. DH5α *E. coli* were from Invitrogen. Trypsin/Lys-C mix was from Promega. Analysis of radiolabeled oligonucleotides was carried out using a Storm 860 Phosphorimager and ImageQuant 7.0 TL software. LC-MS/MS analysis was conducted using Waters Acquity/ Xevo-G2 UPLC-MS system. ESI-MS was conducted using a Thermoquest LCQ Deca. MALDI-TOF MS was conducted using a Bruker Autoflex III MALDI TOF. Sanger sequencing was conducted by the Genetic Resources Core Facility at Johns Hopkins. Fluorescence anisotropy measurements were conducted using an AVIV Biomedical Model ATF 107 spectrofluorometer at the Center for Molecular Biophysics at Johns Hopkins University. Photolyses were carried out in a Rayonet photoreactor fitted with 16 lamps having a maximum output at 350 nm. Pol θ catalytic core (residues 1792-2590) was expressed and purified as previously described.[1,2] Polymerase active site titration of wild type Pol θ was reported previously.[3] All oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) and desalted by C_{18} Sep Pak. Modified oligonucleotides were characterized by ESI-MS or MALDI-TOF MS. Phosphoramidites of photochemical precursors to pC4-AP, DOB, and 5'-dRP were synthesized as previously described and incorporated into oligonucleotides by solid-phase synthesis.[4–6]

Preparation of Oligonucleotide Complexes. For lyase experiments, oligonucleotides containing the appropriate photochemical precursor were 3'-^{32}P-labeled. The oligonucleotide (100 pmol) was incubated (37 °C, 2 h) with α-^{32}P-ATP (30 μCi) and terminal transferase (40 units) in a 50 μL reaction in the terminal transferase and $CoCl_2$ buffers provided by the manufacturer. For primer extension experiments, the primer strand was 5'-^{32}P-labeled. The primer (100 pmol) was incubated (37 °C, 1.5 h) with γ-^{32}P-ATP (20 μCi) and T4 polynucleotide kinase in the reaction buffer provided by the enzyme manufacturer. Following incubation, unincorporated radionuclides were removed by Sephadex G-25 spin column. Ternary complexes **1-3** were prepared by mixing 3'-^{32}P-labeled oligonucleotide with the appropriate template and flanking strand in a 1:1.5:3 ratio in phosphate buffered saline (10 mM sodium phosphate, 100 mM NaCl, pH 7.2), heating to 95 °C, and slowly cooling to 25 °C. Primer-template complex **5** was prepared in the same fashion, but the 5'-^{32}P-labeled primer strand and the template were mixed in a 1:1.5 ratio. Ternary complex **6** used for fluorescence anisotropy measurements was prepared by annealing the fluorophore-labeled template with the other two strands in a 1:1.5:1.5 ratio. For lyase experiments, pC4-AP, DOB, and 5'-dRP were generated immediately before the start of the experiment by photolysis (350 nm, 10 min) of the ternary complex containing the appropriate photochemical precursor. For every experiment, complete photolysis was confirmed by treatment with NaOH (0.1 M, 15 min, 37 °C), which gave quantitative cleavage of 5'-dRP, DOB, and pC4-AP.

Steady-state kinetic analysis of dA incorporation in 5 by Pol θ. Polymerase reactions were conducted with Pol θ (432 pM for wild type Pol θ and 5 nM for K2383A and K2383R), 5'-^{32}P-**5** (50 nM), and a range of dATP concentrations (0.25 μM-100 μM for wild type and 62.5-5000 μM for mutants) at 25 °C in reaction buffer (10 mM Tris HCl pH 8, 25 mM KCl, 10 mM MgCl2, 1 mM BME). The concentration range for dATP and the reaction time were selected so that reactions

proceeded no further than 25% (single-hit conditions). In a typical experiment, a 2 × DNA-polymerase solution was prepared by mixing 5'-^{32}P-**5** (500 nM, 18 μL), 10 × reaction buffer (18 μL), 10 × Pol θ (4.32 nM for wild type or 50 nM for mutants, 18 μL) in storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME), and H_2O (36 μL). The 2 × DNA-enzyme solution (3 μL) was mixed with the appropriate 2 × dNTP solution (3 μL) to initiate the reaction. Reactions were incubated for 20 s for wild type Pol θ, 1.5 min for K2383R, and 4 min for K2383A, after which, they were quenched with 95% formamide loading buffer containing 25 mM EDTA (6 μL). An aliquot (4 μL) was analyzed by 20% denaturing PAGE run at 55 W for approximately 3.5 h. The gel was analyzed by phosphorimaging, and the data were fit to the Michaelis-Menten equation. The k_{cat} was determined by dividing V_{max} by the active concentration of enzyme. Active site titrations could not be carried out on mutant proteins due to extremely slow reaction rates. Hence, the active fraction was assumed to be equal for mutant and wild type proteins.

Analysis of Pol θ lyase activity under multiple turnover conditions. Ternary complexes 3'-^{32}P-**1-3** (100 nM) were incubated with Pol θ (2.5 nM) at 37 °C in a reaction buffer consisting of 50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol. In a typical experiment, a 10 × solution of DNA (500 nM) in 1 × phosphate buffered saline (10 mM sodium phosphate 100 mM NaCl, pH 7.2) was prepared and photolyzed (350 nm, 10 min) to generate the appropriate abasic site (dRP, DOB, or pC4-AP). The 10 × DNA solution (3 μL) was added to a solution of H_2O (21 μL) and 10 × reaction buffer (3 μL). A 10 × solution of Pol θ (3 μL) in storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) was added and the reaction was incubated at 37 °C. To account for the background reaction in the absence of enzyme, a control sample was treated in the exact same fashion except instead of adding Pol θ to the reaction, the

same volume of Pol θ storage buffer (3 μL) was added. Aliquots were removed from the reaction and frozen on dry ice at 2.5, 5, 7.5, 10, 20, and 30 min for **1** and 10, 20, 30, 45, and 60 min for **2** and **3**. At the end of the experiment, a 500 mM solution of $NaBH_4$ was prepared in H_2O and immediately added (1 μL) to each aliquot to quench the reaction. The reduction of the substrate was nearly instantaneous, but residual $NaBH_4$ sometimes interfered with mobility during gel electrophoresis, so the reactions were incubated at room temperature for 1.5 h with occasional centrifugation on a bench-top centrifuge to allow for complete reaction of residual $NaBH_4$. Alternatively, ethanol precipitation could be used to remove excess $NaBH_4$ without the necessity of a prolonged incubation, but this was typically not employed. Following quenching of the reactions, samples were mixed with an equal volume (5 μL) of 95% formamide containing 10 mM EDTA and trace bromophenol blue and xylene cyanol and subjected to 20% denaturing polyacrylamide gel electrophoresis at 55 W for approximately 4 h. The gel was exposed to a phosphor storage cassette and imaged using phosphorimaging. The fraction of product formed in the background reaction without enzyme was subtracted from each time point, and the resulting fraction of product formed by enzymatic reaction was plotted as a function of time. Reactions were conducted in triplicate for each experiment and each experiment was conducted at least twice.

Comparison of single turnover lyase kinetics for dRP excision by Pol θ variants. Ternary complex 3'-^{32}P-**3** (50 nM) were incubated with a large excess (1 μM) of Pol θ (wild type, K2383A, K2383R, K2575A/K2577A, or K2383A/K2575A/K2577A) at 37 °C in reaction buffer consisting of 50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol. Aliquots (4 μL) were removed and frozen on dry ice at 1, 2, 5, 10, 15, 20 min for the wild type; 2, 5, 10, 20, and 30 min for K2383R mutant; 2, 5, 10, 15, and 20 min for K2575A/K2577A and 5, 10, 15, 30, and 60 min for K2383A and K2383A/K2575A/K2577A. The experimental procedure was the same as

for multiple turnover conditions, including accounting for noncatalyzed reaction, except for the noted differences in concentration and reaction times. The fraction of product was plotted as a function of reaction time and the data were fit to the equation fraction cleaved = (maximum fraction cleaved) ($1-e^{-kobs*time}$). Reactions were conducted in triplicate for each experiment and each experiment was conducted at least twice.

Comparison of single turnover lyase kinetics for dRP, DOB, and pC4-AP excision by wild type Pol θ. Experimental conditions were the same as the previous section except aliquots were removed at 20 s, 40s, 1 min, 2 min, 5 min, and 10 min for pC4-AP (3'-^{32}P-**1**); 45 s, 90 s, 2 min, 5 min, 7.5 min, and 10 min for DOB (3'-^{32}P-**2**); and 1, 2, 5, 10, 15, and 20 min for dRP (3'-^{32}P-**3**).

Inhibition of Pol θ Lyase Activity by pC4-AP. Pol θ (5 nM) was incubated with pC4-AP (3'-^{32}P-**3**, 100 nM) in reaction buffer buffer (50 mM HEPES pH 7.5, 20 mM KCl, 2 mM DTT, 1 mM EDTA) at 25 °C. A 10 × solution of 3'-^{32}P-**3** was prepared by photolysis (10 min, 350 nm) and 7 μL of this solution was added to a microcentrifuge tube containing H_2O (49 μL) and 10 × reaction buffer (7 μL). The reaction was initiated by the addition of a 10 × solution of Pol θ (7 μL, 350 fmol) in storage buffer (20 mM Tris•HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) bringing the volume of the reaction to 70 μL. To account for the background reaction in the absence of enzyme, a control sample was treated in the exact same fashion except instead of adding Pol θ to the reaction, the same volume of Pol θ storage buffer was added. Aliquots (4 μL) were removed from the reactions at 5, 10, 15, and 20 min and frozen on dry ice. Immediately after removing the aliquot at 20 min, an aliquot (350 fmol, 1 μL) of Pol θ in storage buffer was added to the reaction. Aliquots were removed at 25, 30, 35, and 40 min and frozen on dry ice. Another aliquot of Pol θ in storage buffer (350 fmol, 1 μL) was added immediately after removing the aliquot at 40 min. Aliquots were removed from the reaction at 45, 50, 55, and 60 min and frozen on dry ice. Reactions

were quenched with $NaBH_4$ (1 μL, 500 mM) and analyzed by polyacrylamide gel electrophoresis and phosphorimaging. The fraction of product formed in the background reaction without enzyme was subtracted from each time point, and the resulting fraction of product formed by enzymatic reaction was plotted as a function of time. Reactions were conducted in triplicate for each experiment and each experiment was conducted at least twice.

Generation of Pol θ mutants. Pol θ variants were generated by site-directed mutagenesis of the Pol θ plasmid reported previously.[1] PCR was conducted with the following primers: primers for the K2383A mutation were 5'-d(CTG AGG CAG CAG GCA GCA CAG ATT TGC TAT GGG ATC A) and 5'-d(TGA TCC CAT AGC AAA TCT GTG CTG CCT GCT GCC TCA G). Primers for the K2383R mutation were 5'-d(CTG AGG CAG CAG GCA AGA CAG ATT TGC TAT GGG ATC A) and 5'-d(TGA TCC CAT AGC AAA TCT GTC TTG CCT GCT GCC TCA G). Primers for the K2575A/K2577A mutation were 5'-d(GTC TGT GAA ATT GAA AGT GGC AAT AGG CGC CAG CTG GGG AGA GC) and 5'-d(GCT CTC CCC AGC TGG CGC CTA TTG CCA CTG CCA CTT TCA ATT TCA CAG AC). The K2575A/K2577A mutant plasmid was generated by PCR amplification of the wild type plasmid using primers for the K2575A/K2577A mutant. The K2383A/K2575A/K2577A mutant plasmid was generated by PCR amplification of the K2383A mutant plasmid using primers for the K2575A/K2577A mutant. PCR was conducted using 50 ng of plasmid and 15 pmol of each primer in a 50 μL reaction using Phusion polymerase according to manufacturer protocol. Briefly, the reaction contained the provided high fidelity buffer (10 μL), dNTPs (1 μL of 10 mM solution), both primers (1 μL for each, 15 μM solution), the parental plasmid (50 ng, 1 μL), Phusion polymerase (1 unit, 0.5 μL), and H_2O (35.5 μL). PCR was conducted with 95 °C initial denaturing (30 s), followed by 18 cycles of 95 °C denaturing (30 s), 55 °C annealing (1 min), 72 °C extension (5 min). Parental plasmid was digested by addition

of Dpn1 (1 μL, 20 units) at 37 °C for 4 h. An aliquot (2 μL) of this mixture was transformed into DH5-α cells (40 μL) by incubation on ice (30 min), heat shock at 42 °C (30 s), and incubation on ice (5 min). SOC medium (200 μL) was added and outgrowth was conducted at 37 °C for 1 h with 250 rpm shaking. A portion (100 μL) of the transformed cells was plated on an LB plate with ampicillin (100 μg/mL). Plates were grown for 16 h at 37 °C. A single colony was picked and resuspended in LB media (5 mL) with ampicillin (100 μg/mL) and grown for 16 h at 37 °C. Plasmids were isolated by Mini prep according to the manufacturer protocol and sequenced to confirm mutagenesis. The mutant proteins were expressed and purified in the same fashion as the wild type.[1,2]

UPLC-MS/MS analysis of Pol θ modification by pC4-AP. A solution of Pol θ (100 μL, 10 μM) was mixed with H_2O (700 μL), 10 × reaction buffer (100 μL, 500 mM HEPES pH 7.5, 200 mM KCl, 10 mM EDTA), and **1** (100 μL, 100 μM) and incubated at room temperature for 30 min. The reaction mixture was concentrated by centrifugation using an Amicon 10K centrifugal filter. To prevent loss of protein, the centrifugal filter was blocked with Pol θ prior to addition of the sample. Blocking was conducted by adding Pol θ (500 μL, 1 μM,) followed by centrifugation (13,000 g, 5 min, 4 °C) and removal of the supernatant. Following blocking of the membrane filter, half of the sample (500 μL) was added to the Amicon centrifugal filter, and centrifugation was carried out (13,000 g, 5 min, 4 °C). The remainder of the sample was added and centrifugation was repeated. The sample was then washed twice with 400 μL of reaction buffer (50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA) and concentrated by centrifugation in the Amicon centrifugal filter to 100 μL. Trypsin/Lys-C mix was reconstituted in the resuspension buffer provided by the manufacturer (20 μL) and added (2 μg, 2 μL) to the sample, which was incubated at 37 °C for 4 h. The digestion mixture was concentrated in a Speed Vac concentrator to 40 μL and a portion (10 μL) was analyzed

by UPLC-MS/MS using an ACQUITY UPLC HSS T3 Column (100 Å, 1.8 μm, 2.1 mm × 100 mm). The flow rate was 0.3 mL/min running a gradient from 85:5:10 water: acetonitrile:1% formic acid to 50:40:10 water: acetonitrile:1% formic acid over 35 min. The MS conditions were as follow. Acquisition Mode = Positive Polarity, Resolution Scan Mode; Scan type = MSe (alternating MS and pseudo MS/MS scans with ramping collision voltage); Scan time = 0.5 sec / scan; Acquired Mass Range (MS) = 160-3000; MSe Collision Energy Ramp = 15-45 V across MSe scan; Cone Voltage = 40 V; Capillary Voltage = 3.0 kV; Extraction voltage = 4 V; Source temp. = 130 °C; Desolvation temp. = 400 °C; Desolvation gas flow = 400 L/h.

Schiff base trapping experiments A solution of Pol θ (wild type, K2383A, K2383R, K2383A/K2575A/K2577A, K2575A/K2577A, 250 nM) and 3'-^{32}P-**3** (50 nM) was incubated in reaction buffer (50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) at 37 °C for 15 s. $NaBH_4$ (2.5 mM) was added and the solution was incubated at 37 °C for 1 h. In a typical experiment, a 10 × solution of 3'-^{32}P-**3** (500 nM) in 1 × phosphate buffered saline (10 mM sodium phosphate 100 mM NaCl, pH 7.2) was prepared by photolysis (350 nm, 10 min). The 10 × solution of 3'-^{32}P-**3** (2 μL) was added to a solution of H_2O (12 μL) and 10 × reaction buffer (2 μL). A 10 × solution (2 μL) of Pol θ in storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) was added, and the reaction was incubated at 37 °C for 15 s. A solution of $NaBH_4$ (25 mM) was prepared in H_2O and added (2 μL) to each reaction. Reactions were incubated at 37 °C for 1 h and then quenched by addition of 5 μL of 5× SDS loading buffer (200 mM Tris HCl pH 8, 5 % SDS, 40 % glycerol). Samples were resolved by SDS-PAGE (5% stacking, 10% resolving) run for approximately 40 min at 190 V. The gel was exposed to phosphor storage cassette and imaged by phosphorimaging.

Fluorescence anisotropy measurements. Anisotropy measurements were conducted using a solution of dichloro diphenyl fluorescein-labeled ternary complex **6** (1 nM) and Pol θ (varying concentrations) in a reaction buffer (50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol). Samples also contained 10 % Pol θ storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) by volume. In a typical experiment, a sample (300 μL) was prepared by mixing Pol θ (30 μL, 2 μM) in storage buffer with 10 × reaction buffer (30 μL), **6** (30 μL, 10 nM), and H_2O (210 μL). The concentration of Pol θ in this solution, termed solution A, is 200 nM. Samples containing varying concentrations of Pol θ were prepared by serial dilution with solution B. Solution B (10 mL) was prepared by mixing H_2O (7.95 mL) with 10 × reaction buffer (1 mL), Pol θ storage buffer (1 mL), and **6** (200 nM, 50 μL). By mixing solution A (150 μL) with solution B (150 μL), the concentration of Pol θ was decreased to 100 nM, while the concentration of **6**, reaction buffer, and storage buffer remained unchanged. An aliquot (150 μL) of this new solution was then mixed with solution B (150 μL) to prepare a new solution containing 50 nM Pol θ. Serial dilutions were repeated such that, samples contained Pol θ concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.57 nM for wild type Pol θ; 300, 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 nM for K2383A and K2383R; 100, 50, 25, 12.5, 6.25, 3.13, 1.57 nM for K2575A/K2577A; and 200, 100, 75, 50, 37.5, 25, 12.5, 6.25 nM for K2575A/K2577A/K2383A. Samples were incubated at 25 °C for 1 h and fluorescence anisotropy (A) was measured using a portion (125 μL) of each sample with PMT voltage of 800 mV, 8 nm slit width, and 535 nm excitation and 556 nm emission. Fluorescence anisotropy was measured for **6** in the absence of polymerase (A_0), and the change in anisotropy ($A-A_0$) was calculated for each sample and plotted against the concentration of Pol θ. The data were fit to the Hill equation $A=A_{max}([enzyme]^n/K_d{}^n+n)$ where n is the Hill coefficient using Origin 7.0.

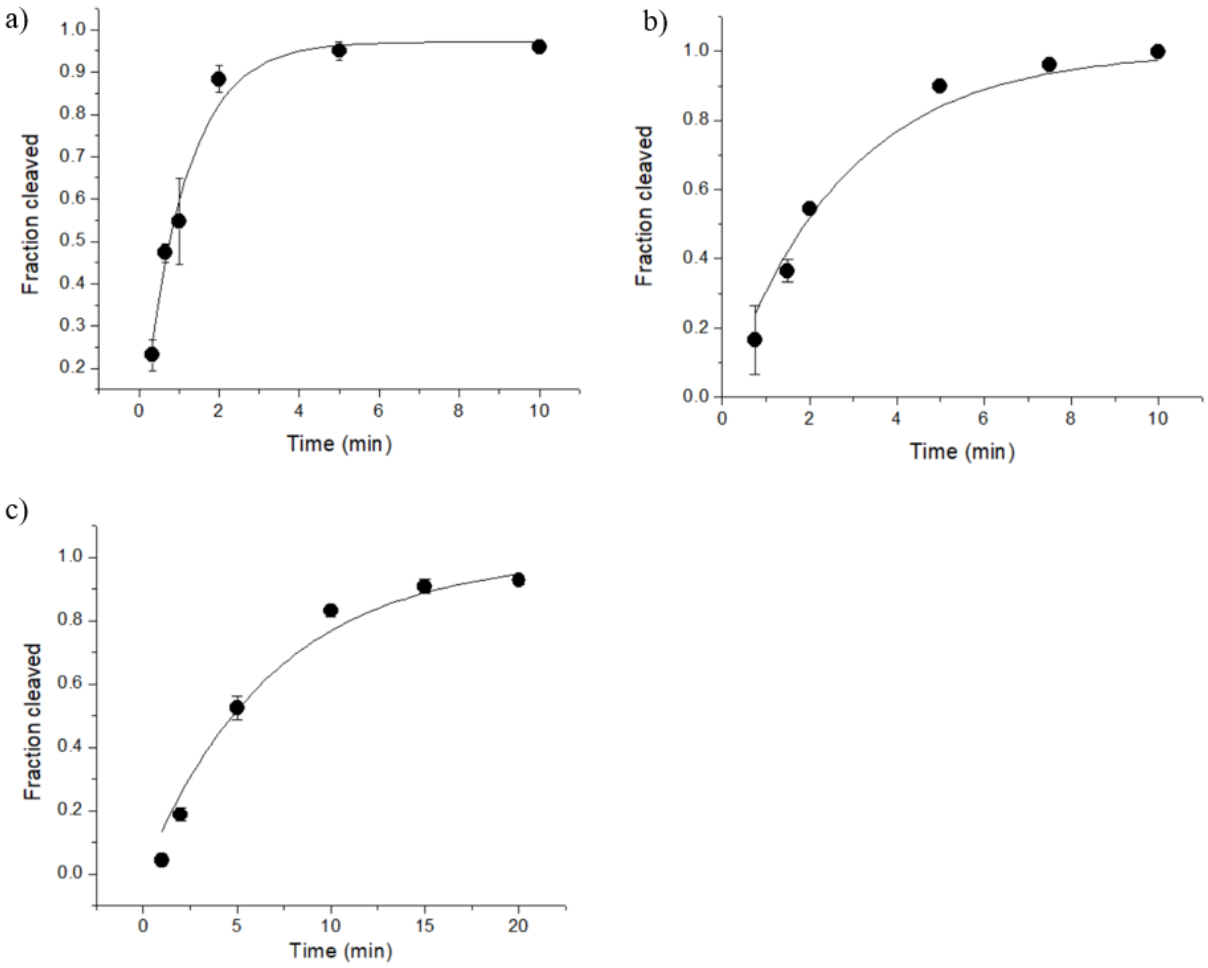
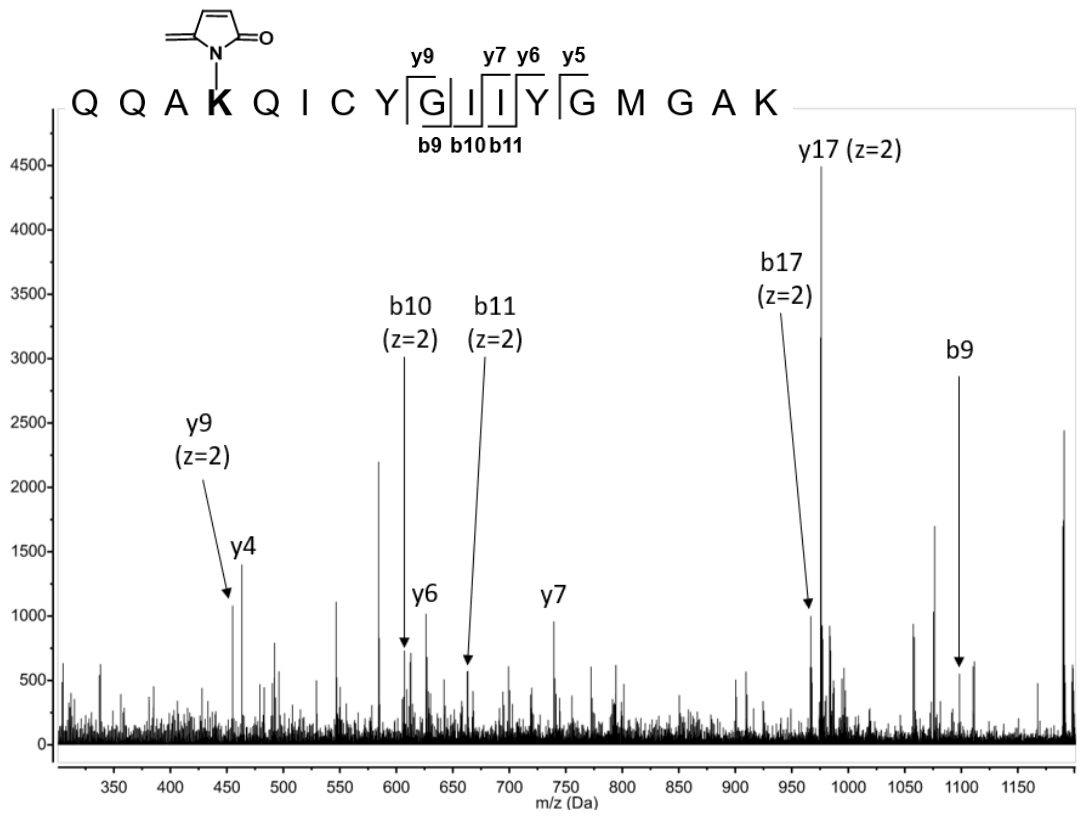


Figure S1. Representative kinetics plots for single turnover kinetics measuring excision by Pol θ of a) pC4-AP (3'-^{32}P-**1**) b) DOB (3'-^{32}P-**2**) and c) dRP (3'-^{32}P-**3**). Data are the average ± standard deviation of three replicates.



b	found	predicted	y	found	predicted
1			17	1949.944	1949.9613
2			16		
3			15		
4			14		
5			13		
6			12		
7			11		
8			10		
9	1098.4962	1098.5038	9	909.4584	909.4863
10	1211.5826	1211.5878	8		
11	1324.643	1324.6719	7	739.3467	739.3808
12			6	626.2722	626.2967
13			5	463.2239	463.2334
14			4		
15			3		
16			2		
17	1931.9026	1931.9507	1		

Figure S2. Annotated MS/MS of modified peptide **4**

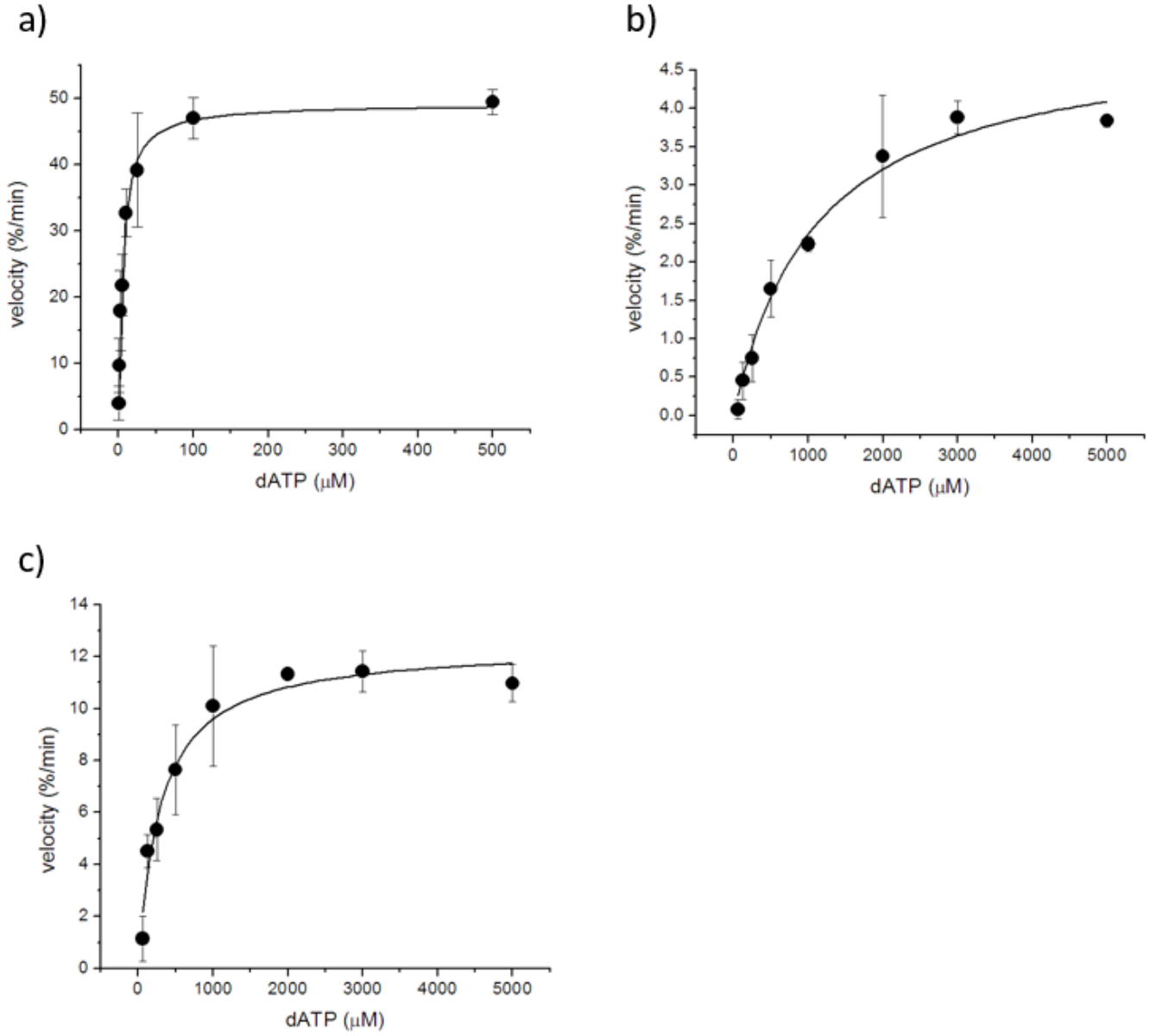


Figure S3. Sample kinetic plots for dA insertion on 5'-^{32}P-**5** by Pol θ variants. a) wild type b) K2383A c) K2383R. Data are the average ± standard deviation of three replicates.

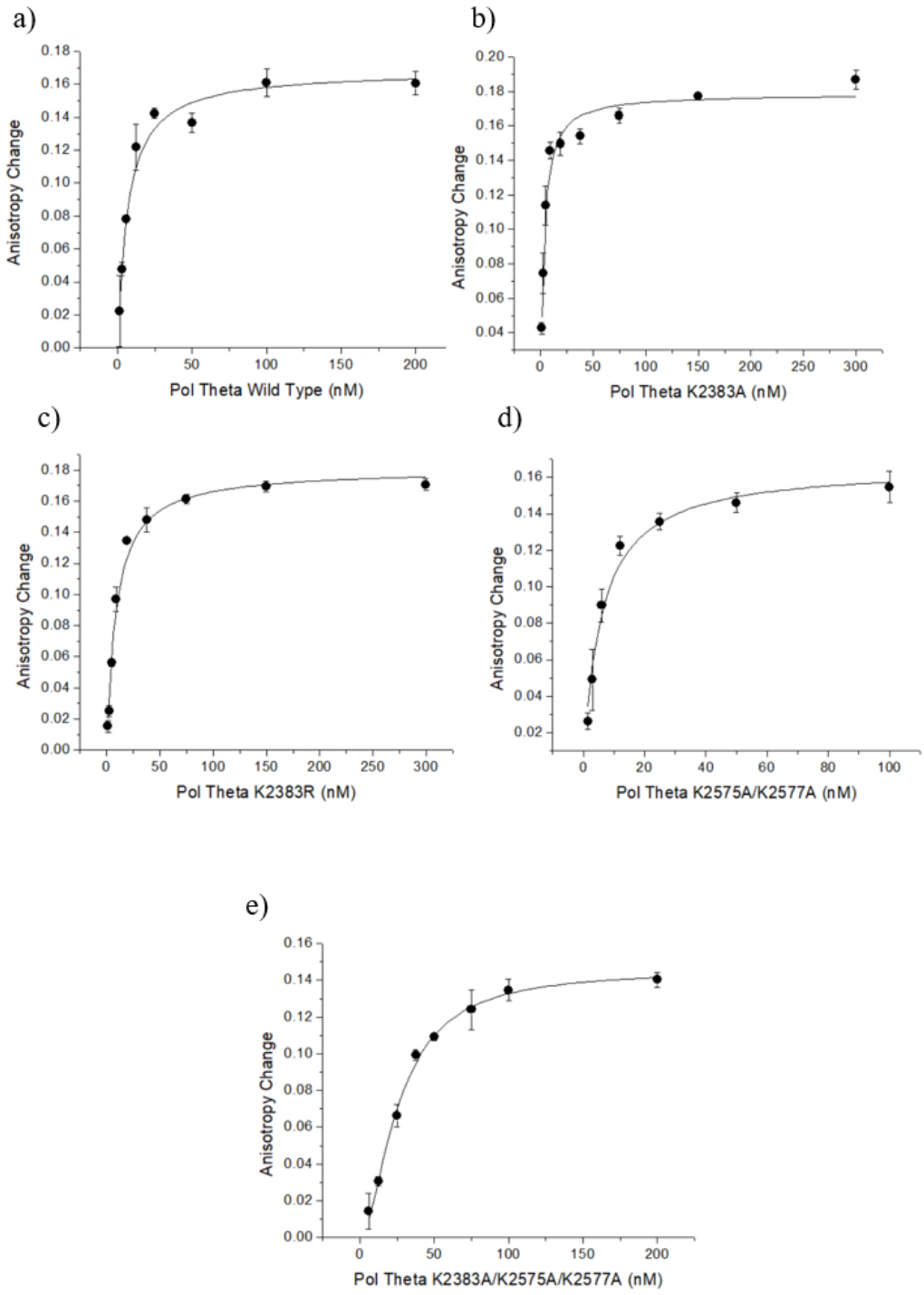


Figure S4. Plots for fluorescence anisotropy experiments using Pol θ variants and **6**. a) wild type b) K2383A c) K2383R d) K2575A/K2577A e) K2383A/K2575A/K2577A. Data are the average ± standard deviation of three experiments.

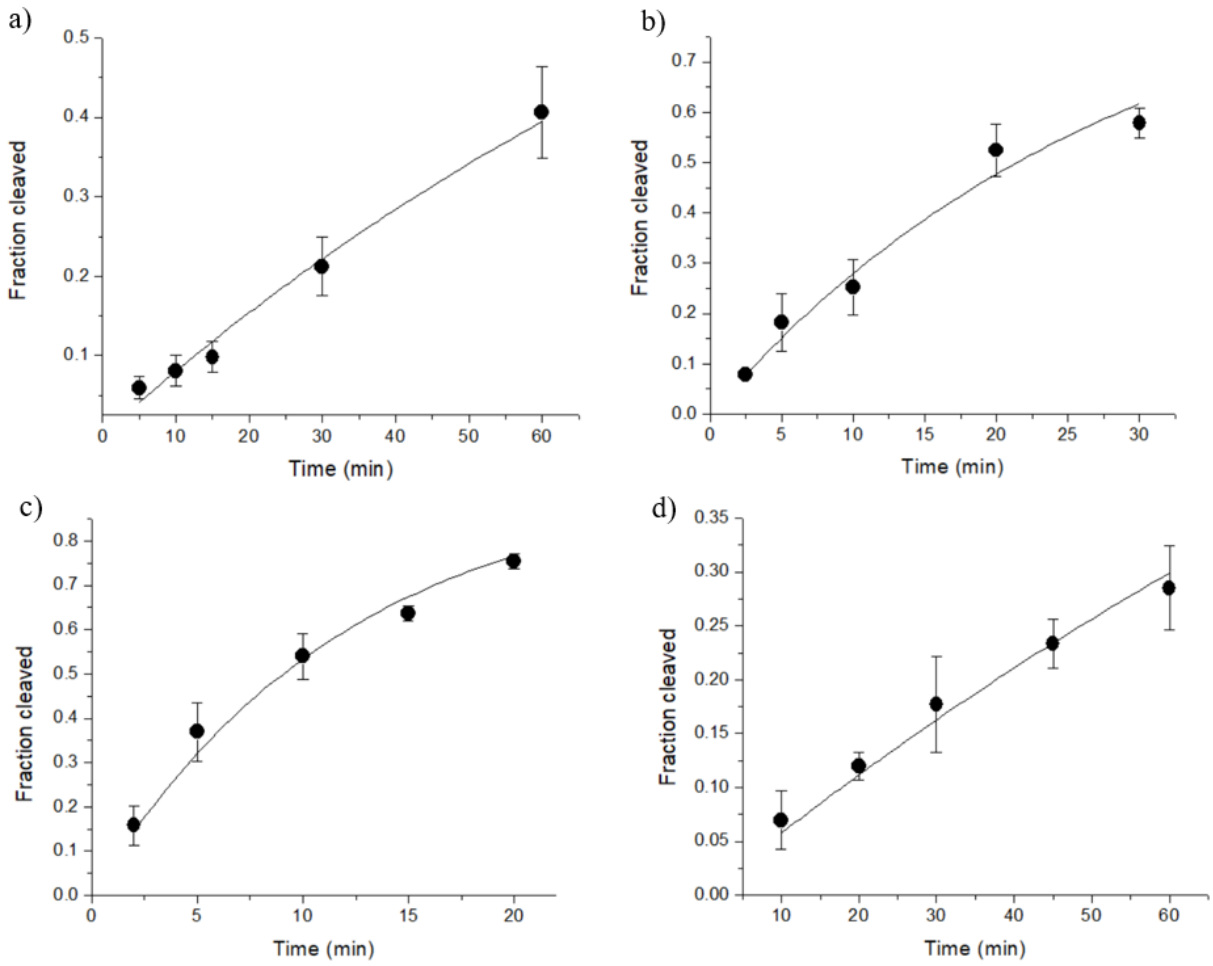


Figure S5. Sample kinetic plots for excision of dRP (3'-^{32}P-**1**) by Pol θ variants. A) K2383A b) K2383R c) K2575A/K2577A d) K2383A/K2575A/K2577A. Data are the average ± standard deviation of three replicates.

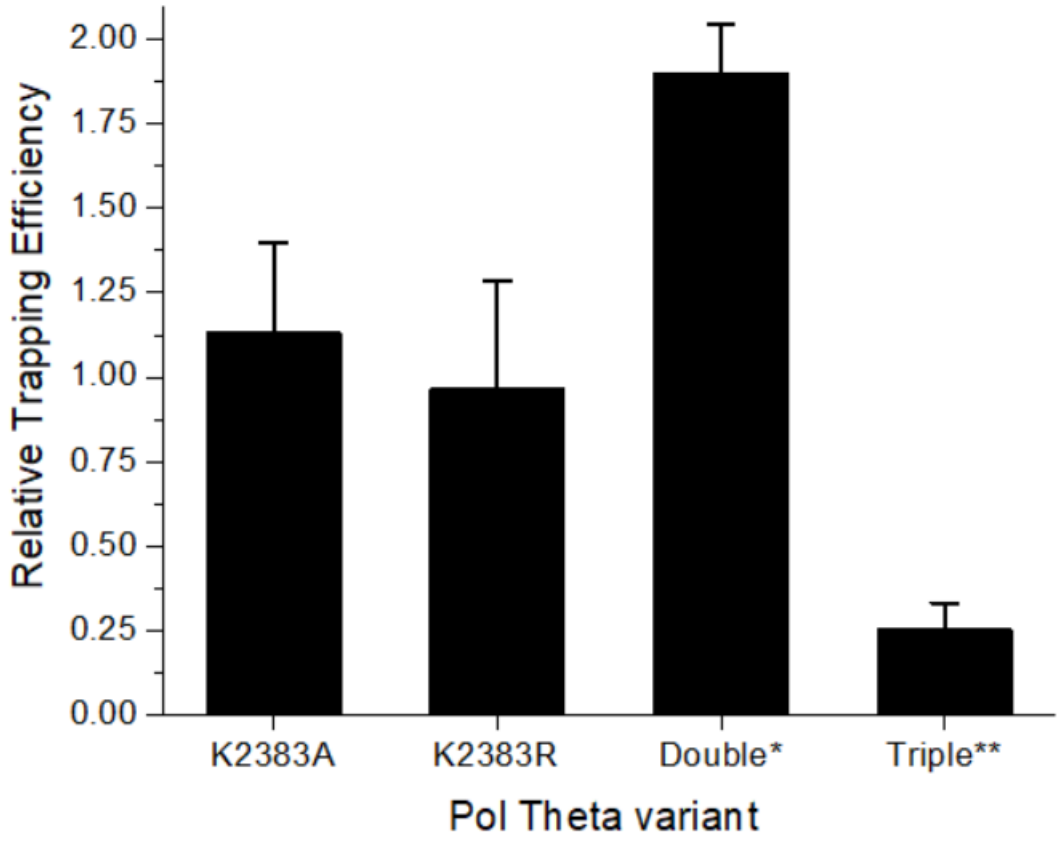


Figure S6. Trapping efficiency (relative to wild type) of Pol θ variants with dRP (3'-^{32}P-**3**) in the presence of $NaBH_4$. *Double mutant = K2575A/K2577A. **Triple mutant = K2383A/K2575A/K2577A. Data are the average ± standard deviation of at least three independent experiments.

5’- **PX**C CGT AAT GCA GTC T-3’

P: PO_4^{2-}

Mass (calc): 4920.3

Mass observed: 4921.0

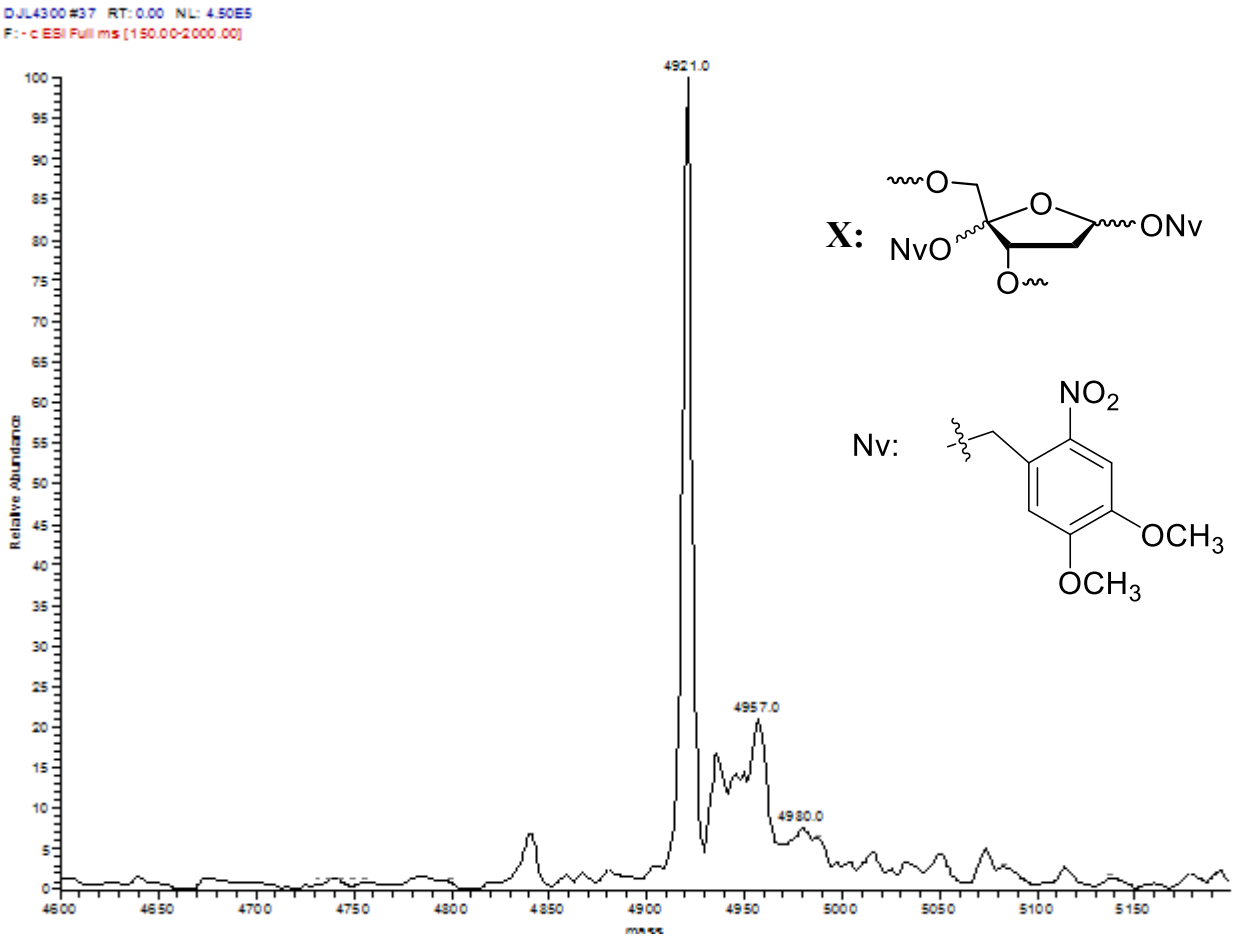


Figure S7. ESI-MS of pC4-AP precursor oligonucleotide used for **1**.

5’- **X**C CGT AAT GCA GTC T-3’

Mass (calc): 4811.2

Mass observed: 4810.0

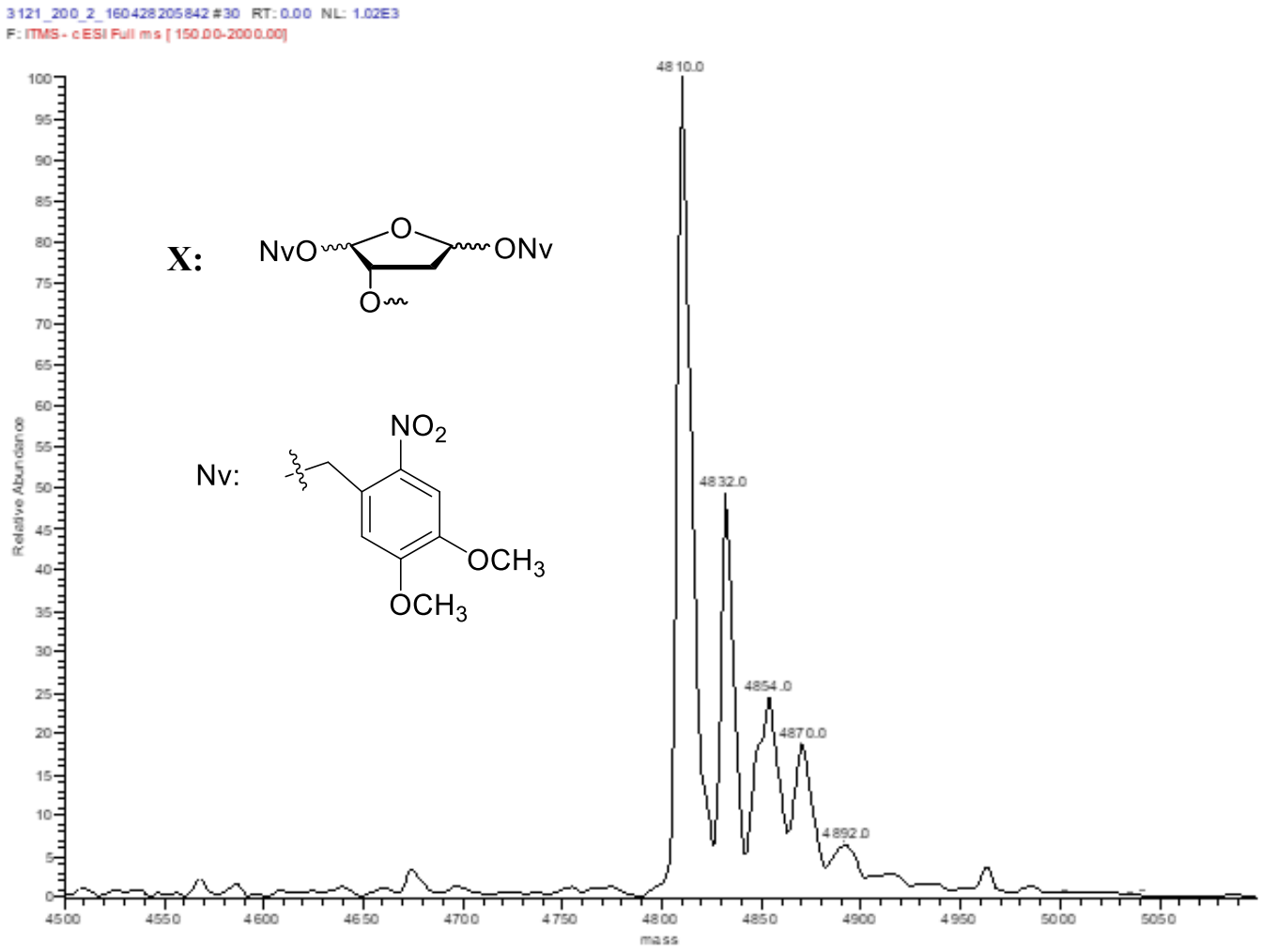


Figure S8. ESI-MS of DOB precursor oligonucleotide used for **2**. *m/z* = 4832 corresponds to M + Na.

5’- **PF**C CGT AAT GCA GTC T-3’

P: PO_4^{2-}

Mass (calc): 4498.9

Mass observed: 4499.0

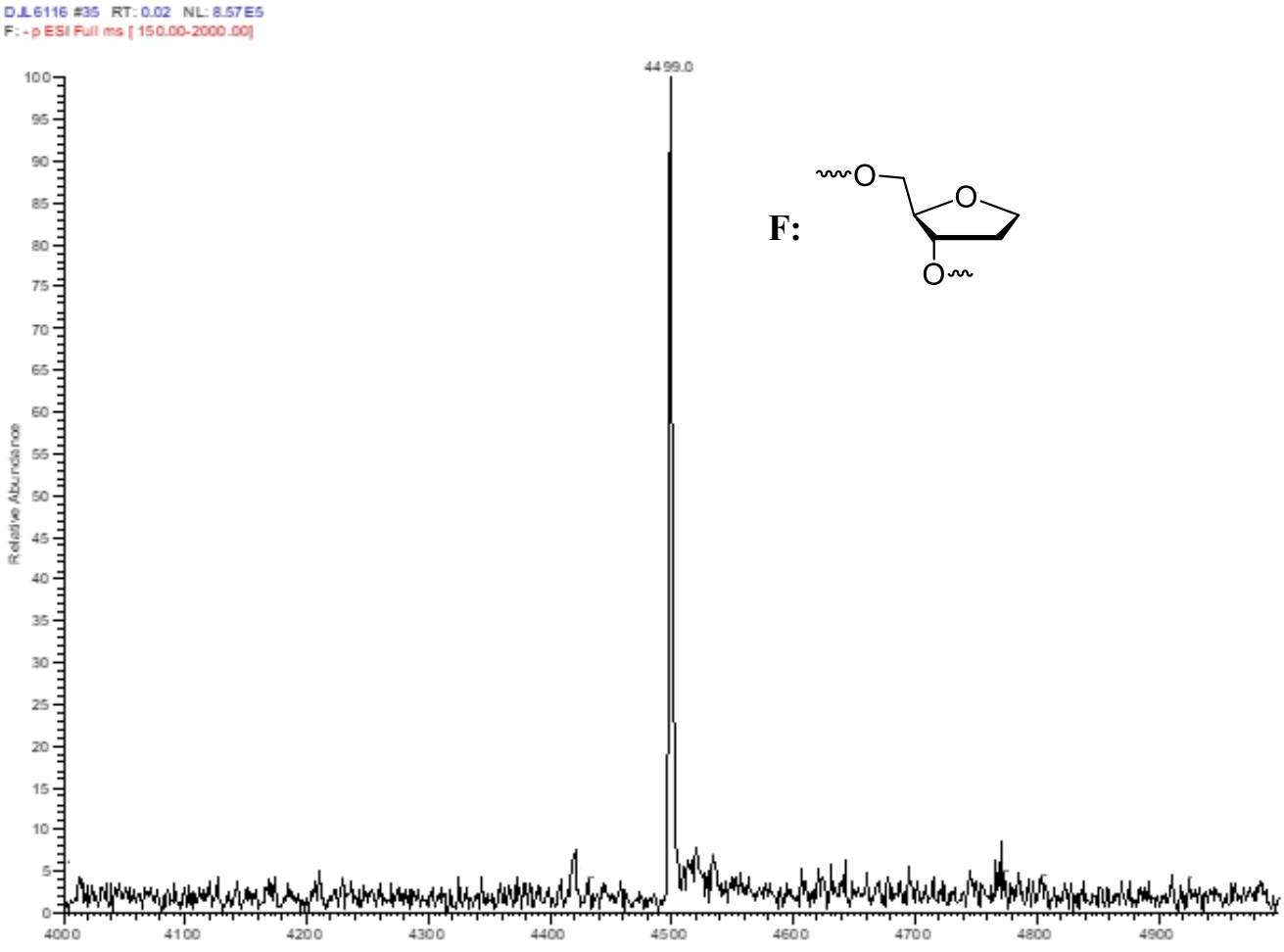


Figure S9. ESI-MS of F-containing oligonucleotide used for **6**.

5’- **X**AG ACT GCA TTA CGG AAA GCG TTA GCC ATT A-3’

Mass (calc): 9998.6

Mass observed: 9996.1

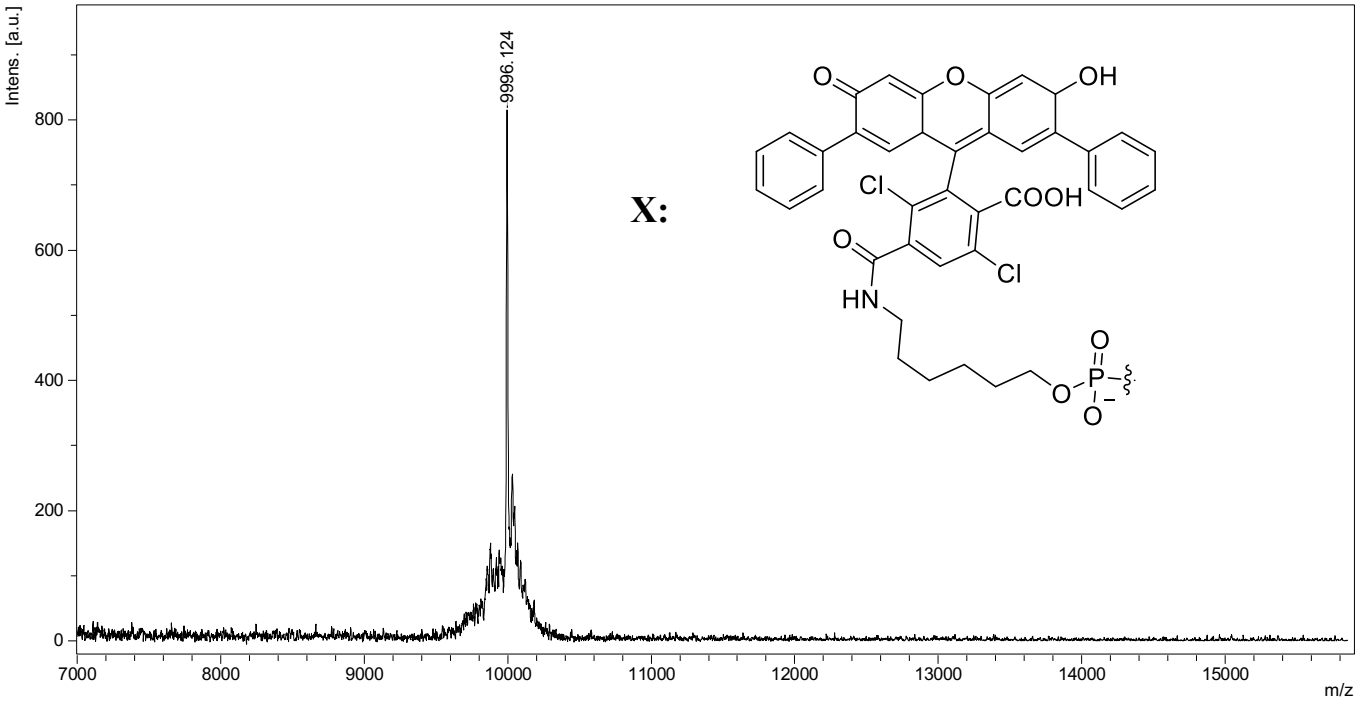


Figure S10. MALDI TOF-MS of dichloro diphenyl fluorescein-containing oligonucleotide used for **6**.

References

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