Supporting Information

Fluorescent molecular rotors detect *O*^{6}-methylguanine dynamics and repair in duplex DNA

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Oligomer synthesis, purification, and folding

Standard DNA phosphoramidites, solid supports, and all necessary reagents were purchased from LinkTech and Sigma-Aldrich. MG-modified oligonucleotides were synthesized on a 1.0 μmol scale using a Bioautomation Co. Mermade 4 DNA synthesizer according to the Trityl-off procedure. Three coupling reactions were performed for the site-specific introduction of the modified nucleoside into oligonucleotides. MG phosphoramidite (N-isobutyryl protected) phosphoramidite were purchased from Glen Research and dissolved in dry acetonitrile (0.1 M) immediately prior to use. The synthesis of the oligonucleotides was monitored by DMT deprotection. Upon completion of the sequences, MG-modified oligonucleotides were cleaved from the solid support and deprotected by 1 mL of 10% DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) in anhydrous methanol in a glass vial. After keeping the solution at r.t for 5 days in the dark, the volume was reduced under vacuum and 1 mL, 10 mM aqueous sodium hydroxide solution was added. The obtained solutions were lyophilized to dryness and purified by HPLC column chromatography using a semi-prep C-18 reverse-phase column (YMCbasic B-22-10P 150 x 10 mm) using a Varian 140 Pro Star HPLC system. The gradient conditions were typically acetonitrile: 0.1 M triethylammonium acetate (TEAA, pH 7.4), 2:98 to 10:90 over 35 minutes and with rate of 3.00 mL/min. Elution was monitored by UV absorption at 260 nm (for MG-modified oligonucleotides). Peaks were collected and twice lyophilized to dryness from water. The purities of MG containing oligonucleotides were found to be >90% (260 nm) according to analytical, reverse-phase chromatography using a Waters XBridge C8, 5 μm 4.6 x 150 mm. A gradient of 5–40% of acetonitrile in 0.1 M triethylammonium acetate (TEAA, pH 7.4), was applied over 35 minutes at 0.4 mL/min. Oligonucleotides were analyzed by LC-MS using a Dionex Ultimate 3000 UHPLC coupled to a Bruker Maxis Impact QTOF in negative ESI mode. Samples were run through a Phenomenex Luna C18(2)-HST column (2.5 μM 120A 2.1 x 100 mm) using a gradient of 90% mobile phase A (100 mM HFIP and 5 mM TEA in H_2O) and 10% mobile phase B (MeOH) to 40% mobile phase A and 60% mobile phase B in 20 minutes. The data was processed and spectra deconvoluted using the Bruker DataAnalysis software version 4.2. Oligonucleotide stock solutions were prepared in deionized water and their concentrations were determined by absorbance at 260 nm using the molar extinction coefficient calculated using a nearest-neighbor model.[1] and the molar extinction coefficient of MG was estimated to be the same as G.[2] Double-stranded oligonucleotides were prepared by diluting the

complementary sequences (1.0 : 1.2 equiv. ratio) in the PBS buffer (pH = 7.4, Na^+ concentration of ≈137 mM) and heating to 95 °C for 5 min, followed by slow cooling to room temperature over 4 h.

Fluorescence spectroscopy

Oligonucleotide stock solutions were diluted into PBS buffer (pH = 7.4, Na+ concentration of ≈137 mM) to a final concentration of 2.0 µM using their extinction coefficient at 260 nm. All measurements were collected on a Molecular Devices SpectraMax M5 in a 1 cm path-length quartz cuvette. Quantum yields were calculated using the most red-shifted absorbance maxima of samples. Quinine hemisulfate (φ_R = 0.546) in 0.5 M H_2SO_4 (n_R = 1.346) was used as the fluorescent standard. Quantum yields were calculated using the equation shown below[2]:

$$\Phi = \Phi_R \frac{F}{F_R} \frac{A_R}{A} \frac{n^2}{{n_R}^2}$$

where φ_R is the quantum yield of the fluorescent standard, F and F_R are the integrated emissions of the sample and reference respectively. A and A_R are the optical densities of the sample and reference respectively (both set to 0.10 ± 0.01). n and n_R are the refractive indexes of the sample and reference respectively.

Protein overexpression and purification

The vector expressing a C-terminally His-tagged version of an inactive variant of human MGMT (MGMT-C145S) was a kind gift from the Wilds laboratory (Concordia University). The plasmids encoding wildtype MGMT (pAG9378) and MGMT-C145A variant (pAG9381) were generated by site-directed mutagenesis and verified by DNA sequencing (Génome Québec Innovation Centre). MGMT and MGMT-C145A were produced in BL21(DE3)GOLD cells (Thermo Scientific). Cells were grown in LB medium supplemented with 100 µM $ZnCl_2$ to an optical density (600 nm) of ~0.7. Protein expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by incubation at either 37°C for 3 h (wildtype) or 16 °C for 18 h (MGMT-C145A). Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl pH 8, 0.5 M NaCl, 5 mM β-mercaptoethanol, 45 mM imidazole, and 5% glycerol) in the presence of protease inhibitors (1 mM benzamidine, 1 mM PMSF, 5 µg/mL leupeptin, and 0.7 µg/mL pepstatin A), and sonicated. The insoluble fraction of the lysate was removed by centrifugation, and the supernatant was loaded onto a HisTrap HP 5 mL column (Cytiva). Contaminants were eluted with 45-60 mM imidazole, whereas MGMT eluted with 240 mM imidazole. The MGMT variants were further purified by cation exchange chromatography using a HiTrap SP HP 5 mL column (GE Healthcare) equilibrated with cation

exchange buffer (20 mM Tris pH 7, 50 mM NaCl, 5 mM β-mercaptoethanol, and 5% glycerol). Purified MGMT and MGMT-C145A were concentrated using Amicon centrifugal devices with a 10 kDa MWCO (Sigma-Aldrich) using storage buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 5 mM DTT, and 15% glycerol).

Repair assays and Proteinase K digests

0.8 µM of duplex DNA was incubated with 0.96 µM of protein in a total volume of 150 µL of activity buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM DTT, pH 7.6) for 30 seconds before a fluorescence spectrum was acquired. Samples were excited with an excitation wavelength of 310 nm and emission spectra were acquired from 260-600 nm. To these samples was added 0.5 µL from 20 mg/mL stocks of Proteinase K (New England Biolabs, Ipswich, MA) and they were allowed to incubate for 3 hours before a second fluorescence measurement was acquired. All measurements were collected on a Molecular Devices SpectraMax M5 in a 1 cm path-length quartz cuvette.

Supplementary tables and figures

Table S1. Synthesized oligonucleotides with their calculated (calc.) extinction coefficients (Ɛ) at 260 nm, and calc. and observed masses and their deviation (δ) in parts-per-million (ppm).

Oligonucleotide	Sequence (5′ → 3′)	ε_{260} ($cm^{-1}M^{-1}$)	Calc. (m/z)	Found (m/z)	δ (ppm)
T	GCGA **T** CGCGCGCTAGCG	156,210	5209.8910	5209.9375	8.9
^{ts}T	GCGA ^{ts}T CGCGCGCTAGCG	163,000	5347.9382	5347.8438	17.7
^{ts}T*	GCGA ^{ts}T CGCGCGCTAGCG	163,000	5347.9382	5347.9375	0.1
MG	CGCTAGCGCGCG MG TCGC	149,000	5199.8957	5200.0781	35.1
MG*	CGCTAGCGCGCG G TCGC	149,000	5185.8800	5185.9453	12.6
MG1	CGCTAGCGCGC MG ATCGC	152,000	5183.9007	5184.0625	31.2
MG3	CGCTAGCGC MG CGATCGC	152,000	5183.9007	5184.0156	22.2
MG5	CGCTAGC MG CGCGATCGC	152,000	5183.9007	5183.9297	22.2
^{ts}C	GCGA ^{ts}C CGC GCG CTA GCG	159,000	5346.3960	5346.8906	92.5
^{ts}C*	GCGA ^{ts}C CGC GCG CTA GCG	159,000	5346.3960	5346.9531	104.2

* = masses observed following incubation with WT MGMT

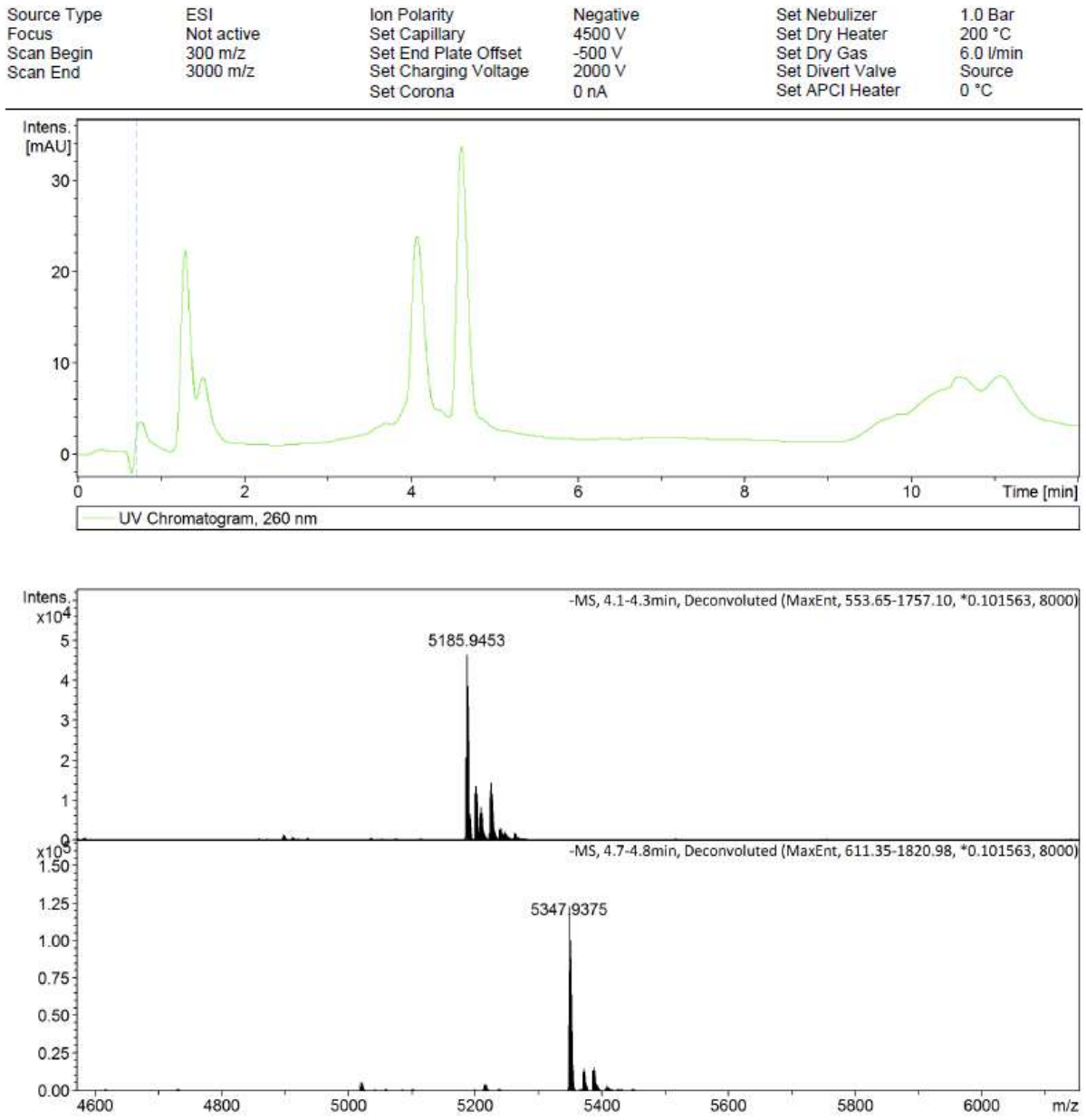

Figure S1. LC-MS analysis of *ds*-^{ts}T:MG duplex after incubation with WT MGMT (1.2 eq.).

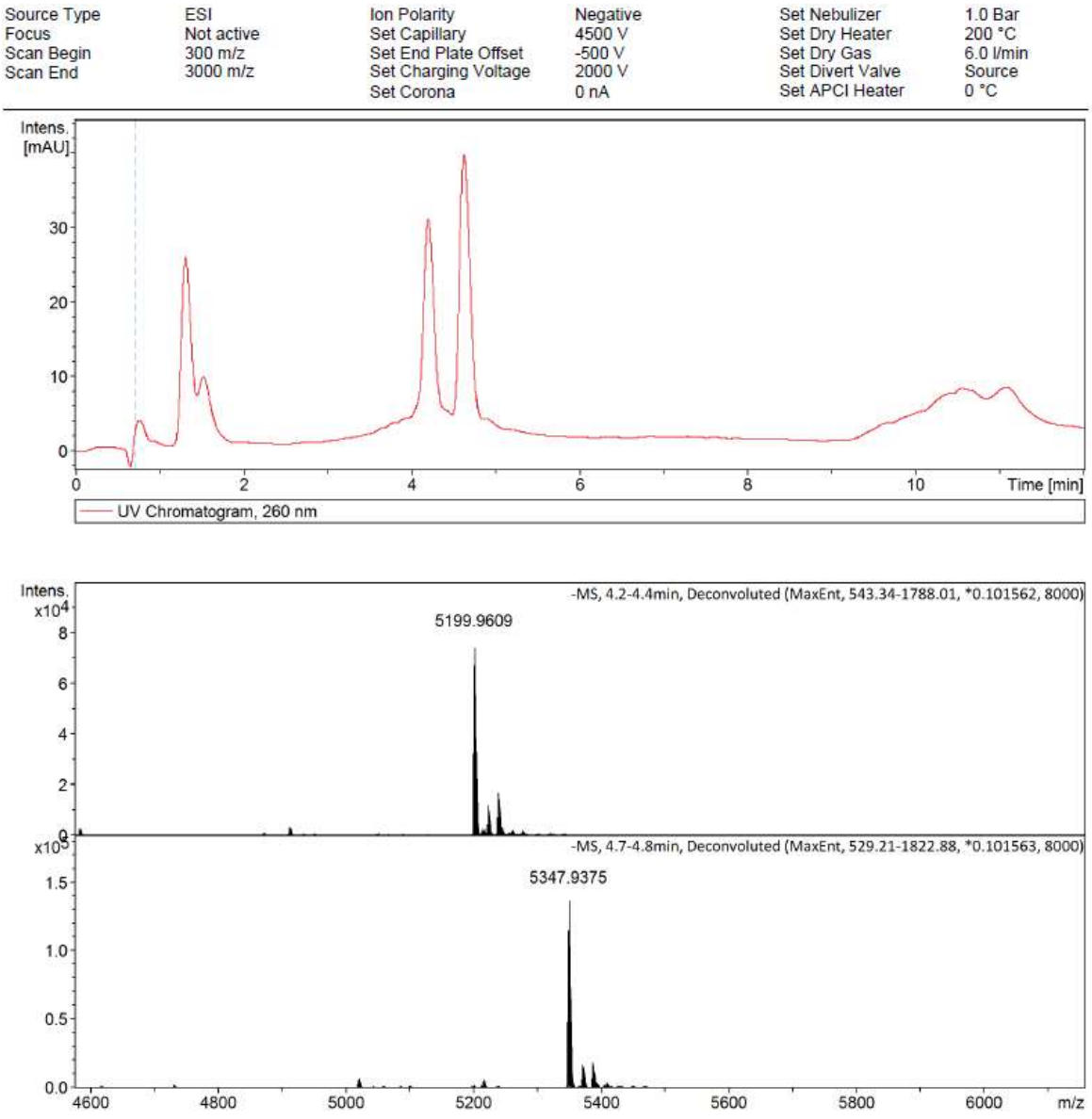

Figure S2. LC-MS analysis of *ds*-^{ts}T:MG duplex after incubation with C145A mutated MGMT (1.2 eq.).

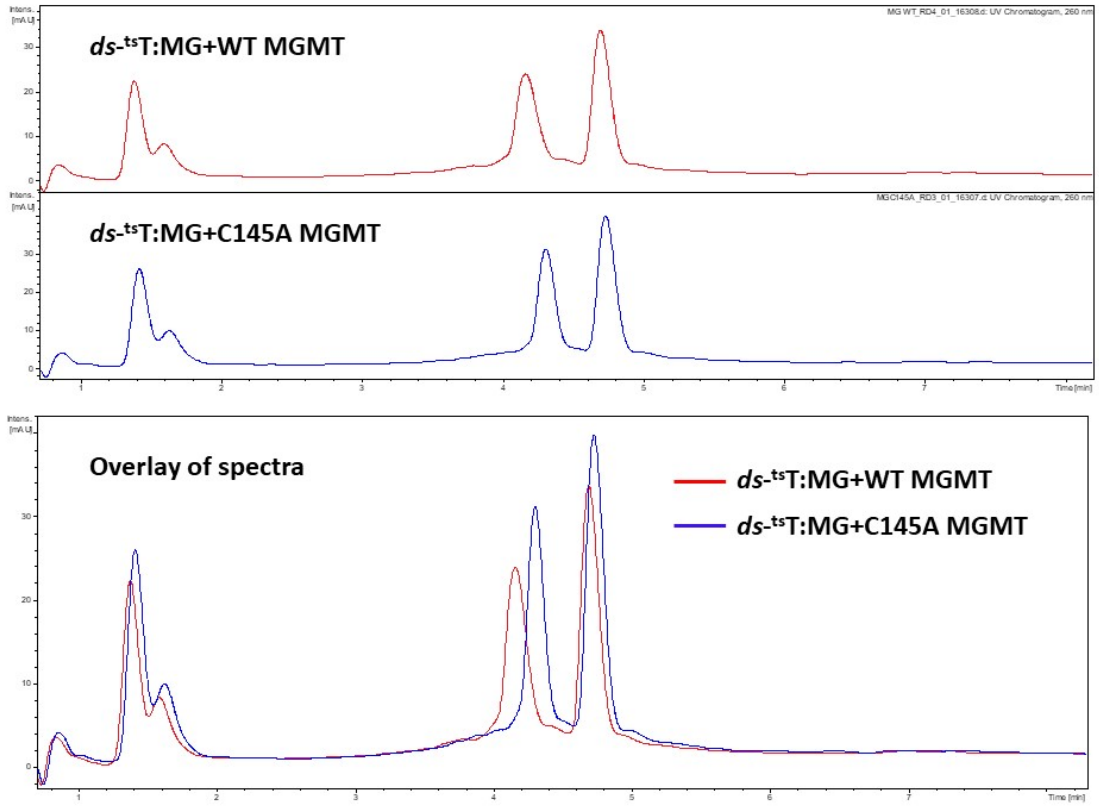

Figure S3. Overlay of chromatographs of *ds*-^{ts}T:MG duplex after incubation with WT or C145A MGMT.

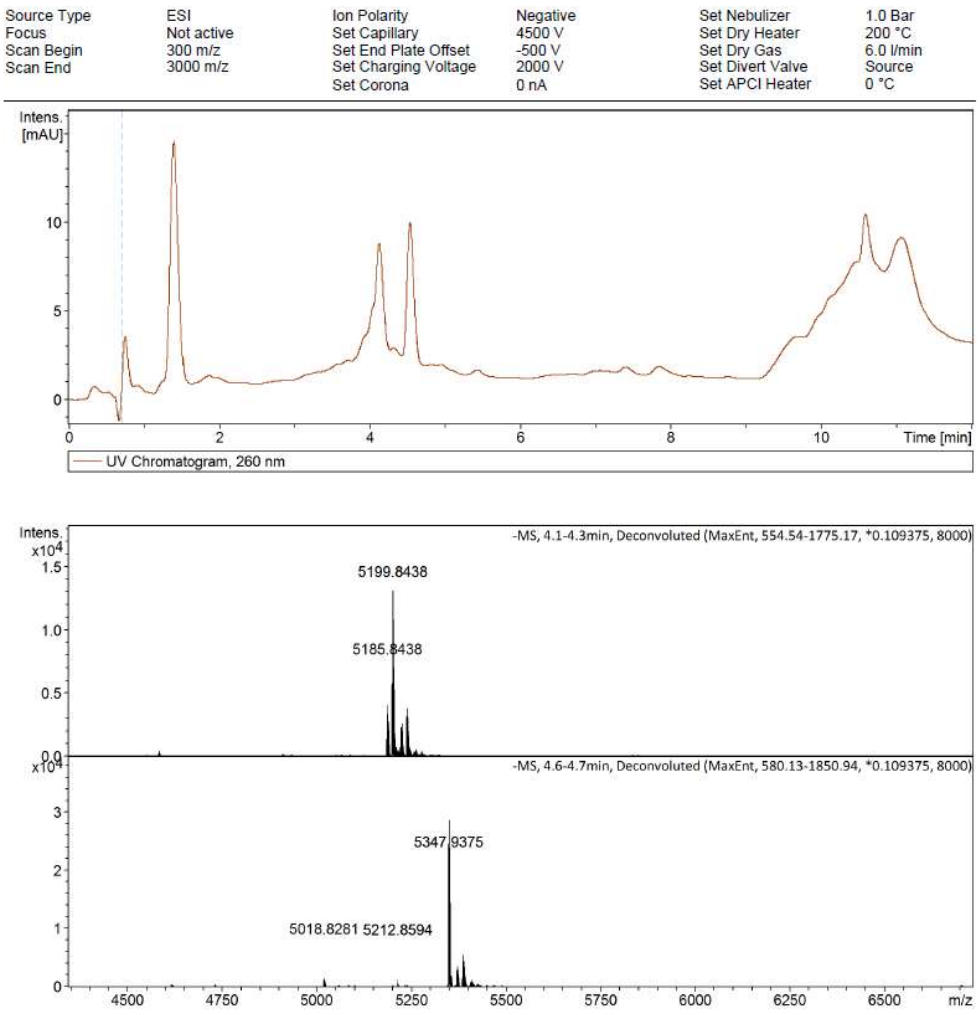

Figure S4. LC-MS analysis of *ds*-^{ts}T:MG duplex (0.8 µM) after incubation with WT MGMT (0.2 eq) for 1 second followed by heat inactivation and analysis.

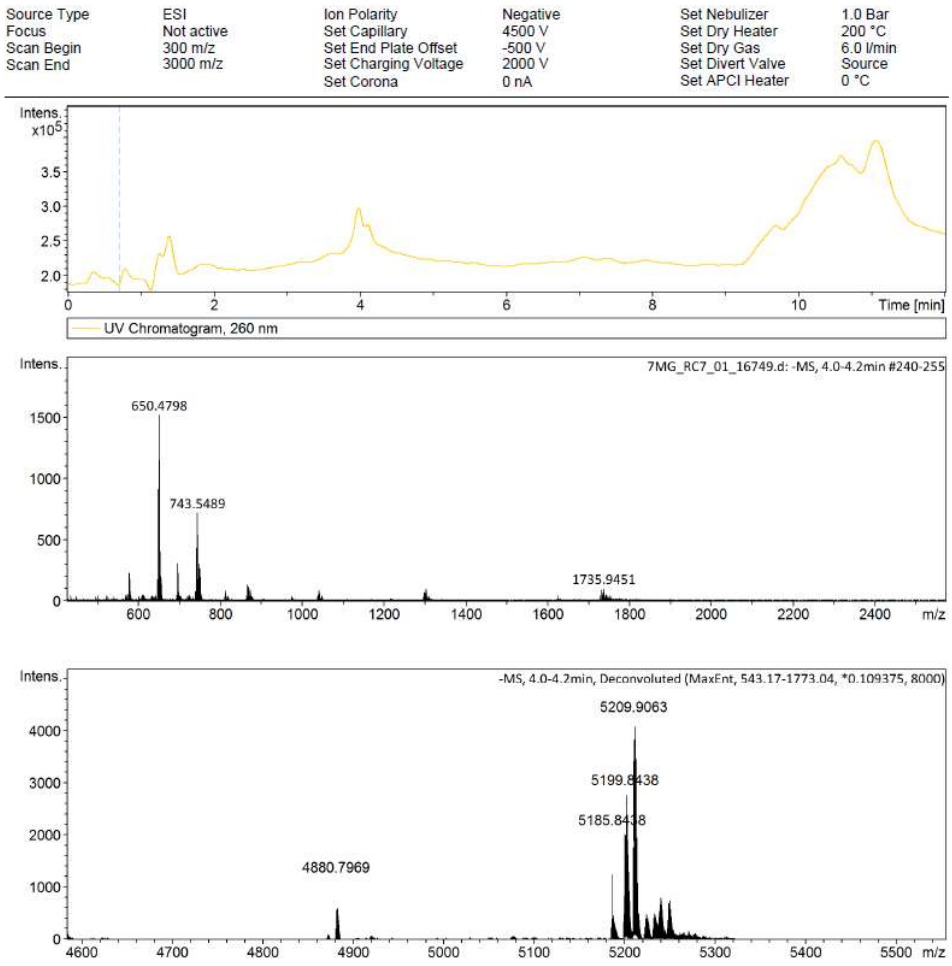

Figure S5. LC-MS analysis of *ds*-**T**:MG duplex (0.8 µM) after incubation with WT MGMT (0.2 eq) for 1 second followed by heat inactivation and analysis.

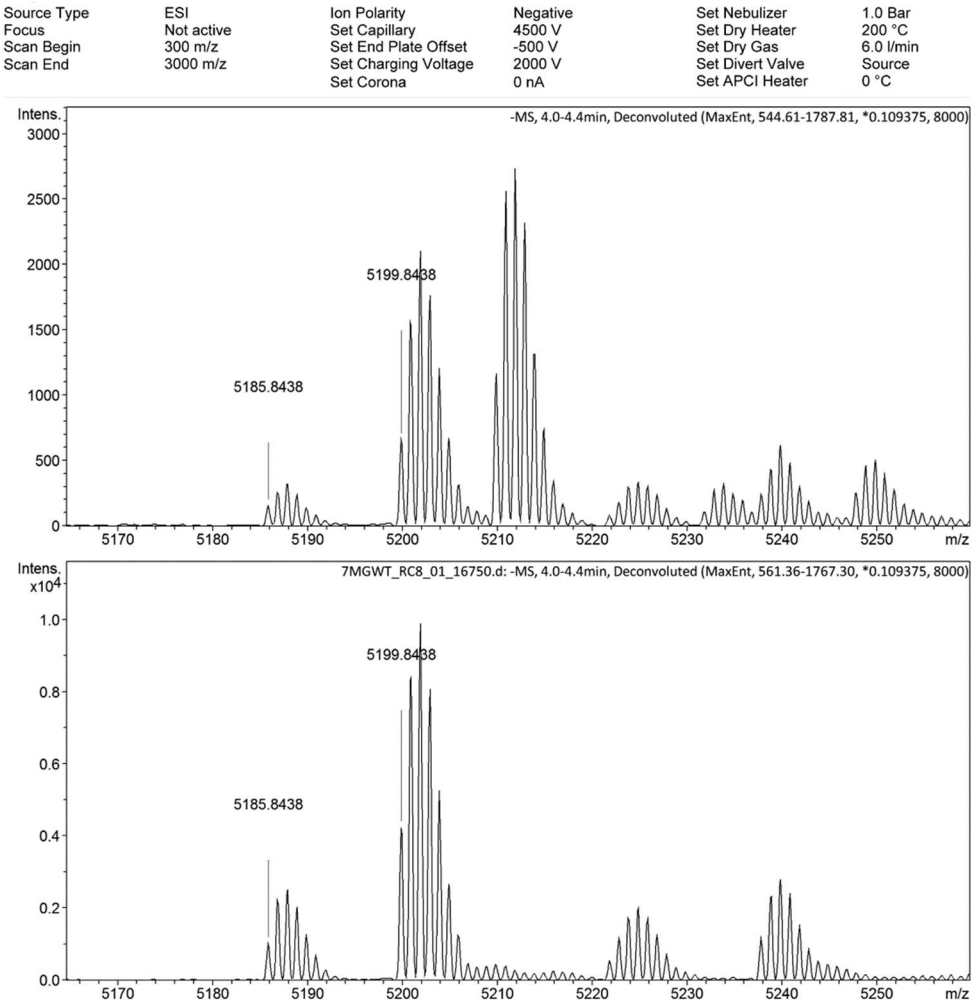

Figure S6. Comparison of integrated LC-MS spectra of *ds*-T:MG duplex (top) and *ds*-^{ts}T:MG duplex (bottom) after incubation with WT MGMT for 1 second followed by heat inactivation and analysis.

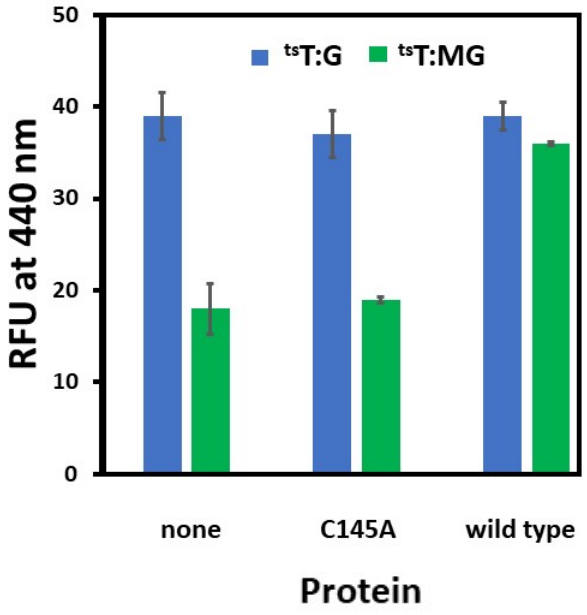

Figure S7. Average fluorescence intensities (Ex. = 310 nm, Em. = 440 nm) of ^{ts}T-containing duplexes (0.8 μM DNA) incubated with 1.2 equivalents of WT or C145A MGMT in buffer for ~30 seconds at room temperature followed by addition of 0.5 μL of Proteinase K (20 mg/mL) for 3 h and monitored by fluorescence. n = 3 independent replicates, error bars represent standard deviations.

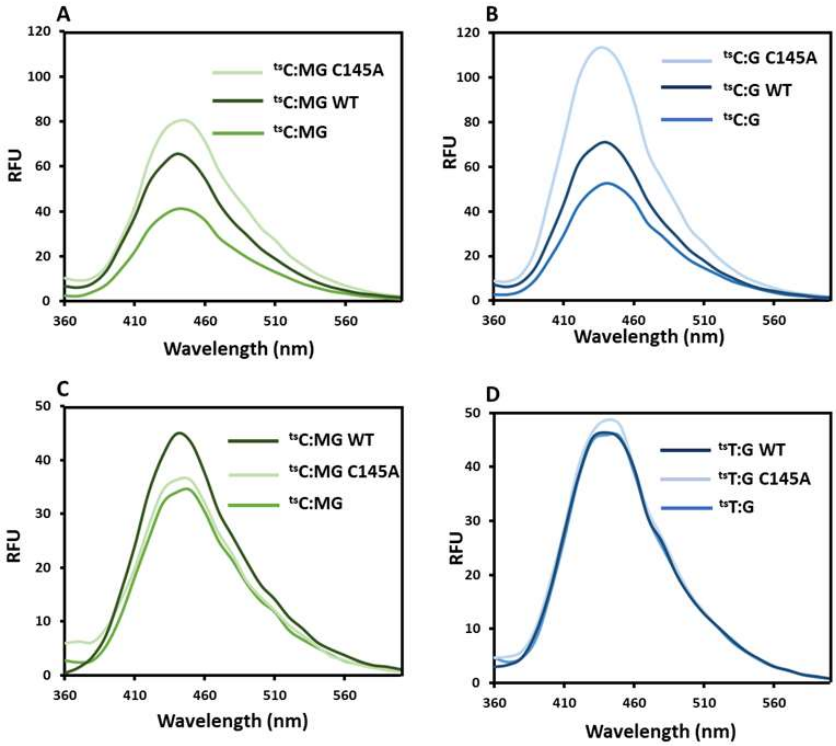

Figure S8. Repair assays of MGMT with ds-^{ts}C:MG or corresponding repair product-ds ^{ts}C:G. (A) – (B): Fluorescence spectra before the addition of Proteinase K. (C) – (D): Fluorescence spectra after the addition of Proteinase K. ^{ts}C containing oligonucleotide sequences. 0.8 µM DNA duplex was incubated with 1.2 equivalents of MGMT in activity buffer for ~30 seconds at room temperature, optionally followed by heat inactivation and addition of 0.5 µL of Proteinase K (20 mg/mL) at room temperature for 3 h and measured for fluorescence (excitation = 310 nm).

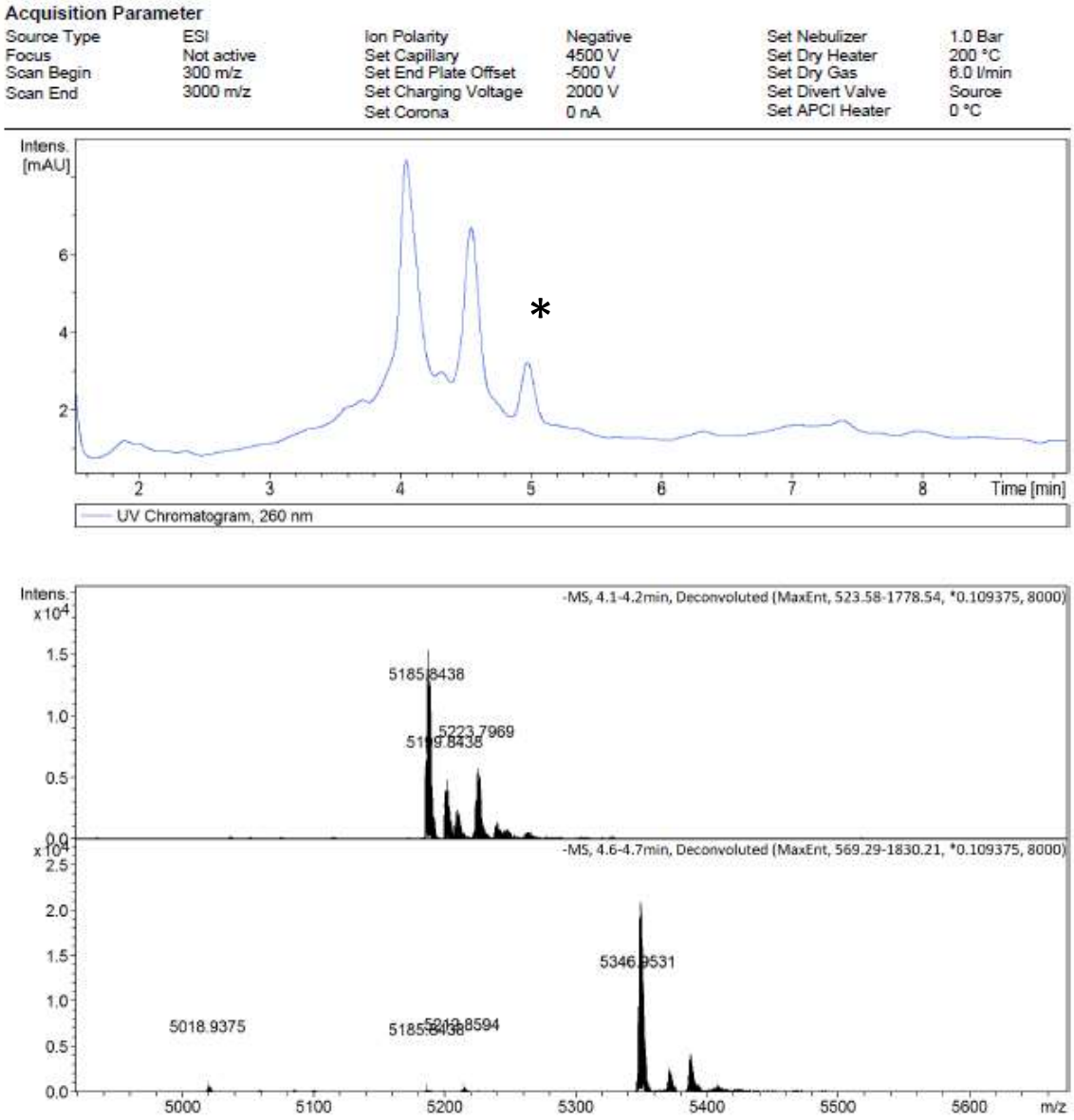

Figure S9. LC-MS analysis of *ds*-^{ts}C:MG duplex after incubation with WT MGMT (1.2 eq.).

* = no detectable mass in peak.

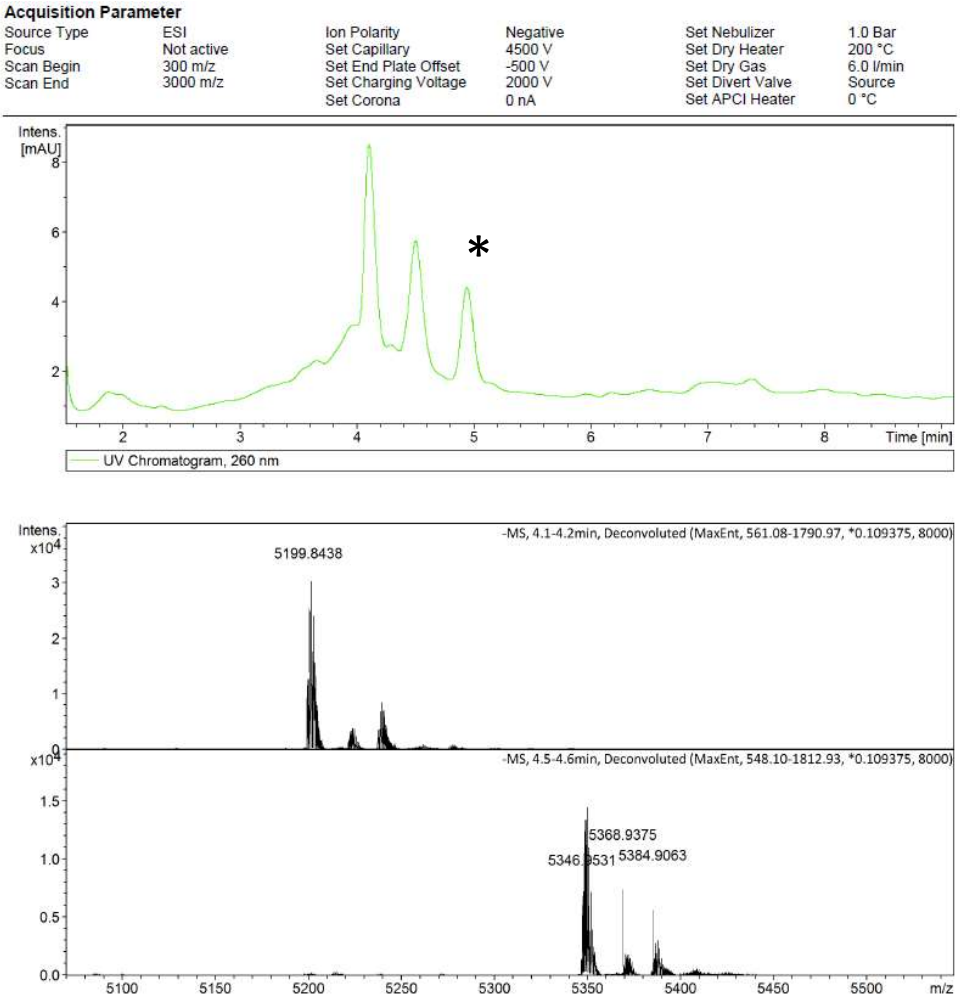

Figure S10. LC-MS analysis of *ds*-^{ts}C:MG duplex after incubation with C145A MGMT (1.2 eq.).
* = no detectable mass in peak.

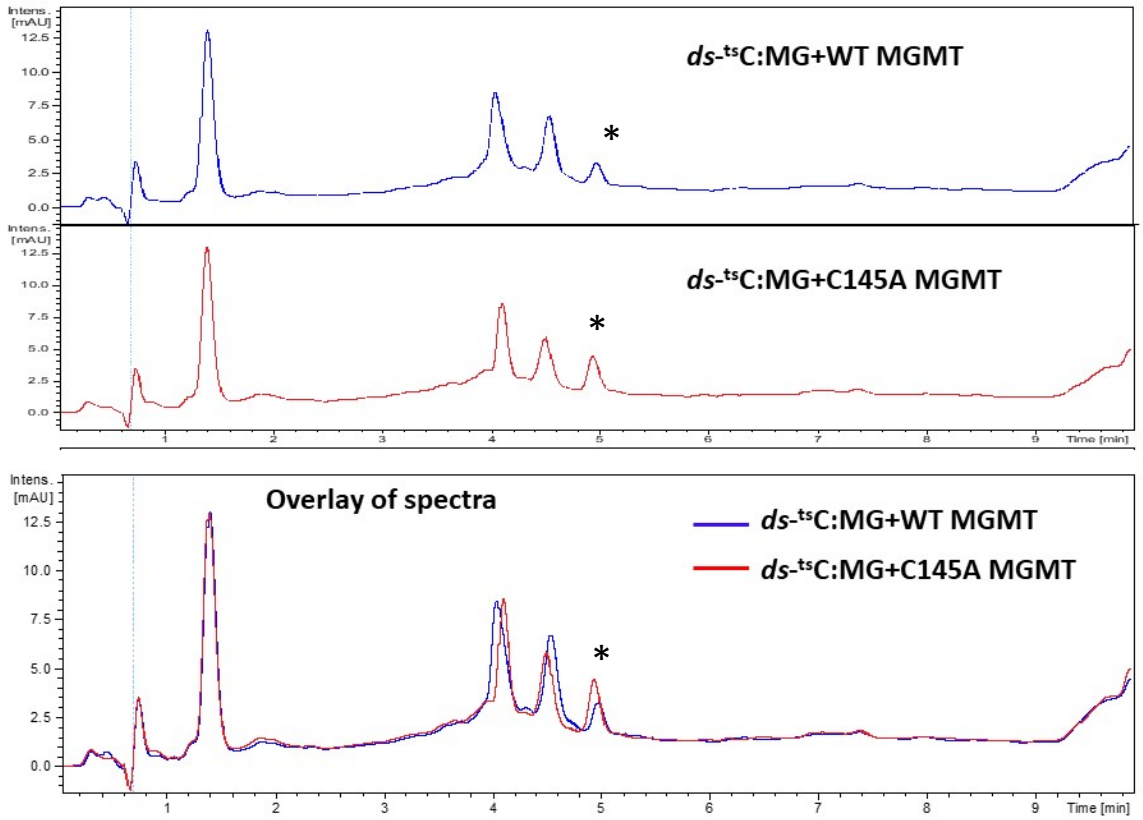

Figure S11. Overlay of chromatographs of *ds*-^{ts}C:MG duplex after incubation with WT or C145A MGMT. * = no detectable mass in peak.

References

[1] 1. C. R. Cantor, M. M. Warshaw, H. Shapiro, *Biopolymers* **1970,** *9,* 1059-1077.

[2] C. W. Abner, A. Y. Lau, T. Ellenberger, L. B. Bloom, *J. Biol. Chem.* **2001,** *276*, 13379-13387.