Supporting Information for

Effects of Tet-induced Oxidation Products of 5-Methylcytosine on Dnmt1- and DNMT3a-mediated Cytosine Methylation

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Materials and Methods

Protein Expression and Purification

The C-terminal fragment of mouse Dnmt1, containing residues 731–1602, was used for enzymatic assay of Dnmt1. Expression and purification of Dnmt1 (731–1602) was as previously described.[1]

To prepare the protein sample for human DNMT3a/DNMT3L complex, the genes encoding DNMT3a and DNMT3L were purchased from Open Biosystems Inc. The sequences corresponding to the C-terminal fragments of DNMT3a (residues 628-912) and DNMT3L (residues 178-380) were subsequently inserted into the BamHI/NotI and NdeI/XhoI sites of a modified pRSFDuet-1 vector (Novagen), in which DNMT3a (628-912) was separated from the preceding His6-SUMO tag by a ubiquitin-like protease (ULP1) cleavage site. To produce the protein, the plasmid was transformed into BL21 (DE3) RIL cell strain (Stratagene). The cells were grown at 37°C until OD_{600} reached approximately 0.6. The temperature was then shifted to 20°C and the cells were induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction, the cells continued to grow overnight. The DNMT3a/DNMT3L complex was first purified through a Ni-NTA column. After removal of the His6-SUMO tag by ULP1 cleavage, the protein complex was further purified through a Heparin column, followed by gel filtration on a 16/60 G200 Superdex column, using buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM DTT, and 5% glycerol.

Preparation of Oligodeoxyribonucleotides Substrates

The modified oligodeoxyribonucleotides (ODNs) were synthesized on a Beckman Oligo 1000S DNA synthesizer (Fullerton, CA, USA) at a 1-μmol scale using phosphoramidite building blocks from Glen Research Co. (Sterling, VA, USA). The ODNs were purified on a Beckman HPLC system with pump module 125 and a UV detector (module 126). A 4.6 × 250 mm Apollo C18 column (5 μm in particle size and 300 Å in pore size; Alltech Associate Inc., Deerfield, IL) was used. Triethylammonium acetate (TEAA, 50 mM, pH 6.6, solution A) and a mixture of 50 mM TEAA and acetonitrile (70/30, v/v, solution B) were employed as mobile phases. The flow rate was 0.8 mL/min. A gradient of 5–20% B in 5 min, 20–45% B in 40 min, and 45-80% B in 5

min was employed for the separation. The purified ODNs were desalted on the same HPLC system with H_2O as mobile phase A and methanol as mobile phase B, and a gradient of 0% B in 20 min, 0–50% B in 1 min, and 50% B in 25 min was used. The unmodified ODNs used in this study were purchased from Integrated DNA Technologies (Coralville, IA), purified and desalted in the same fashion. The identities of the modified ODNs were confirmed by LC-MS/MS analysis (spectra shown in Fig. S1–S5) which were carried out using an Agilent 1100 capillary HPLC pump (Agilent Technologies, Palo Alto, CA) and an LTQ linear ion-trap mass spectrometer (Thermo Electro Inc., San Jose, CA).

Methylation of Cytosine Residues in Duplex DNA

The Dnmt1-mediated methylation assays were carried out at 37°C in triplicate using protocols described previously with minor modifications.[1] A typical reaction mixture contained 300 μM *S*-adenosyl-L-methionine (SAM, New England Biolabs, Ipswich, WA), 60 nM of Dnmt1 and 30 pmol duplex DNA in a 50-μL buffer with 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5% glycerol and 100 μg/mL BSA. The reaction times for Dnmt1 were 30 min, 1 h and 2 h, respectively. The enzyme was subsequently inactivated by heating at 65°C for 20 min and removed by chloroform extraction. The aqueous layer was dried by using a Savant Speed-Vac (Thermo Savant Inc., Holbrook, NY).

The DNMT3a-mediated methylation assays were performed at 37°C in triplicate. A typical 50-μL reaction mixture contained 300 μM SAM, 600 nM of DNMT3a/DNMT3L and 30 pmol duplex DNA in a buffer comprised of 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT and 2.5% glycerol. The reaction was stopped after 6 h using the same method as described above for Dnmt1.

LC-MS/MS Analysis and Data Processing

The extent of cytosine methylation in synthetic duplex DNA was quantified by online LC-MS/MS experiments, which were carried out the same instrument as described above. A 0.5 × 150 mm Zorbax SB-C18 column (particle size 5 μm; Agilent Technologies) was used for the separation of ODNs and the flow rate was 8.0 μL/min. A gradient of 5 min of 5–25% methanol followed by 35 min of 25–50% methanol in 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP;

pH was adjusted to 7.0 by addition of triethylamine) was employed. The voltage for electrospray was 4.0 kV, and the ion transport tube of the mass spectrometer was maintained at 275°C to minimize the formation of HFIP adducts of ODNs.

The specific precursor ions for the methylated and unmethylated sequences were chosen for fragmentation to acquire the tandem mass spectra, where the normalized collision energy was 30%. The integrated peak areas (IAs), which were found in the selected-ion chromatogram (SIC) plotted for the formation of three abundant fragment ions of the ODNs containing a methylated and unmethylated cytosine at CpG site, were used to calculate the percentage of methylation. For instance, we chose the w_6^{2-}, w_7^{2-}, and $[a_6 - G]^{2-}$ ions for the quantification of the methylation levels at sites 1 and 2 of bottom strand, i.e., the ions of *m/z* 921.8, 1086.3 and 822.3 when site 2 was methylated, and the ions of *m/z* 914.7, 1079.3 and 829.3 when site 1 was methylated (Fig. S12). The three fragment ions selected for the quantification of the top strand were w_6^{2-}, w_8^{2-}, w_9^{2-}. The MS/MS of the methylated ODNs were shown in Fig. S6–12. It is worth noting that our LC-MS/MS results also led to the detection of the bottom strand with both sites 1 and 2 being methylated (Fig. S11); this product was included during the calculation of the percentages of methylations at sites 1 and 2. For instance, the methylation level at site 1 reflected the sum of the product methylated only at site 1 and that methylated at both sites.

Supplementary References:

1. J. Song, O. Rechkoblit, T. H. Bestor, and D. J. Patel, Science 2011, 331, 1036-1040.

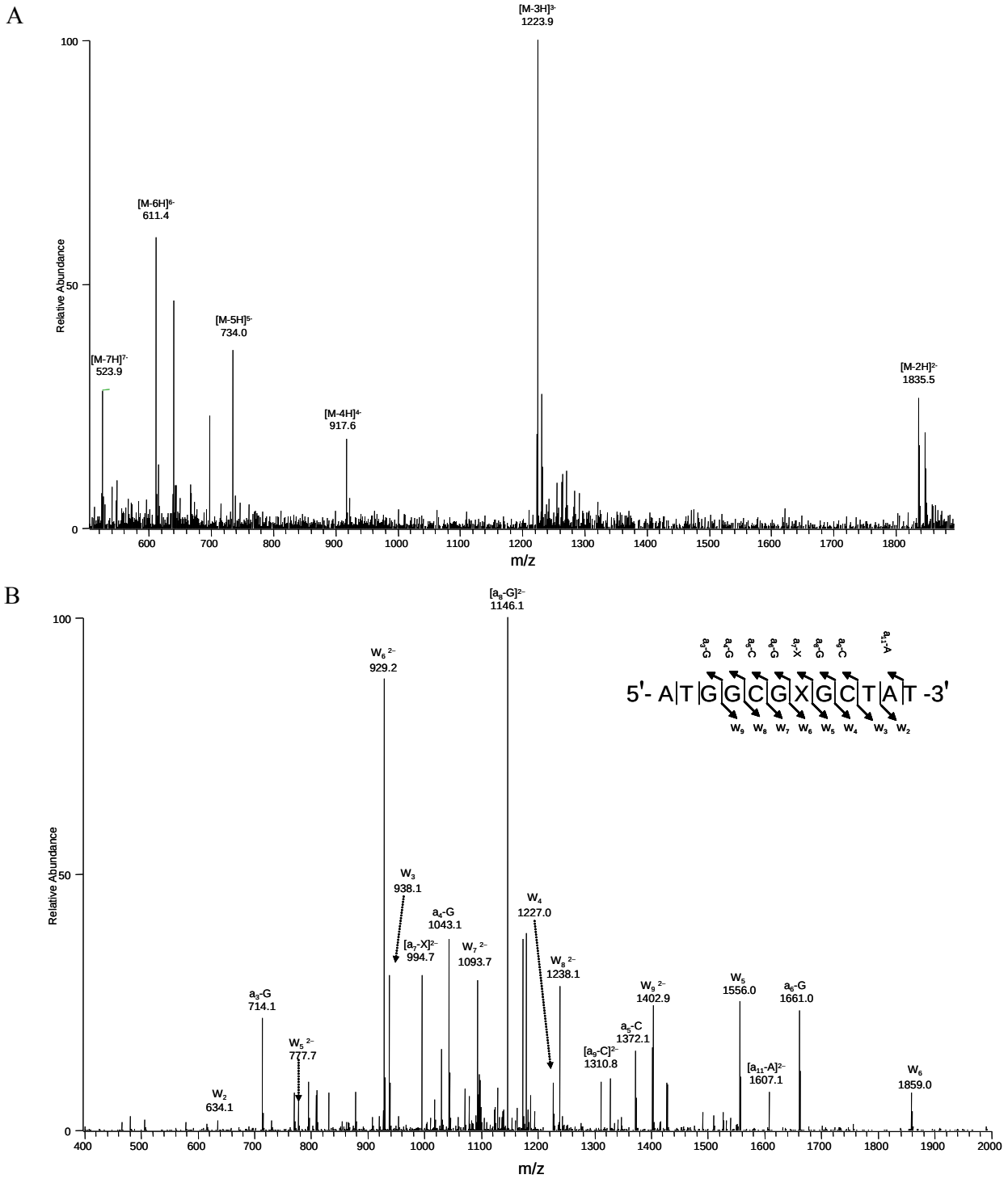

Fig. S1. ESI-MS and MS/MS characterizations of d(ATGGCGXGCTAT), X = 5mC: A. Negative-ion ESI-MS; B. The product-ion spectrum of the $[M-3H]^{3-}$ ion (*m/z* 1223.9).

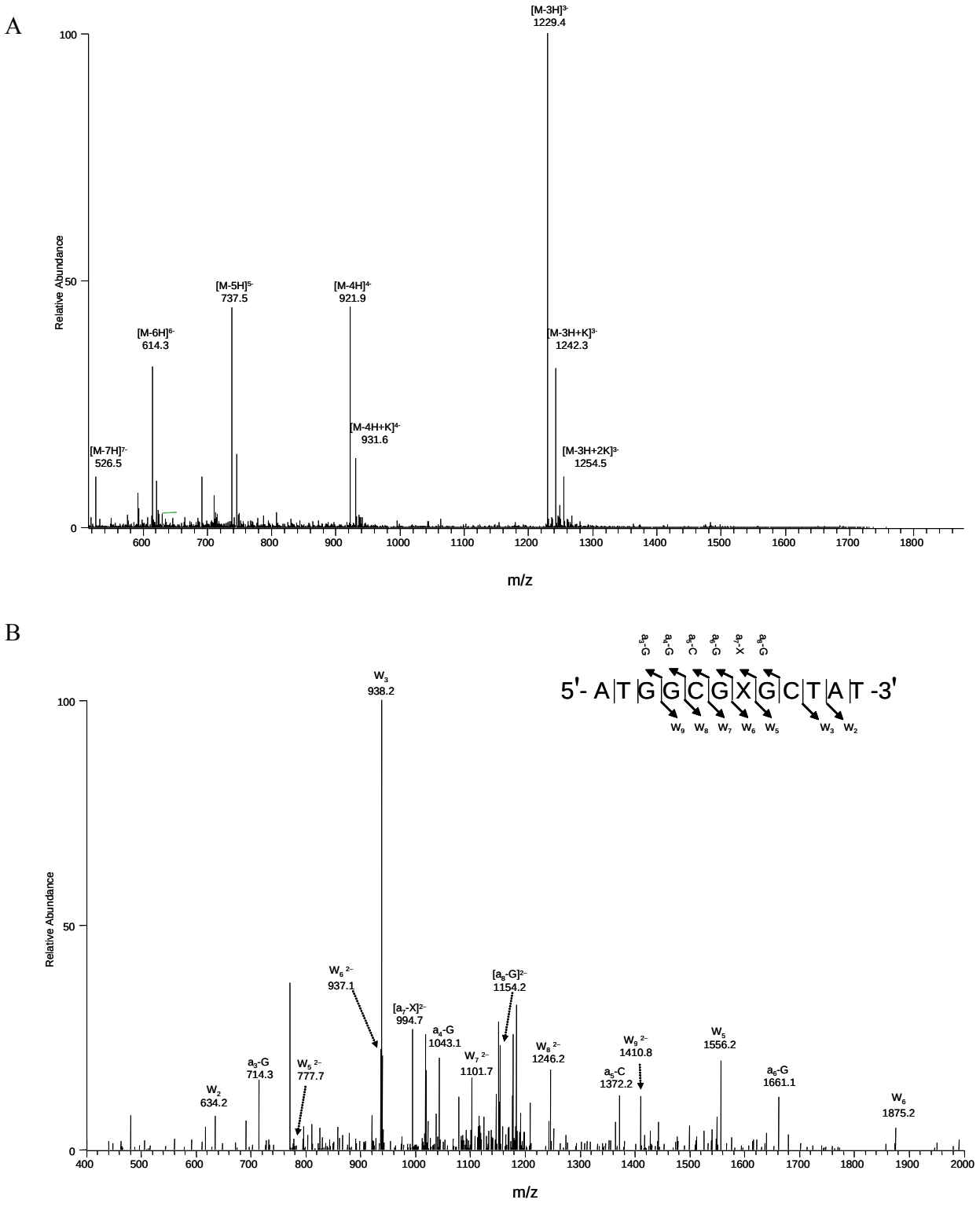

Fig. S2. ESI-MS and MS/MS characterizations of d(ATGGCGXGCTAT), X = 5hmC: A. negative-ion ESI-MS; B. The product-ion spectrum of the $[M-3H]^{3-}$ ion (*m/z* 1229.4).

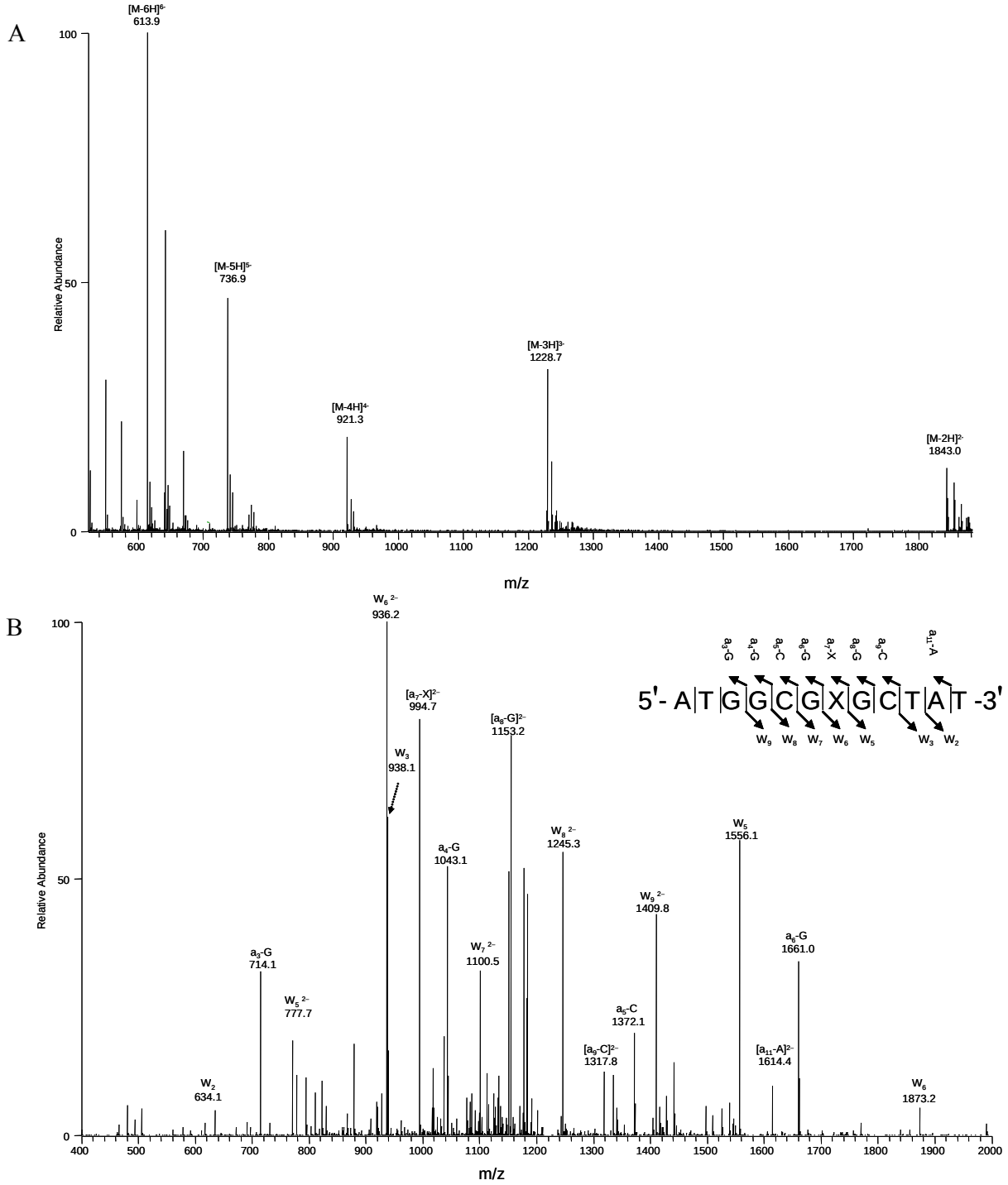

Fig. S3. ESI-MS and MS/MS characterizations of d(ATGGCGXGCTAT), X = 5fC: A. negative-ion ESI-MS; B. The product-ion spectrum of the $[M-3H]^{3-}$ ion (*m/z* 1228.7).

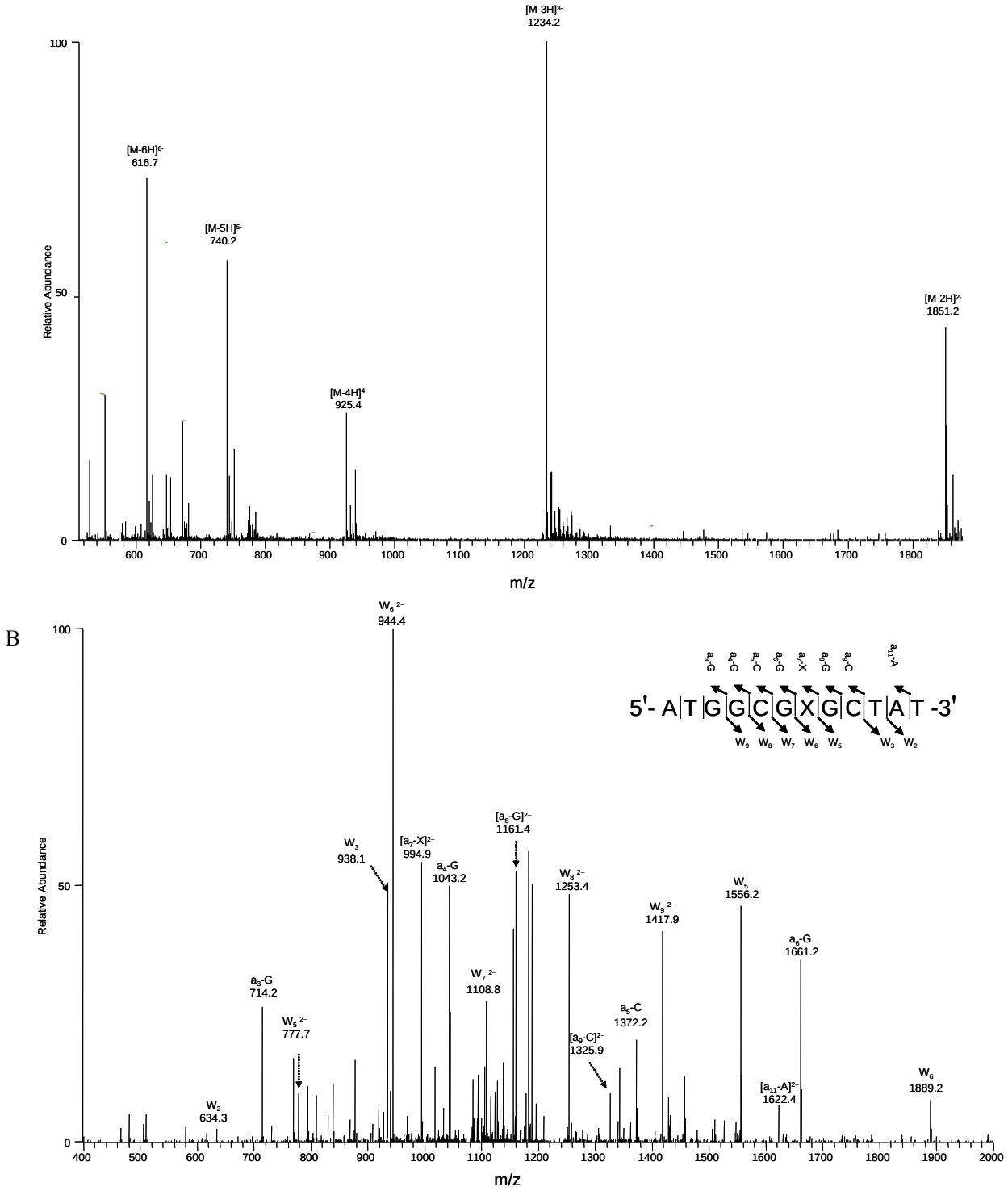

Fig. S4. ESI-MS and MS/MS characterizations of d(ATGGCGXGCTAT), X = 5caC: A. negative-ion ESI-MS; B. The product-ion spectrum of the $[M-3H]^{3-}$ ion (*m/z* 1234.2).

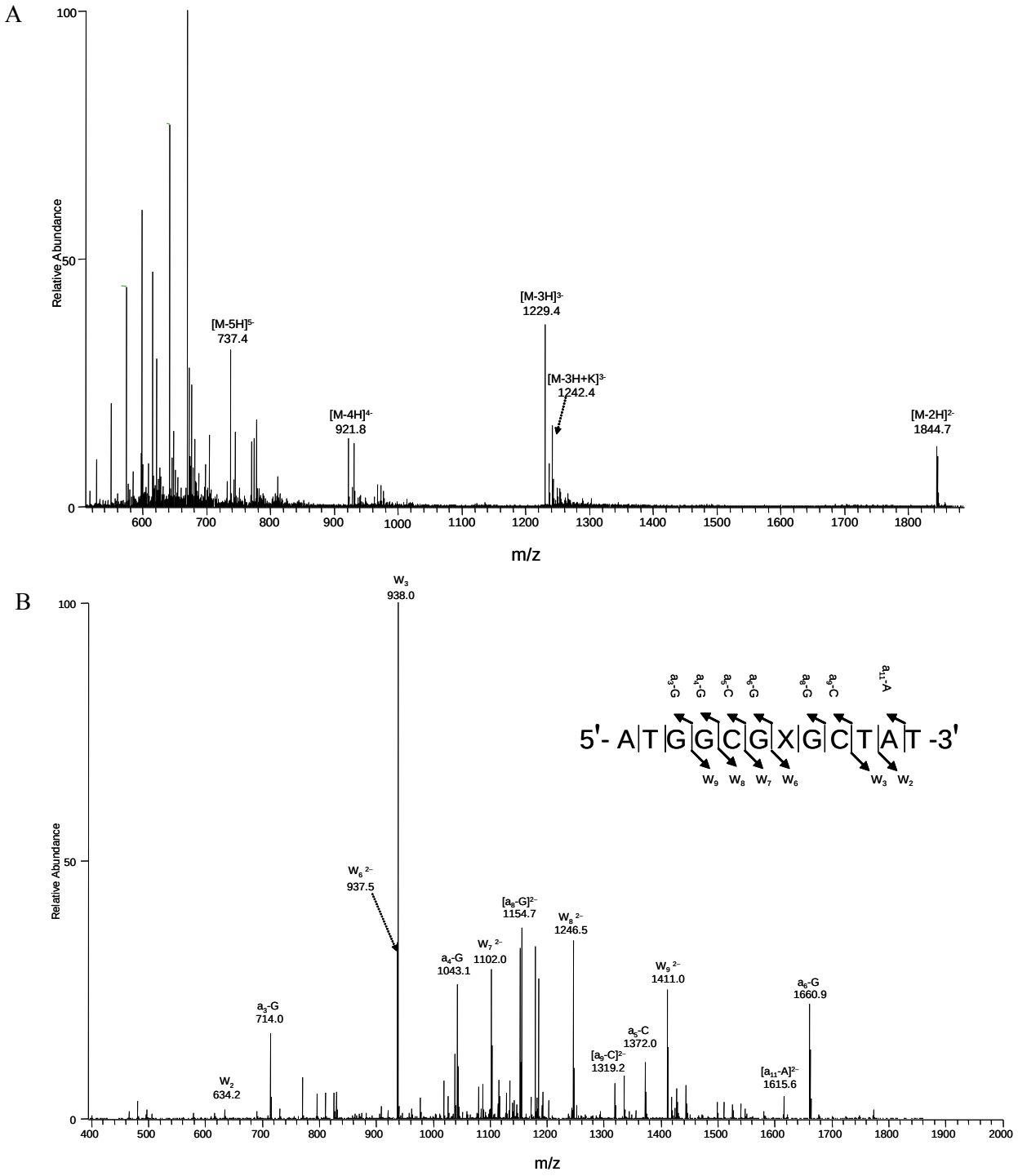

Fig. S5. ESI-MS and MS/MS characterizations of d(ATGGCGXGCTAT), X = 5hmU: A. negative-ion ESI-MS; B. The product-ion spectrum of the $[M-3H]^{3-}$ ion (*m/z* 1229.4).

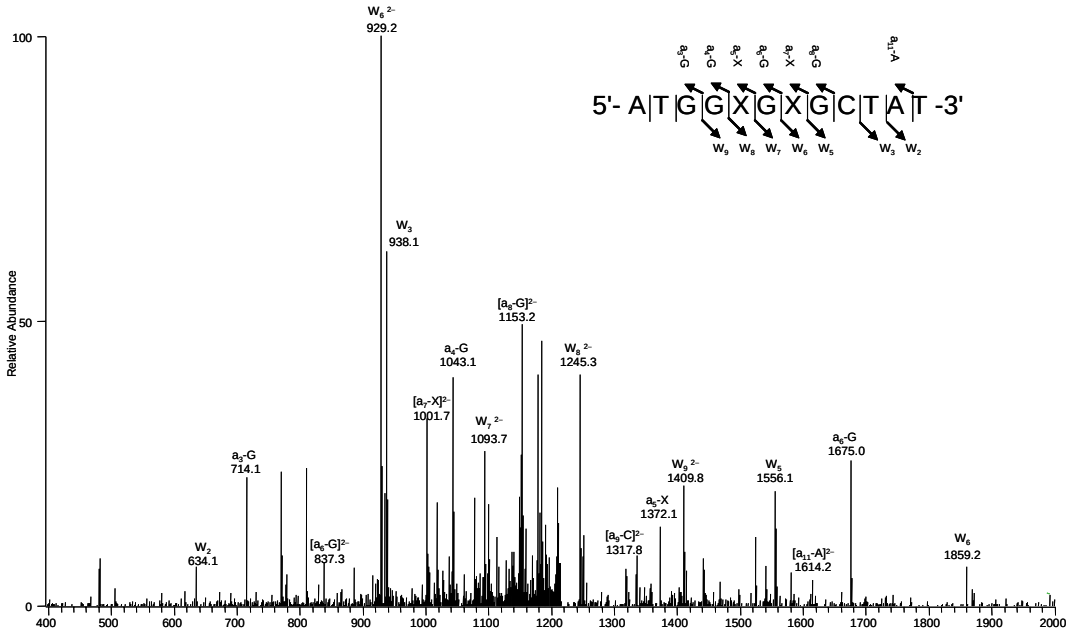

Fig. S6. LC-MS/MS for monitoring the methylation in 5mC-containing strand d(ATGGXGXGCTAT, X = 5mC). Illustrated in figure is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1228.2). The sequence was from 5mC-containing duplex DNA after methylation by Dnmt1.

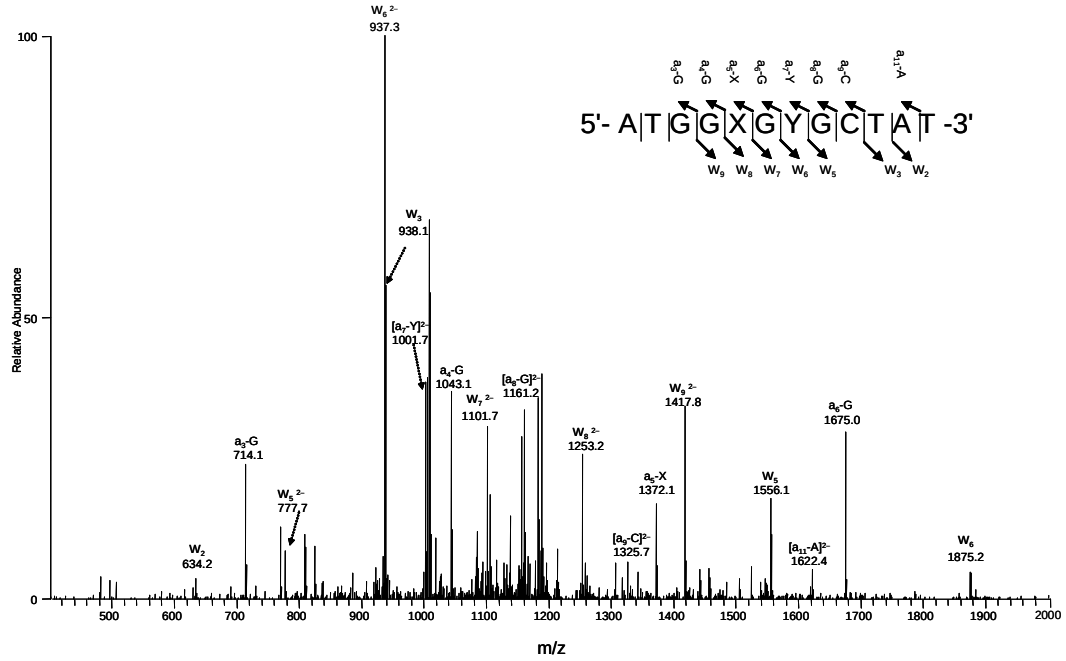

Fig. S7. LC-MS/MS for monitoring the methylation in 5hmC-containing strand d(ATGGXGYGCTAT, X = 5mC, Y = 5hmC). Shown is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1233.5). The sequence was from 5hmC-containing duplex DNA after methylation by Dnmt1.

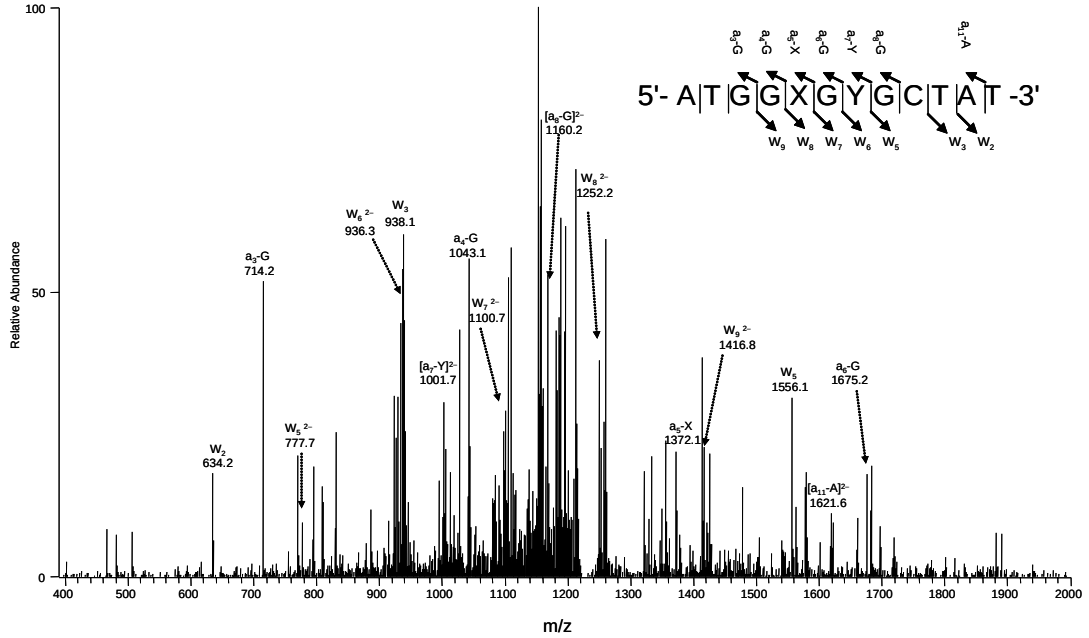

Fig. S8. LC-MS/MS for monitoring the methylation in 5fC-containing strand d(ATGGXGYGCTAT, X = 5mC, Y = 5fC). Displayed is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1232.8). The sequence was from 5fC-containing duplex DNA after methylation by DNMT3a-DNMT3L.

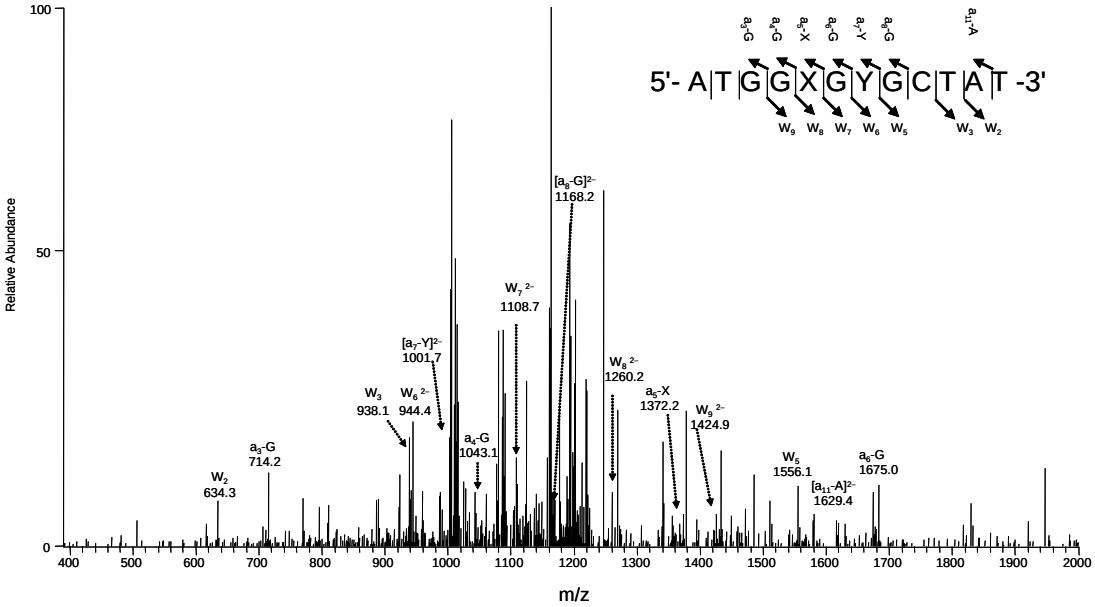

Fig. S9. LC-MS/MS for monitoring the methylation in 5caC-containing strand d(ATGGXGYGCTAT, X = 5mC, Y = 5caC). Shown is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1238.2). The sequence was from 5caC-containing duplex DNA after methylation by Dnmt1.

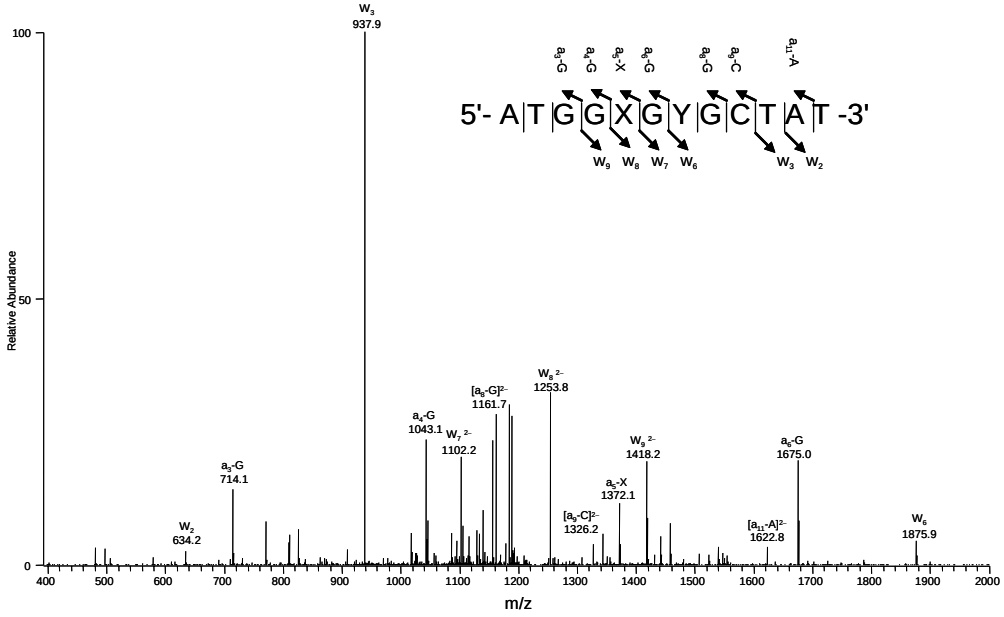

Fig. S10. LC-MS/MS for monitoring the methylation in 5hmU-containing strand d(ATGGXGYGCTAT, X = 5mC, Y = 5hmU). Illustrated in figure is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1233.8). The sequence was from 5hmU-containing duplex DNA after methylation by DNMT3a-DNMT3L.

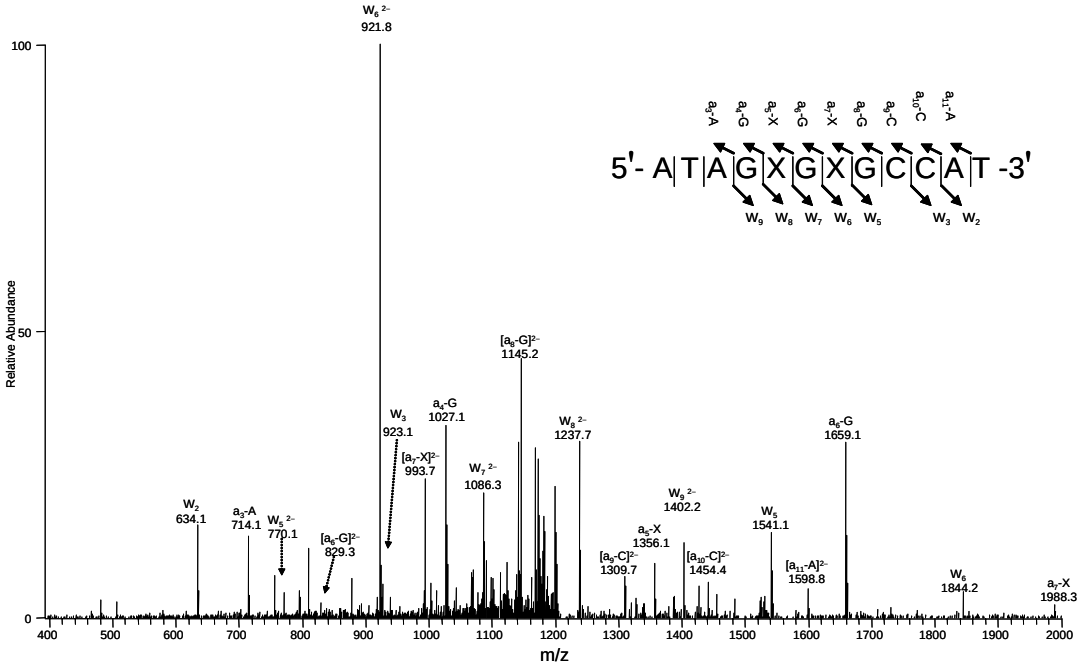

Fig. S11. LC-MS/MS for monitoring the methylation at both CpG sites in the bottom strand d(ATAGXGXGCCAT, X = 5mC). Illustrated in figure is the MS/MS of the $[M-3H]^{3-}$ ion (*m/z* 1218.2). The sequence was from 5mC-containing duplex DNA after methylation by Dnmt1.

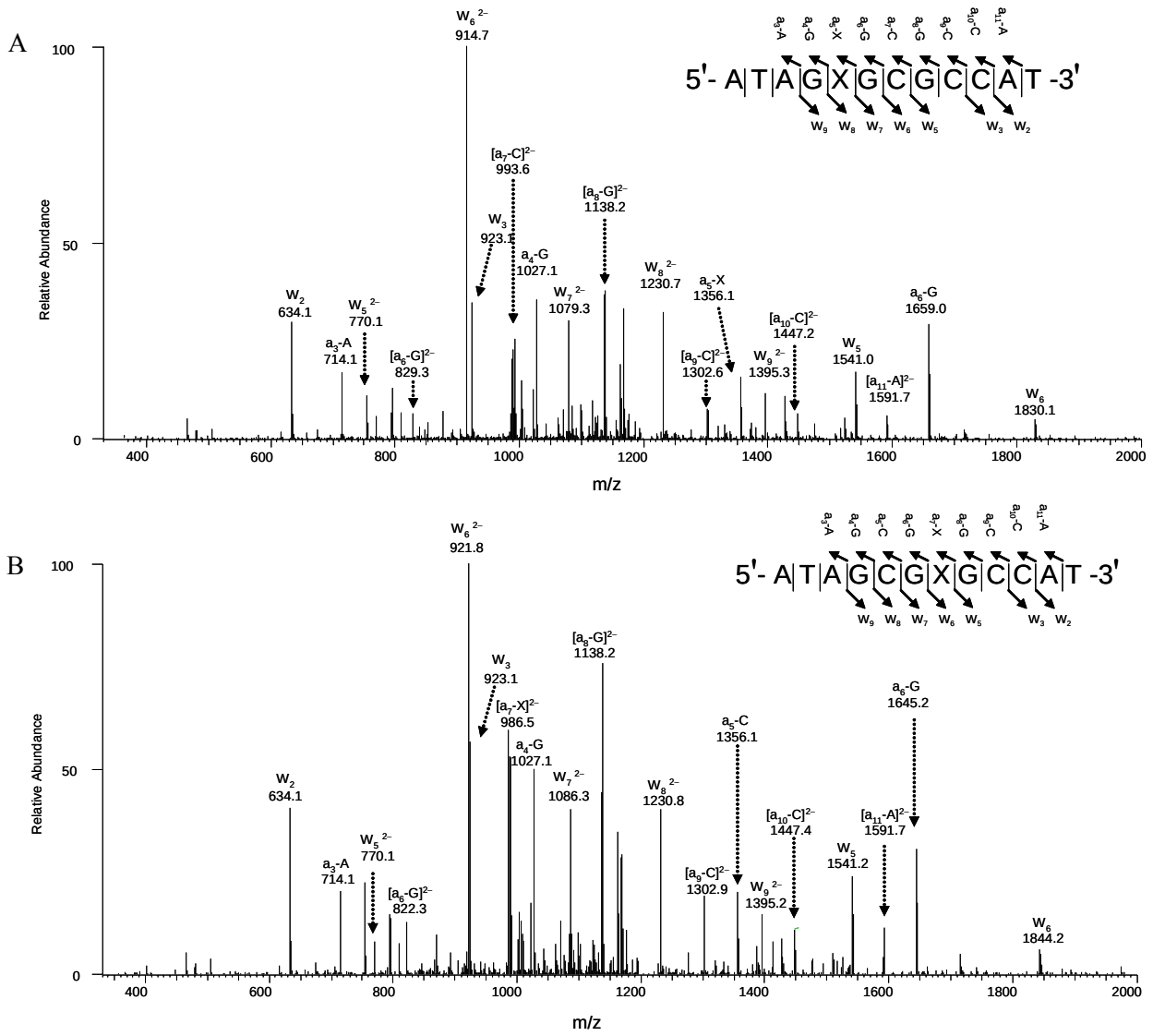

Fig. S12. LC-MS/MS for monitoring the methylation at different CpG sites in the bottom strand. Illustrated in A and B are the MS/MS of the $[M - 3H]^{3-}$ ions (*m/z* 1213.2) of two different ODNs (X = 5mC). A. The sequence which was methylated by Dnmt1 at CpG site 1 was from 5mC-containing duplex DNA. B. The sequence which was methylated by Dnmt1 at CpG site 2 was from 5hmU containing duplex DNA.

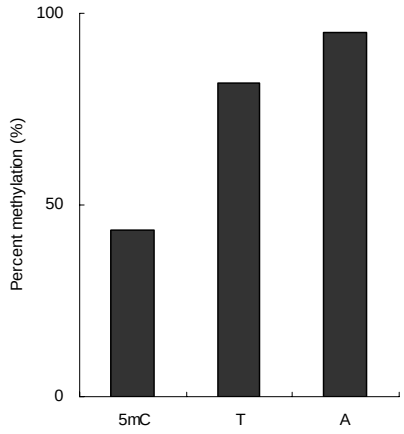

Fig. S13. Levels of cytosine methylation in different substrates methylated by Dnmt1. 5mC represent the methylation rate at site 1, while T and A represent the methylation rate at site 2. The reaction were carried out at 37 °C using 20 nM Dnmt1 in 20 min.