Supplementary Information

Sequence-selective osmium oxidation of DNA: Efficient distinction between 5-methylcytosine and cytosine

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Experimental Section

General. The reagents for 5-methylcytosine (M) oxidation were purchased from Wako, and the reagents for DNA synthesizer such as A, G, C, T, M-β-cyanoethyl phosphoramidite, and Oligo-Affinity Support (PS) were purchased from Applied Biosystems, or GLEN RESERCH. Oligodeoxynucleotides (ODNs) were purchased from Gene Design. HPLC was performed on a cosmosil 5C-18AR or CHEMCOBOND 5-ODS-H column (10 × 150 mm) with a Gilson Chromatography Model 305 using a UV detector Model 118 at 254 nm. HotStarTaq Master Mix Kit was purchased from QIAGEN. Micro Bio-Spin Chromatography Columns used for desalting were purchased from BIO-RAD.

Preparation of ^{32}P-5'-end-labeled oligodeoxynucleotides. The ODNs (400 pmol-strand) were 5'-end-labeled by phosphorylation with 4 μL of [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase using a standard procedure. The 5'-end-labeled ODN was recovered by ethanol precipitation and further purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE).

Osmium oxidation of oligodeoxynucleotides. ^{32}P-5'-End-labeled ODNs were hybridized with the complementary strand or the bulge forming strand (guide DNA) in 1 mM EDTA, 100 mM Tris-HCl buffer (pH = 7.7). Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The ODN to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. A reaction mixture was ethanol-precipitated with the addition of 15 μL of 3 M sodium acetate (pH = 5.0), 10 μL of salmon sperm DNA (1 mg/mL), and 1 mL of cold ethanol. The precipitated ODN was washed with 150 μL of 80% cold ethanol and dried *in vacuo*. The

precipitated ODN was resolved in 50 μL of 10% piperidine (*v*/*v*), heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness, and then resuspended in 5–20 μL of 80% formamide loading buffer (a solution of 80% formamide (*v*/*v*), 1 mM EDTA, 0.1% xylenecyanol, and 0.1% bromophenol blue). The samples (1 μL, 3-10 kcpm) were loaded onto 15% denatureing 19:1 acrylamide-bisacrylamide gel containing 7 M urea, electrophoresed at 1900 V for approximately 1 h, and transferred to a cassette and stored at –80°C with Fuji X-ray film.

Judgment of cytosine methylation status using osmium oxidation and real-time PCR (Fig. 3(b)). We prepared a p53 gene fragment (exon 5, codons 166–186) as a target DNA (100 fmol), 5'-GCTAT-CTGAGCAGCGCTCATGGTGGGGGCAG(M/C)GCCTCACAACCTCCGTCATGTGCTGTGA-3', and guide DNAs tethered to an Oligo-Affinity Support (a polystyrene bead, PS), 5'-TCACAGCACATGACG-GAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC-PS-3' (fullmatch) and 5'-TC-ACAGCACATGACGGAGGTTGTGAGGCCTGCCCCCACCATGAGCGCTGCTCAGATAGC-PS-3' (bulge). Hybridization of these strands was achieved by heating a solution containing 1 mM EDTA, 100 mM Tris-HCl buffer (pH = 7.7) at 90°C for 5 min and slowly cooling to room temperature. The ODN to be examined was incubated in a solution of 10 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. The ODN tethered to beads was washed with 3 M sodium acetate (pH = 6.0) for three times, then added to 7 M urea solution. After incubation at 55 °C for 20 min, the supernatant was recovered, and this operation was repeated twice. The supernatant was ethanol-precipitated. The recovered ODN was resolved in 50 μL of 10% piperidine (*v*/*v*), and heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness. After addition of 20 μL water, the sample solution was desalted by Micro Bio-Spin Chromatography Columns, and dried *in vacuo*. Process of PCR amplification was monitored by the fluorescence of TaqMan probe on the LightCycler (Roche Diagnostics). We prepared primers, 5'-GCTATCTGAGCAGCGCTCATG-3' and 5'-TCACAGCACAT- GACGGAGG-3', and a TaqMan probe, 5'-FAM-AGGCGCTGCCCCCACCATGA-TAMRA-3'. PCR amplifications were performed in a solution of sample DNA, 0.5 μM primers and 0.1 μM TaqMan probe in 20 μL of 2× HotStarTaq Master Mix containing 400 μM each of dATP, dCTP, dGTP, and dTTP and 5 units of HotStarTaq DNA polymerase. Amplifications were performed in glass capillaries as follows: denaturaion at 94 °C for 30 seconds, annealing with fluorescence monitoring at 50 °C for 30 seconds, and extension at 72 °C for 1 minute for 40 cycles. As filters for fluorescence detection, a 470 ± 20 nm band pass filter for excitation and a 530 ± 10 nm band pass filter for detector were used.

Product analysis. The reaction mixture was prepared in a 50 μL total volume containing 100 μM **ODN(M)**, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA, 100 mM Tris-HCl buffer at pH 7.7 and 10% acetonitrile. After addition of 25 μL of 10 mM potassium osmate solution, the sample solution was incubated at 37 °C for 6 h. After desalting by Micro Bio-Spin Chromatography Columns, osmate complex was purified by reverse phase HPLC on a 5-ODS-H column (10 × 150 mm, elution with a solvent mixture of 0.1 M triethylamine acetate, pH 7.0, linear gradient over 30 min from 5% to 15% acetonitrile at a flow rate 3.0 mL/min). The purified osmate complex was treated with sodium sulfite (50% saturated solution) for 3 h at 50 °C. The resulting ODN was purified by reverse phase HPLC. The molecular weight of ODN obtained by MALDI-TOF mass measurement was in good agreement with the expected values (See Fig. S3).

3',5'-Bis(*tert*-butyldimethylsilyl)-5-methyl-2'-deoxycytidine *cis*-glycol. To a solution of 3',5'-bis(*tert*-butyldimethylsilyl)-5-methyl-2'-deoxycytidine (470 mg, 1.0 mmol) in pyridine (5 mL) was added osmium tetroxide (254 mg, 1.0 mmol), and stirred at room temperature for 1 h. Sodium bisulfite (450 mg) in 2:1 water-pyridine (15 mL) was then added to the reaction mixture, and the mixture was stirred at room temperature for 30 min. The reaction mixture was extracted with chloroform and water. The organic layer was washed with brine, dried over sodium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (chloroform : methanol = 10 : 1, 1% aqueous ammonia) to yield two *cis*-glycol isomers (68.5 mg for 5*R*,6*S* form, 11.4 mg for *5S,6R* form, total yield 16%).

The configuration of each isomer was assigned by NOESY. For both isomers, a cross-peak was found between H-6 and the methyl proton. On the other hand, no NOE effect was detected between H-6 and H-1'. These observations indicated that two hydroxyl groups were in the *cis* orientation and that the base moiety was in the *anti* conformation around the glycosyl bond. For the minor isomer strong NOE cross-peaks were detected between H-6 and H-2', and H-6 and H-3', whereas the corresponding cross-peak was too weak to be discriminated from the noise for the major product. From the data on thymine glycol shown by Vaishnav *et al*. (Y. Vaishnav *et al*., *J. Biomol. Struct. Dyn.* **1991**, *8*, 935–951), the configuration of the major product and the minor product were identified as 5*R*,6*S* and *5S,6R* isomers, respectively: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.40 (t, 1H, J = 6.8 Hz, H-1', *5S,6R*), 6.19 (dd, 1H, J = 6.0, 7.7 Hz, H-1', 5*R*,6*S*), 5.16 (s, 1H, H-6, 5*R*,6*S*), 4.87 (s, 1H, H-6, 5*R*,6*S*), 4.38 (dt, 1H, J = 2.9 Hz, H-3', *5S,6R*), 4.37 (dt, 1H, J = 2.9, 5.9 Hz, H-3', 5*R*,6*S*), 3.80–3.77 (m, 1H, H-4', *5S,6R*), 3.79 (quartet, 1H, J = 3.1 Hz, H-4', 5*R*,6*S*), 3.73 (dd, 1H, J = 3.5, 11.0 Hz, H-5', 5*R*,6*S*), 3.70–3.68 (m, 2H, H-5', *5S,6R*), 3.69 (dd, 1H, J = 3.3, 11.2 Hz, H-5', 5*R*,6*S*), 2.26 (ddd, 1H, J = 6.2, 7.5, 13.5 Hz, H-2'α, 5*R*,6*S*), 2.15 (ddd, 1H, J = 4.8, 7.1, 13.4 Hz,

H-2'α, *5S,6R*), 2.07 (ddd, 1H, J = 3.1, 5.9, 13.2 Hz, H-2'β, 5*R*,6*S*), 2.06–1.89 (m, 1H, H-2'β, *5S,6R*), 1.413 (s, 3H, CH_3, *5S,6R*), 1.405 (s, 3H, CH_3, 5*R*,6*S*), 0.89 (s, 9H, *t*-Bu, 5*R*,6*S*), 0.88 (s, 9H, *t*-Bu, *5S,6R*), 0.87 (s, 9H, *t*-Bu, *5S,6R*), 0.86 (s, 9H, *t*-Bu, 5*R*,6*S*), 0.08 (s, 6H, CH_3Si, *5S,6R*), 0.07 (s, 6H, CH_3Si, 5*R*,6*S*), 0.062 (s, 3H, CH_3Si, *5S,6R*), 0.058 (s, 3H, CH_3Si, *5S,6R*), 0.05 (s, 3H, CH_3Si, 5*R*,6*S*), 0.4 (s, 3H, CH_3Si, 5*R*,6*S*); 5*R*,6*S* product: ^{13}C NMR (100 MHz, $CDCl_3$) δ 86.6, 84.1, 77.8, 72.0, 63.2, 39.0, 26.0, 25.8, 22.7, 18.4, 18.0, –4.6, –4.9, –5.46, –5.50: MS (FAB), *m/z* 504 $[M + H]^+$; HRMS (FAB) calculated for $C_{22}H_{46}O_6N_3Si_2$ $[M + H]^+$ 504.2939, found 504.2925.

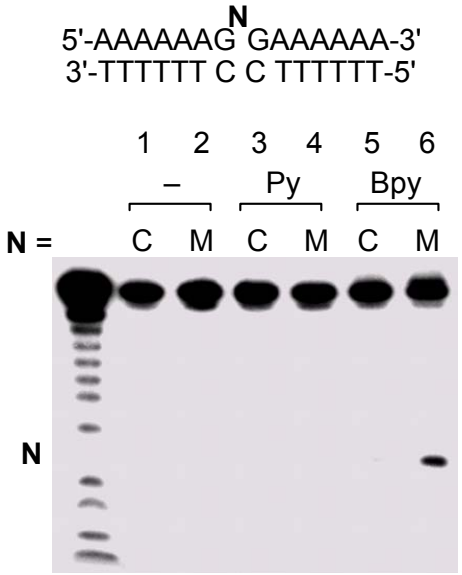


Fig. S1. Effect of bipyridine on M-selective oxidation. **ODN(N)** was incubated in a solution of 10 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM ligand (pyridine or 2,2'-bipyridine), 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. Subsequently, the reaction sample was washed using ethanol precipitation, and then treated with hot piperidine (90 °C, 20 min). The products were analyzed using a polyacrylamide gel electrophoresis. Lane 1, no ligand, N = C; lane 2, no ligand, N = M; lane 3, pyridine, N = C; lane 4, pyridine, N = M; lane 5, bipyridine, N = C; lane 6, bipyridine, N = M.

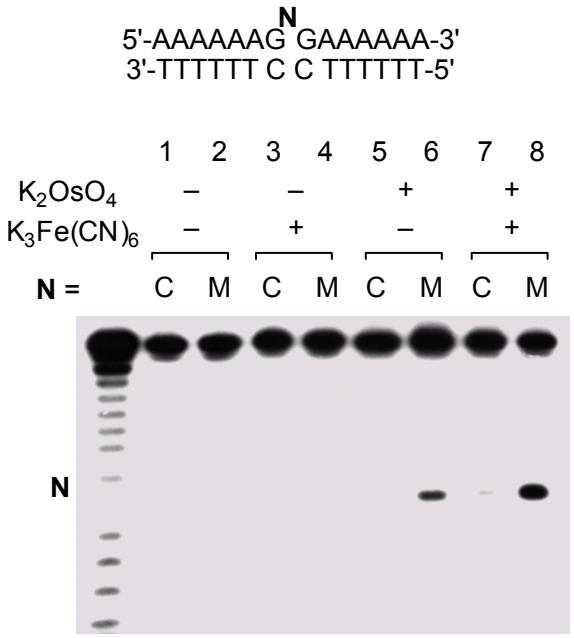


Fig. S2. Effect of potassium hexacyanoferrate(III) on M-selective oxidation. **ODN(N)** was incubated in a solution of 10 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. Subsequently, the reaction sample was washed using ethanol precipitation, and then treated with hot piperidine (90 °C, 20 min). The products were analyzed using a polyacrylamide gel electrophoresis.

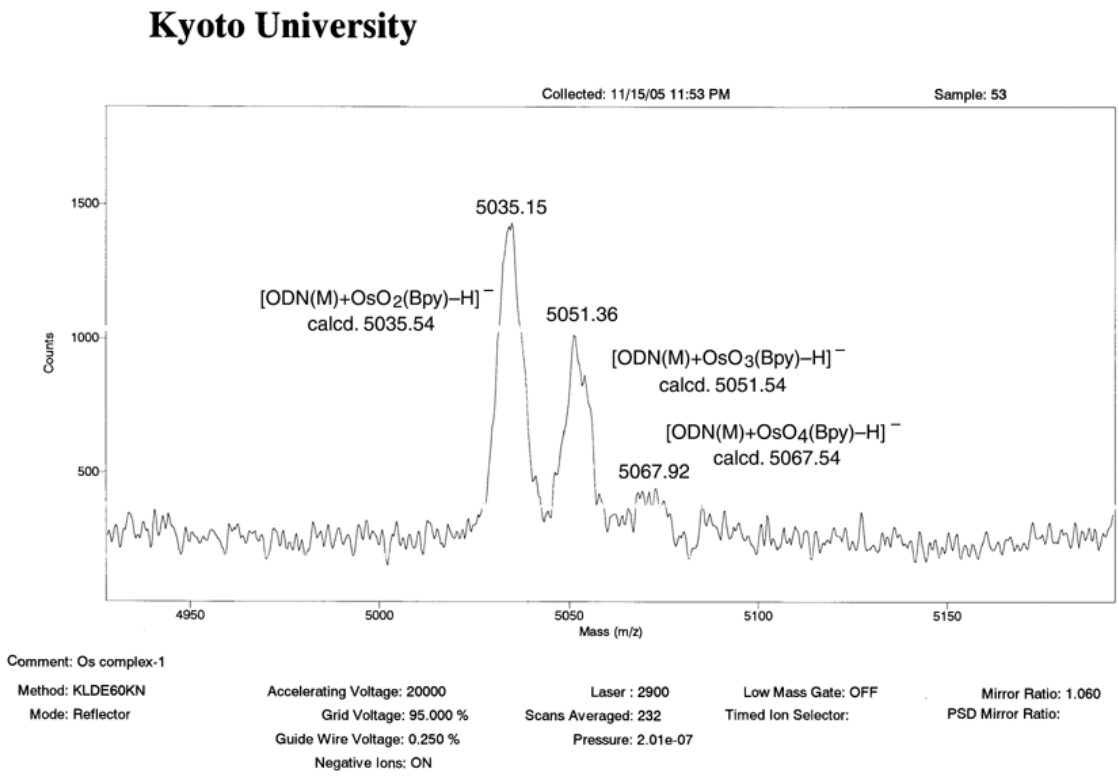
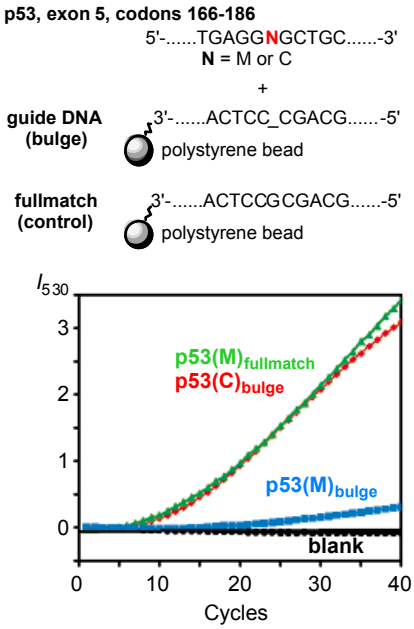


Fig. S3. Mass spectrum of **ODN(M)**-Os adduct. The mass was determined with a MALDI-TOF MS (acceleration voltage 21 kV, negative ion mode) with 2',3',4'-trihydroxyacetophenone as matrix, using T 8-mer ($[M - H]^-$ 2370.61) and T 27-mer ($[M - H]^-$ 8150.33) as an internal standard.



Color ver. of Fig. 3 (b). Real-time PCR amplification. DNA samples were incubated in a solution of 10 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7) and 10% acetonitrile at 0 °C for 5 min. The target DNA was removed from supports by washing with 7 M urea, and then treated with hot piperidine (90 °C, 20 min). The process of PCR amplification of the treated DNA samples was monitored by the fluorescence of the TaqMan probe (λ_{exc} = 470 nm, λ_{em} = 530 nm). PCR amplifications were performed, using 2 μL of 1 μM TaqMan probe and 5 units of HotStarTaq DNA polymerase, over 40 cycles at 94 °C for 0.5 min, to 50 °C for 0.5 min, to 72 °C for 1 min.