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Supplementary Information

Alkylating immobilization linker for immunochemical epigenetic assessment

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1. General information

Physical data were measured as follows: ^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker NMR spectrometer with DMSO-d_6 as the solvent and tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). All exchangeable protons were detected by their disappearance on the addition of D_2O. Mass spectra were recorded on a JEOL JMS-700 MStation at the Open Research Facilities Station, TIA Central Office, National Institute of Advanced Industrial Science and Technology (AIST). Analytical thin layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ precoated plates (Merck). The silica gel used for column chromatography was Wakogel C-200 (particle size 75-150 μm) or Wakogel 100 C18 (Wako Pure Chemical Industries). DNA concentrations were determined on a NanoDrop 1000 (Thermo Fisher Scientific, Inc.). UV-vis spectra were recorded on a UV-vis spectrophotometer UV-1800 (Shimadzu Corp.). Capillary electrophoresis was performed on a Bioanalyzer 2100 (Agilent Technologies Inc.) with DNA 1000 LabChips.

2. Synthesis of linker molecule L1

13-Bis(2-hydroxyethyl)amino-4,7,10-trioxatridecan-1-yl 1,2-dithiolane-3-valeroylamide (4).

1,1'-carbonyldiimidazole (585 mg, 3.60 mmol) was added to a mixture of α-lipoic acid (**1**) (620 mg, 3.00 mmol) and triethylamine (1.00 mL, 7.20 mmol) in acetonitrile (30 mL), and the mixture was stirred at room temperature for 100 min. A solution of diethyleneglycol bis(3-aminopropyl)ether (**2**) (2.62 mL, 12.0 mmol) in acetonitrile (30 mL) was added dropwise to the mixture over 15 min, and stirring was continued for a further 60 min. The solvent was removed in vacuo, and the residue was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed 5 times with H_2O, and then with brine. After drying with Na_2SO_4, it was concentrated in vacuo to give 13-amino-4,7,10-trioxatridecan-1-yl 1,2-dithiolane-3-valeroylamide (**3**) in a pale yellow form (FAB-MS calcd. for $C_{18}H_{37}N_2O_4S_2$ $[M+H]^+$: 409.2189, found: 409.2190). Then the crude compound **3** was dissolved in DMF (35 mL), and 2-bromoethanol (2.09 mL, 29.5 mmol) and diisopropylethylamine (5.14 mL, 29.5

mmol) were added. The whole was stirred at room temperature for 20 h. 2-Bromoethanol (1.05 mL, 14.8 mmol) and diisopropylethylamine (2.58 mL, 14.8 mmol) were added to the mixture, which was then heated at 60 °C for 24 h. The solvent was removed in vacuo, and the residue was purified with silica gel column chromatography (MeOH in $CHCl_3$) to give **4** (930 mg, 63%) as a yellow syrup.

FAB-MS calcd. for $C_{22}H_{45}N_2O_6S_2$ $[M+H]^+$: 497.2714, found: 497.2710; ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ: 7.80 (br t, 1 H, N*H*, *J* = 5.5 Hz), 5.24 (br s, 2 H, O*H*×2), 3.74 (t, 4 H, C*H*$_2$×2, *J* = 5.3 Hz), 3.61 (m, 1 H, C*H*), 3.53-3.51 (m, 6 H, C*H*$_2$×3), 3.49-3.46 (m, 4 H, C*H*$_2$×2), 3.39 (t, 2 H, C*H*$_2$, *J* = 6.4 Hz), 3.24 (t, 4 H, C*H*$_2$×2, *J* = 5.3 Hz), 3.22-3.17 (m, 1 H, C*H*$_2$*a*), 3.14-3.11 (m, 1 H, C*H*$_2$*b*), 3.07 (q, 2 H, C*H*$_2$, *J* = 6.8 Hz), 2.41 (m, 1 H, C*H*$_2$*a*), 2.05 (t, 2 H, C*H*$_2$, *J* = 7.4 Hz), 1.92 (m, 2 H, C*H*$_2$), 1.87 (m, 1 H, C*H*$_2$*b*), 1.69-1.63 (m, 1 H, C*H*$_2$*a*), 1.61 (t, 2 H, C*H*$_2$, *J* = 6.8 Hz), 1.58-1.47 (m, 5 H, C*H*$_2$×2, C*H*$_2$*b*), 1.34 (m, 2 H, C*H*$_2$); ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ: 172.24 (C), 70.13 (CH_2), 70.07 (CH_2), 70.06 (CH_2), 69.92 (CH_2), 68.48 (CH_2), 67.50 (CH_2), 61.32 (CH_2), 58.33 (CH_2), 56.53 (CH), 54.98 (CH_2), 40.30 (CH_2), 38.48 (CH_2), 36.08 (CH_2), 35.58 (CH_2), 34.48 (CH_2), 29.79 (CH_2), 28.68 (CH_2), 25.43 (CH_2).

13-Bis(2-chloroethyl)amino-4,7,10-trioxatridecan-1-yl 1,2-dithiolane-3-valeroylamide (L1).

Methanesulfonyl chloride (0.43 mL, 5.55 mmol) was added dropwise to a solution of **4** (920 mg, 1.85 mmol) and triethylamine (1.55 mL, 11.1 mmol) in CH_2Cl_2 (30 mL) at 0 °C, and the mixture was stirred at 0 °C for 1 h, and then at room temperature for 14 h. The mixture was partitioned between $CHCl_3$ and H_2O, and the separated organic layer was washed with H_2O, followed by brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by using silica gel column chromatography (EtOH in $CHCl_3$) to give **L1** (270 mg, 27%) as a pale yellow solid.

FAB-MS calcd. for $C_{22}H_{43}Cl_2N_2O_4S_2$ $[M+H]^+$: 533.2036, found: 533.2017; ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ: 7.74 (br t, 1 H, N*H*, *J* = 5.5 Hz), 3.59 (m, 1 H, C*H*), 3.58 (t, 4 H, C*H*$_2$×2, *J* = 6.9Hz), 3.52-3.50 (m, 4 H, C*H*$_2$×2), 3.47-3.46 (m, 4 H, C*H*$_2$×2), 3.42 (t, 2 H, C*H*$_2$, *J* = 6.3 Hz), 3.39 (t, 2 H, C*H*$_2$, *J* = 6.4 Hz), 3.21-3.16 (m, 1 H, C*H*$_2$*a*), 3.14-3.09 (m, 1 H, C*H*$_2$*b*), 3.07 (q, 2 H, C*H*$_2$, *J* = 6.7 Hz), 2.80 (t, 4 H, C*H*$_2$×2, *J* = 6.9 Hz), 2.57 (t, 2 H, C*H*$_2$, *J* = 6.9 Hz), 2.41 (m, 1 H, C*H*$_2$*a*), 2.04 (t, 2 H, C*H*$_2$, *J* = 7.3 Hz), 1.86 (m, 1 H, C*H*$_2$*b*), 1.67-1.63 (m, 1 H, C*H*$_2$*a*), 1.62-1.54 (m, 6 H, C*H*$_2$×3),

1.53-1.47 (m, 1 H, CH_2*b*), 1.34 (q, 2 H, CH_2, J = 7.7 Hz); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 172.15 (C), 70.19 (CH_2), 70.16 (CH_2), 69.93 (CH_2), 69.89 (CH_2), 68.50 (CH_2), 68.33 (CH_2), 56.52 (CH), 55.98 (CH_2), 50.68 (CH_2), 42.88 (CH_2), 40.28 (CH_2), 38.47 (CH_2), 36.08 (CH_2), 35.59 (CH_2), 34.49 (CH_2), 29.79 (CH_2), 28.69 (CH_2), 27.78 (CH_2), 25.43 (CH_2).

3. ODN crosslink

Methylated and unmethylated oligodeoxyribonucleotides (ODNs) were synthesized and purified via high-performance liquid chromatography by GeneDesign, Inc. A solution of ODN in 10 mM Tris-HCl and 1.0 mM EDTA (TE buffer) was heated at 95 °C for 5 min and then cooled to room temperature to prepare a DNA duplex. 0 – 500 μM **L1** in DMSO was added to 5.0 μM ODN (20 μM of GpNpC) in TE buffer and then incubated at 37 °C for 30 min. To remove the unreacted **L1**, gel filtration was carried out on a desalting column (NAP5, GE Healthcare Bio-Sciences Corp.) using TE buffer (or running buffer for SPR analysis). The DNA-**L1** was characterized as described below.

4. Denaturating polyacrylamide gel electrophoresis (PAGE)

The ODN samples were mixed with an equal volume of loading buffer containing 90 mM Tris, 90 mM boric acid, 2 mM EDTA, 12% Ficoll Type 400, 7 M urea, 0.01% bromophenol blue, and 0.05% xylene cyanol. The samples were heated at 70 °C for 3 min and then loaded on a 15% polyacrylamide gel containing 7 M urea (Thermo Fisher Scientific Inc.) with a standard ladder marker (10 bp DNA ladder; Thermo Fisher Scientific, Inc.). Electrophoresis was performed in running buffer containing 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.3) at 55 °C. The ODN sample in the gel was stained with SYBR green dye.

5. Circular dichroism (CD) spectrum measurement

CD spectra were recorded using a spectropolarimeter (J-720; JASCO Corp., Ltd.). The quartz cuvette had an optical path length of 10.0 mm. A solution was prepared that contained ODN-**L1** purified with a desalting column using TE buffer (pH 8.0),

and the spectra were measured at 20 °C. The CD spectra of the samples were corrected by subtracting the corresponding spectra of the buffers in the absence of DNA.

6. Surface plasmon resonance (SPR) analysis

SPR analysis was performed on a Biacore T200 (GE Healthcare Bio-Sciences Corp.). Bare gold sensor chips (SIA kit Au; GE Healthcare Bio-Sciences Corp.) were dipped in piranha solution (3:1 volume of concentrated sulfuric acid and 30% hydrogen peroxide) for 15 min, and then rinsed with copious amounts of water prior to use. A bare gold sensor chip was docked into the instrument, and then the immobilization of DNA-**L1** on the gold surface was monitored as follows. 5.0 ng/μL of DNA samples in running buffer A containing 10 mM HEPES (pH 7.4), 150 mM NaCl, and 3.0 mM EDTA were degassed with Ar bubbling, and then injected onto the chip at 25 °C for 10 min at a flow rate of 10 μL/min. After immobilization, the running buffer A was injected onto the chip for 5 min.

To detect 5-mC in the target DNA immobilized on a gold surface, an affinity analysis of an antibody-antigen interaction was performed as follows. Two types of running buffer were used: running buffer A for immobilization, and running buffer A with 0.05 % Tween 20 (running buffer A') for immunoassay. The immobilization of DNA-**L1** on the bare gold surface was first carried out by injecting DNA in running buffer A until the immobilized amount reached the required value (2000 RU for ODN; 1000 RU for λDNA). After immobilization, 0 – 50 nM anti-mC antibody (Epigentek Group, Inc.) in running buffer A was injected for 2 min at a flow rate of 30 μL/min, followed by running buffer A' for 3 min. The sensor surface was regenerated by the injection of 50 mM NaOH for 30 sec at a flow rate of 60 μL/min. It was confirmed that the antibody was removed from the DNA.

7. UV-vis spectra of ODN-L1

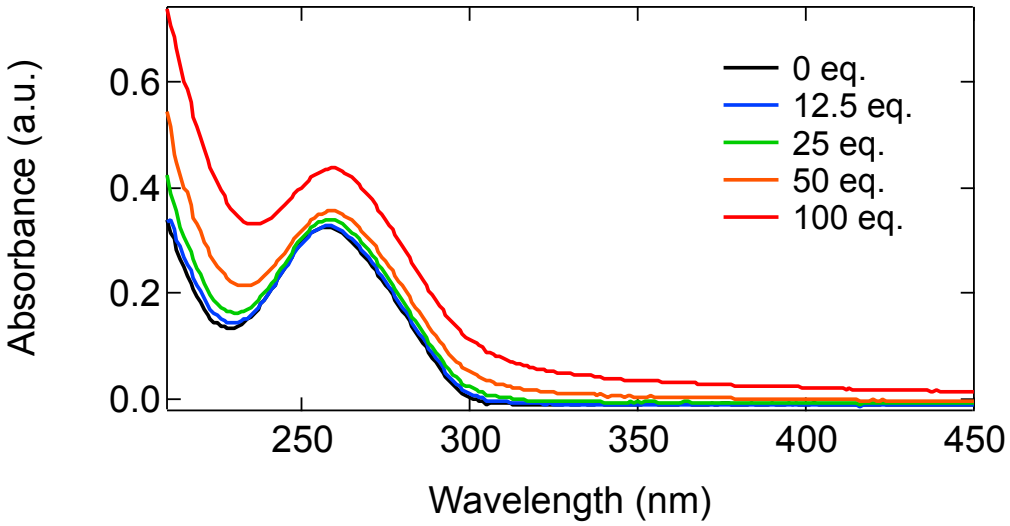

Figure S1. UV-vis spectra of ODN solution after being treated with different concentrations of **L1**. A solution was prepared containing ODN-**L1** purified with a desalting column using TE buffer (pH 8.0), and the spectra were measured at room temperature.

8. Specificity

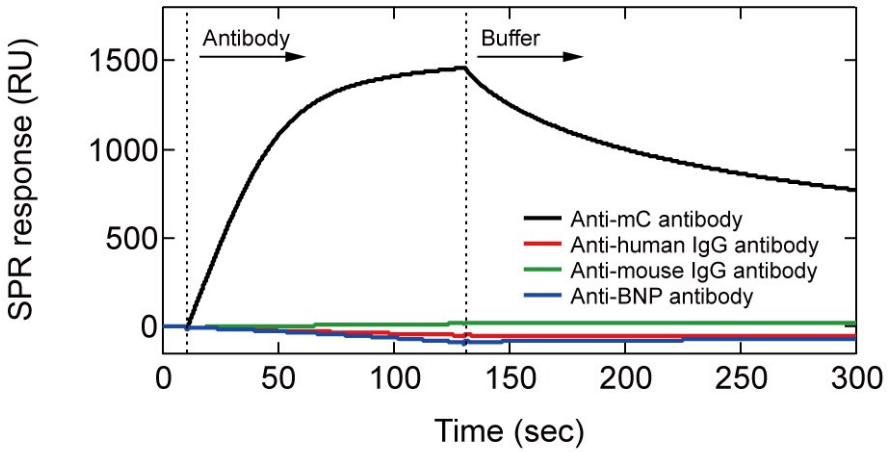

Figure S2. SPR sensorgrams displaying the binding response between anti-mC antibody and 5-mC in immobilized ODN as a function of time, where the subtracted SPR responses of the sample and reference cells are plotted. Methylated ODN-**L1** mixtures containing 100% 5-mC were immobilized on a bare gold sensor chip, which was treated with 3.6 μg/mL of anti-mC antibody in running buffer at 25 °C. The broken lines indicate the starting points of the antibody and buffer injection. The ODN-**L1** immobilization amount was 2000 RU.

9. Kinetic analysis

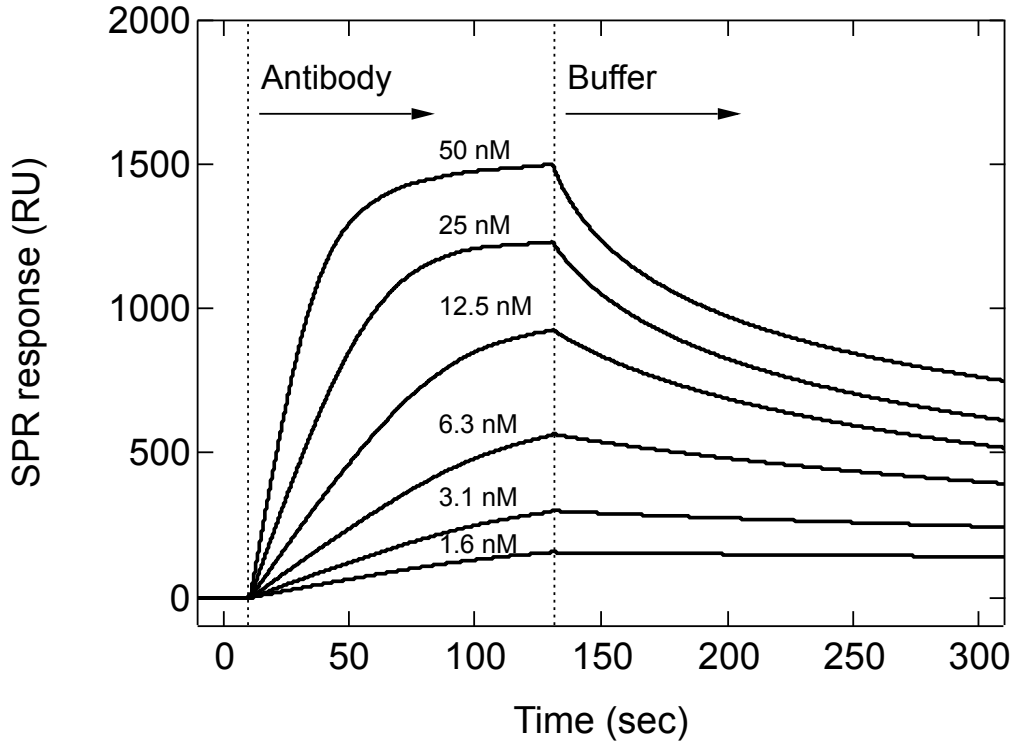

Figure S3. SPR sensorgrams displaying the binding response between anti-mC antibody and 5-mC in immobilized ODN as a function of time, where the subtracted SPR responses of the sample and reference cells are plotted. Methylated ODN-**L1** mixtures containing 100% 5-mC were immobilized on a bare Au sensor chip, which was treated with 0 – 50 nM of anti-mC antibody in running buffer at 25 °C. The broken lines indicate the starting points of the injection of the antibody and buffer. The immobilization amounts of ODN-**L1** were 2000 RU.

10. Immobilization of λDNA

Methylated λDNA was prepared as follows: 5.0 μg of unmethylated λDNA (Promega, Corp.) was added to 10 units of CpG methyltransferase M.Sss I (New England BioLabs, Inc.) with 0.64 μM *S*-adenosylmethionine as a methyl donor in a buffer supplied by the manufacturer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), and incubated at 37 °C for 1 h. Then, restriction enzyme AluI (New England BioLabs, Inc.) in the same buffer containing 100 μg/mL BSA was added to the methylated λDNA and incubated at 37 °C for 15 min to obtain 144 DNA fragments. The methylated λDNA fragments were purified by phenol/chloroform extraction and ethanol precipitation. The fragmentation of purified λDNA was confirmed with capillary electrophoresis. Unmethylated λDNA fragments were obtained by the above procedure without a methylation process. Next, 62.5 μM **L1** in DMSO was added to 30 ng/μL λDNA (about 3.0 μM of GpNpC) in TE buffer and then incubated at 37 °C for 30 min. To remove the unreacted **L1**, gel filtration was carried out on a desalting column using a running buffer for SPR analysis. Our preliminary experiments showed that the λDNA-**L1** yield was the highest under the above condition, where we used 20 eq. of **L1** relative to the GpNpC sequence concentration.

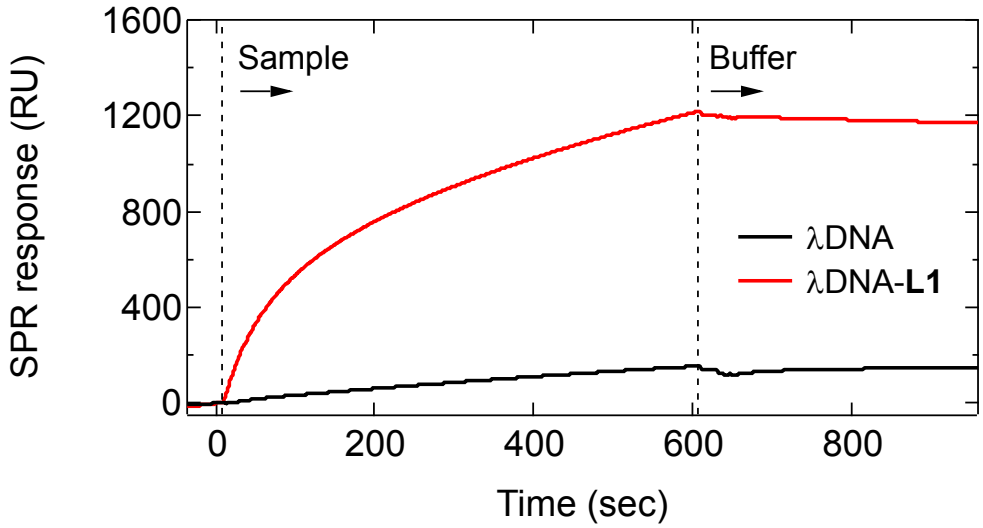

Figure S4. SPR sensorgram displaying the immobilizing response of λDNA-**L1** on a gold substrate as a function of time. 5.0 ng/μL λDNA samples were injected onto a bare gold sensor chip in the running buffer. The broken lines indicate the starting points of DNA and buffer injection.

11. SPR-based immunoassay for genomic λDNA

Table S1. Comparison of SPR results for methylated λDNA and ODN.

DNA	M_w [Da]	total 5-mC	Immmobilized amounts on sensor chip		SPR responses of antibody binding	
			DNA [ng/mm²]	5-mC [pmol/mm²]	total 5-mC [RU]	one 5-mC [RU·mm²/mol]
λDNA	3.23×10^7	6224[a]	1.0[b]	19.3	322 [c]	1.67×10^{13}
ODN	1.66×10^4	4	2.0[b]	48.2	1450 [d]	3.00×10^{13}

[a] λDNA has 3112 CpG sites, which are fully methylated by CpG methyltransferase *M.SssI*.
[b] 1000 RU represents a surface concentration of 1.0 ng/mm² [1].
[c] Obtained from Figure 5b
[d] Obtained from Figure 4

Reference

[1] A. Kobori, S. Horie, H. Suda, I. Saito, K. Nakatani, *J. Am. Chem. Soc.* **2004**, *126*, 557–62.