Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Detection of single methylated cytosine using junction-forming DNA probes

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Experimental details

Reagents

Ex Taq (*Taq* DNA polymerase) and 10,000× SYBR Green I were purchased from Takara (Kyoto, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were purchased from Wako Pure Chemical (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan), or Sigma. The ssDNAs were synthesized by standard phosphoramidite chemistry and were purified by high-performance liquid chromatography, except for PCR primers, which were purified by solid-phase extraction using a reversed-phase resin. The sequences of target DNAs, probes and primers used in this study are listed in Supplementary Table 1.

The sodium bisulfite ($NaHSO_3$) solution, adjusted to pH 5.0 and 4 M, was prepared from an aqueous solution of sodium bisulfite and NaOH. Diethylamine was added to the aqueous solution of *O*-carboxymethylhydroxylamine (CMH, H_2NOCH_2COOH) hemihydrochloride to adjust the pH to 5.0 and the concentration to 3 M. A SYBR Green mix (10×) was prepared by mixing 10 μL of 10,000× SYBR Green I, 100 μL of TWEEN 20, 1 mL of a 10 mg/mL BSA aqueous solution, 5 mL of dimethyl sulfoxide and 3.9 mL of sterilized water.

Modification by sodium bisulfite and CMH

Target DNA (DNA1, DNA1′, or DNA2) with or without appropriate DNA probes was denatured in a 1× HEPES buffer (pH 6.0, 50 mM HEPES–NaOH, containing either 125 mM or 1.0 M NaCl) at 95°C for 5 min and then cooled on ice. To the DNA solutions, an 8× or 4× concentrated HEPES buffer, bisulfite solution (pH 5.0, 4 M) and CMH solution (pH 5.0, 3 M)

were added, and the final concentrations were adjusted to 1.0 M bisulfite and 1.0 M CMH in a 1× HEPES buffer. After the mixture was incubated at 25°C for several hours, DNA was recovered by ethanol precipitation, dried and dissolved in sterilized water.

Primer extension

Modification of 0.5 pmol (50 nM) of DNA1 in the absence or presence of 2.0 pmol (200 nM) of FM-TWJ probes 1 and 2 was performed for 5 h in a 1× HEPES buffer (125 mM NaCl). The recovered DNA was subsequently mixed with 2.5 pmol of 5′-FAM-labeled 18mer primer, 2 U of *Ex Taq* and 10 nmol of each dNTP in 50 μL of a 1× *Ex Taq* buffer. Primer extension was performed in a thermal cycler under the following conditions: 94°C for 1 min and 40 cycles of 94°C for 30 s and 52°C for 30 s. After *Ex Taq* was deactivated by addition of 5 μL of 0.5 M EDTA–NaOH (pH 8.0), DNA samples were ethanol-precipitated, dissolved in a loading buffer (24 μL of formamide and 6 μL of a 1× TBE buffer), heated at 95°C for 5 min and then cooled on ice.

All DNA samples dissolved in the loading buffer were run on 19% polyacrylamide gels (19:1 monomer/bis ratio) containing 7 M urea. TBE buffer (1×) was used as the electrophoresis buffer. A 5′-FAM-labeled DNA complementary to the 56mer target DNA1 (5′-FAM-labeled cDNA1) was used to prepare Maxam–Gilbert A+G ladder markers. The gels were scanned using a Typhoon 9410 (GE Healthcare) system. The fluorescent images were analysed using software (ImageQuant tool) provided with the Typhoon 9410 system.

Quantitative PCR assay

Modification of the target DNA (DNA1, DNA1′, or DNA2) in the absence or presence of appropriate DNA probes was performed for 3 h in a 1× HEPES buffer (1.0 M NaCl) as

previously described. After ethanol precipitation and dissolution in sterilized water, the recovered DNA was used as a template for quantitative PCR.

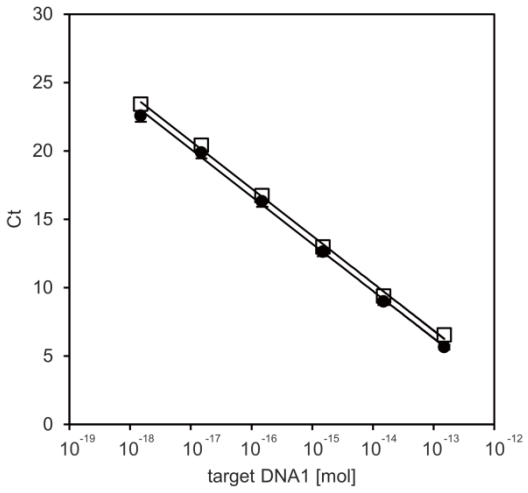
Quantitative PCR was performed using a 96-well plate format with a Prism 7700 sequence detection system (ABI). The PCR mixtures contained 0.5 U of *Ex Taq*, 0.2 mM of each dNTP, 150 nM of each primer, 2 mM of $MgSO_4$ and a 1× SYBR Green mix in 25 μL of a 1× *Ex Taq* buffer. PCR conditions were as follows: 50°C for 5 min, 95°C for 10 min and 50 cycles of 95°C for 5 s, 54°C (64°C for target DNA2) for 10 s and 72°C for 15 s. The amplification process was monitored by the fluorescence of SYBR Green I (ex. 470 nm, em. 510 nm). The cycle number at which plot of the SYBR Green I fluorescence crossed the threshold was defined as the Ct (cycle of threshold). A 10-fold serial dilution series of non-incubated target DNAs (DNA1, DNA1′, or DNA2) was used to generate the standard curves. The Ct values for each dilution were measured in triplicate and were plotted against the logarithm of the initial amount of the non-incubated targets. Each standard curve was generated by a linear regression of the plotted points.

Supplementary Table 1. DNA sequences used in this study.

Oligo name	Sequence
56mer target DNA1 (X = C or M)	5'-CTGATGAACATACTATCTTGCGCTCGGXGGCTGCGGAGAATTGAATAAGCTGGTAC-3'
56mer target DNA1' (X = C or M)	5'-CTGATGAACATACTATCTTGCGCTCGCXGGCTGCGGAGAATTGAATAAGCTGGTAC-3'
5'-FAM-labeled-cDNA1	5'-FAM-GTACCAGCTTATTCAATTCTCCGCAGCCGCCGAGCGCAAGATAGTATGTTCACAG-3'
5'-FAM-labeled 18mer primer	5'-FAM-GTACCAGCTTATTCAATT-3'
FM-TWJ probe 1	5'-CCACCGCTCTGCCGAGCGCAAGATAGTATGTTCATCAG-3'
FM-TWJ probe 2 (mismatched TWJ probe 2)	5'-GTACCAGCTTATTCAATTCTCCGCAGCMAGAGCGGTGG-3'
FM-TWJ probe 1'	5'-CCACCGCTCTGGCGAGCGCAAGATAGTATGTTCATCAG-3'
mismatched TWJ probe 1	5'-CCACCGCTCTMGCGAGCGCAAGATAGTATGTTCATCAG-3'
bulge probe	5'-GTACCAGCTTATTCAATTCTCCGCAGCMGCGAGCGCAAGATAGTATGTTCATCAG-3'
mismatch probe	5'-GTACCAGCTTATTCAATTCTCCGCAGCMMGCGAGCGCAAGATAGTATGTTCATCAG-3'
PCR primer 1 (forward)	5'-GTACCAGCTTATTCAATT-3'
PCR primer 2 (reverse)	5'-CTGATGAACATACTATCT-3'
54mer target DNA2 (X = C or M)	5'-AGCGTCCCCTTGCCTGGAAAGATACCGXGGTCCCTCCAGAGGATTTGAGGGACA-3'
54mer cDNA2	5'-TGTCCCTCAAATCCTCTGGAGGGACCGCGGTATCTTTCCAGGCAAGGGGACGCT-3'
FM-TWJ probe 3	5'-CCACCGCTCTGCGGTATCTTTCCAGGCAAGGGGACGCT-3'
FM-TWJ probe 4	5'-TGTCCCTCAAATCCTCTGGAGGGACMAGAGCGGTGG-3'
PCR primer 3 (forward)	5'-CCCTTGCCTGGAAAGATACC-3'
PCR primer 4 (reverse)	5'-TGTCCCTCAAATCCTCTGGAG-3'

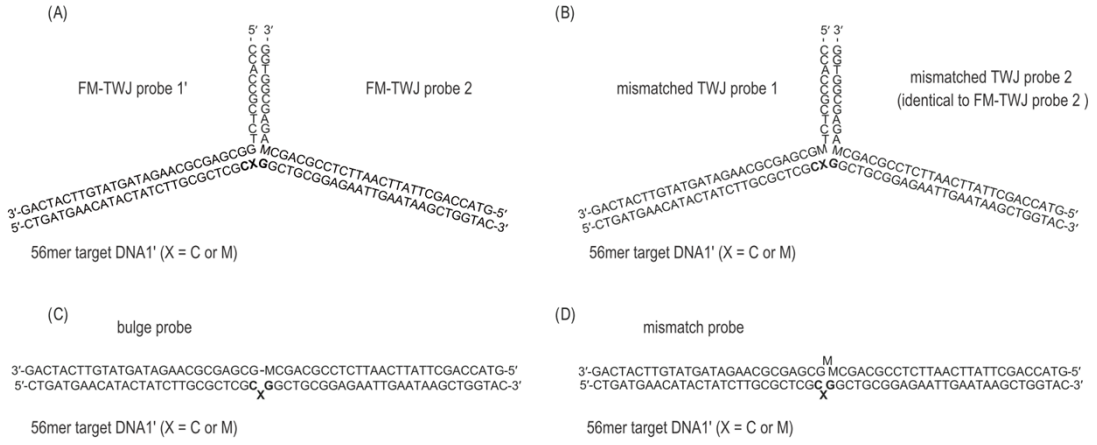
Underlined letters in target DNAs indicate primer regions for primer extension and/or PCR amplification.

Supplementary Fig. 1. Standard curve for quantification of unmodified DNA1.



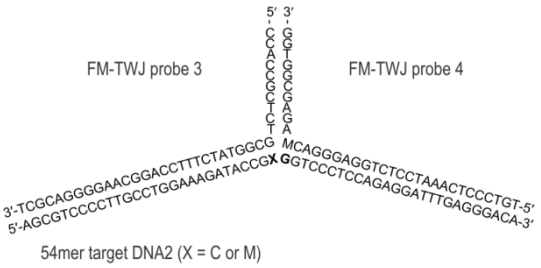
Linear relationship between the Ct values and the 10-fold serial dilutions of non-incubated DNA1 (X = C; square, X = M; circle).

Supplementary Fig. 2. Secondary structures formed by the 56mer target DNA1′ and appropriate probes.



Bold letters denote the CXpG of interest. (A) Fully matched TWJ formed by DNA1′ and FM-TWJ probes 1′ and 2. (B) TWJ with a mismatch at the branching point formed by DNA1′ and the mismatched TWJ probes 1 and 2. (C) Single-base bulge formed by DNA1′ and the bulge probe. (D) Single-base mismatch formed by DNA1′ and the mismatch probe. To avoid modification of probes at the branching point or at the bulge/mismatch, some Cs in probes were substituted for Ms.

Supplementary Fig. 3. A fully matched TWJ formed by the 54mer target DNA2 and FM-TWJ probes 3 and 4.



Bold letters denote the XpG of interest. To avoid modification of the FM-TWJ probe 4 at the branching point, a C was substituted for an M.