Electronic Supplementary Information

A dumbbell probe-based dual signal amplification strategy for sensitive detection of multiple DNA methyltransferases

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EXPERIMENTAL SECTION

Materials. All the oligonucleotides (Table S1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) except that circular templates 1 and 2 were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). The DNA adenine methyltransferase (Dam), CpG Methyltransferase (M.SssI), Vent (exo-) DNA polymerase, Nb.BtsI, DpnI, T4 DNA Ligase, Exonuclease I (Exo I), Exonuclease III (Exo III), Endonuclease IV (Endo IV), T4 polynucleotide kinase (PNK), uracil DNA glycosylase (UDG) and deoxynucleotide solution mix (dNTP) were purchased from New England Biolabs (Beverly, MA, USA). Restriction endonuclease GlaI (GlaI) were purchased from SibEnzyme Ltd (Novosibirsk, Russia). The 5-fluorouracil, bovine serum albumin (BSA), and immunoglobulin G (IgG) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Lung adenocarcinoma cell lines (A549 cells) were obtained from the Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Table S1. Sequences of the oligonucleotides [a]

name	sequence (5'-3')
dumbbell probe	CAG TTC CTC **GAT C**TT GAT AGG GAG ACA A**GA TC**G AGG AAC TGG TGT GTC *G^{m}CG C*GA AAG AAG AAA GAC AGA AAT TTC *G^{m}CG C*GA CAC AC
circular template 1	CTA TCA AGA TAA GCA GTG TCG TCA TAG CCC TAC TAC TCA TCT TGT CTC C
circular template 2	TGT CTT TCT TCT TTC GCT ATG CAG TGT TCT GGA TAG TGA TGC TAG GGC GAA ATT TC
signal probe 1	CAT AGC CXT ACT ACT
signal probe 2	GGA TAG TXA TGC TAG

[a] In dumbbell probe, the boldface regions indicate the recognition sequence of Dam, and the italic regions indicate the recognition sequence of M.SssI, and the mC indicates 5-methylcytosine. In circular templates 1 and 2, the underlined regions indicate the recognition sequence of Nb.BtsI. In signal probes 1 and 2, the "X" indicates the abasic site mimic.

Preparation of dumbbell probes. The 2 μL of 100 μM dumbbell probe, 1.5 μL of 10× T4 DNA Ligase buffer (500 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, pH 7.5) were incubated at 95 ℃ for 5 min with a final volume of 10 μL, followed by slowly cooling to room temperature. Then the DNA ligase reaction was performed in 15 μL of reaction mixture containing 100 U of T4 DNA Ligase and 10 μL of the above mixture at 16 ℃ overnight, followed by inactivation at 65 ℃ for 10 min. Subsequently, the exonuclease digestion reaction was performed in 20 μL of reaction mixture containing 10 U of Exo I, 10 U of Exo III, 1× NEBuffer 1 (10 mM Bis Tirs Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0), and 1× Exonuclease I reaction buffer (67 mM glycine–KOH, 6.7 mM $MgCl_2$, 10 mM b-ME, pH 9.5) and 15 μL of DNA ligase reaction solution at 37 ℃ for 30 min, and the reaction was terminated by incubation at 80 ℃ for 20 min. The obtained dumbbell probes were stored at 4 ℃ prior to use.

Methylation and cleavage of dumbbell probes. The methylation and cleavage reactions were performed in 20 μL of reaction mixture containing 500 nM dumbbell probe, 1× SEBuffer Y (33 mM Tris-Ac, 10 mM $MgAc_2$, 66 mM KAc, 1 mM DTT, pH 7.9), 8 U of DpnI, 10 U of GlaI, 160 μM SAM and various concentrations of Dam and M.SssI. The mixture was incubated at 37 ℃ for 2 h, and then the reaction was terminated by incubation at 80 ℃ for 20 min.

Rolling circle amplification reaction. After methylation and cleavage reactions, 10 μL of products were added to 30 μL of annealing reaction mixture containing 50 nM circular template 1, 50 nM circular template 2, 5 mM $MgCl_2$, 10 mM Tris-HCl. The mixture was heated at 95 ℃ for 5 min, followed by slowly cooling to room temperature.

Then the RCA reaction was performed in 50 μL of reaction mixture containing 30 μL of annealing products, 1 U of Vent (exo-) DNA polymerase, 10 U of Nb.BbtsI and 1× Cutsmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $MgAc_2$, 100 μg/mL BSA, pH 7.9) at 55 °C for 1 h. The reaction was terminated by heating at 80 °C for 20 min.

Endo IV-assisted signal amplification and fluorescence measurement. The 30 μL of RCA products, 500 nM signal probe 1, 500 nM signal probe 2, 1× NEBuffer 3 (100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), and 10 U of Endo IV were added to the reaction solution with a final volume of 50 μL, followed by incubation at 37 ℃ for 90 min. The reaction was terminated by incubation at 80 ℃ for 20 min.

We measured the fluorescence intensity of reaction products (50 μL) using F-7000 spectrometer (Hitachi, Japan). The fluorescence intensities at 567 nm for Cy3 and 668 nm for Cy5 were used for data analysis, respectively. The emission spectra were recorded over the wavelength range of 550 – 650 nm for Cy3 and 650 - 750 nm for Cy5, with a slit width of 5 nm for both excitation and emission. The $F - F_0$ value is used to calculate the concentration of MTase based on the obtained regression equation, where F_0 and F are the fluorescence intensity in the absence and presence of MTase, respectively.

Gel Electrophoresis. The products of methylation and cleavage reactions were analyzed by 12% non-denaturating polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (9 mM Tris-HCl, pH 8.3, 9 mM boric acid, 0.2 mM EDTA) at a 110 V constant voltage for 50 min by using an illumination source of Epi-blue (460 – 490 nm excitation) and a 516 – 544 nm filter for SYBR Gold fluorophores. The products of dual DNA amplification reaction were analyzed by 12% non-denaturating polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer at a 110 V constant voltage for 30 min. The fluorescent DNA fragments of reaction products were analyzed by using an illumination source of Epi-green (520 – 545 nm excitation) and a 577 – 613 nm filter for Cy3 fluorophores, and an illumination source of Epi-red (625 – 650 nm excitation) and a 675 – 725 nm filter for Cy5 fluorophores.

Inhibition Assay. Various concentrations of 5-fluorouracil were incubated with 500 nM dumbbell probes in 1×

SEBuffer Y (33 mM Tris-Ac, 10 mM $MgAc_2$, 66 mM KAc, 1 mM DTT, pH 7.9) at 37 ℃ for 15 min. Then 40 U/mL Dam, 40 U/mL M.SssI, 160 μM SAM, 8 U of DpnI, and 10 U of GlaI were added to the mixture and incubated at 37 °C for 2 h, and the subsequent experiments were carried out according to the procedures of MTase assay described above. The relative activity (*RA*) of DNA MTase was quantitatively calculated according to equation S1.

$$RA\ (\%) = \frac{F_i - F_0}{F_t - F_0} \times 100\% \qquad (S1)$$

where F_0, F_t, F_i are the fluorescence intensity in the absence of DNA MTase, in the presence of DNA MTase, and in the presence of both DNA MTase and inhibitor, respectively. The IC_{50} value of inhibitor was obtained from the curve-fitting equation.

Cell Culture and Preparation of Cell Extracts. The lung adenocarcinoma cell line (A549 cells) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2. The 1×10^6 cells were collected and washed twice with 1× PBS (pH 7.4). The number of cells was measured by Countstar automated cell counter (IC1000, Wilmington, DE, USA). The nuclear extracts of A549 cells were prepared by using the nuclear extract kit (ActiveMotif, Carlsbad, CA, USA) according to the manufacturer's protocol. The obtained supernatant was subjected to recovery assay.

Detection of MTase activity in human serum and cell extracts. The 20 μL of sample containing 5% human serum (or 5 μL of cell extracts), 500 nM dumbbell probe, 1× SEBuffer Y, 8 U of DpnI, 10 U of Gla I, 160 μM SAM, and various concentrations of DNA MTase was incubated at 37 ºC for 2 h, followed by inactivation at 80 ℃ for 20 min. Then the DNA MTase activity was calculated based on the fitting equation (inset of Fig. 3A for Dam and Fig. 3B for M.SssI). The recovery ratio (*R*) is calculated based on equation S2:

$$R(\%) = \frac{C_m}{C_0} \times 100\% \qquad (S2)$$

where C_m and C_0 represent the MTase concentration in the presence and absence of human serum (or A549 cell extracts), respectively.

RESULTS AND DISCUSSION

Optimization of methylation and MTases-mediated cleavage reaction. The methylation and cleavage reaction involves four kinds of enzymes (i.e., Dam, DpnI, M.SssI and GlaI) and three different buffers (i.e., 1× Dam buffer, 1× NEBuffer 2, and 1× SEBuffer Y). The 1× Dam buffer (10 mM EDTA, 50 mM Tris-HCl, 5 mM 2-mercaptoethanol, pH 7.5) is for Dam, and 1× NEBuffer 2 (50 mM NaCl，10 mM Tris-HCl，10 mM $MgCl_2$，1 mM DTT, pH 7.9）is for DpnI and M.SssI, and 1× SEBuffer Y (33 mM Tris-Ac, 10 mM $MgAc_2$, 66 mM KAc, 1 mM DTT, pH 7.9) is for GlaI. To obtain the optimal reaction buffer, we investigated the assay performance using Dam buffer, NEBuffer 2, SEBuffer Y and different mixture buffers (NEBuffer 2 + Dam buffer, Dam buffer + SEBuffer Y, NEBuffer 2+ SEBuffer Y, Dam buffer + NEBuffer 2+ SEBuffer Y), respectively. As shown in Fig. S1A, the highest F/F_0 value is obtained using SEBuffer Y for Cy3 and Cy5, respectively. Thus, SEBuffer Y is used in the subsequent research. To obtain the best performance of DNA MTase assay, the methylation reaction time was optimized. As shown in Fig. S1B, the F/F_0 value enhances with the reaction time and reaches the plateau at 2 h for Cy3 and Cy5, respectively. Therefore, the methylation reaction time of 2 h is used in the subsequent research. We further optimized the amounts of DpnI and GlaI, respectively. As shown in Fig. S1C, the F/F_0 value of Cy3 enhances with the increasing amount of DpnI and reaches the plateau at 8 U. As shown in Fig. S1D, the F/F_0 value of Cy5 enhances with the increasing amount of GlaI and reaches the highest value at the amount of 10 U, followed by the decrease beyond the amount of 10 U. Therefore, 8 U of DpnI and 10 U of GlaI are used in the subsequent experiments.

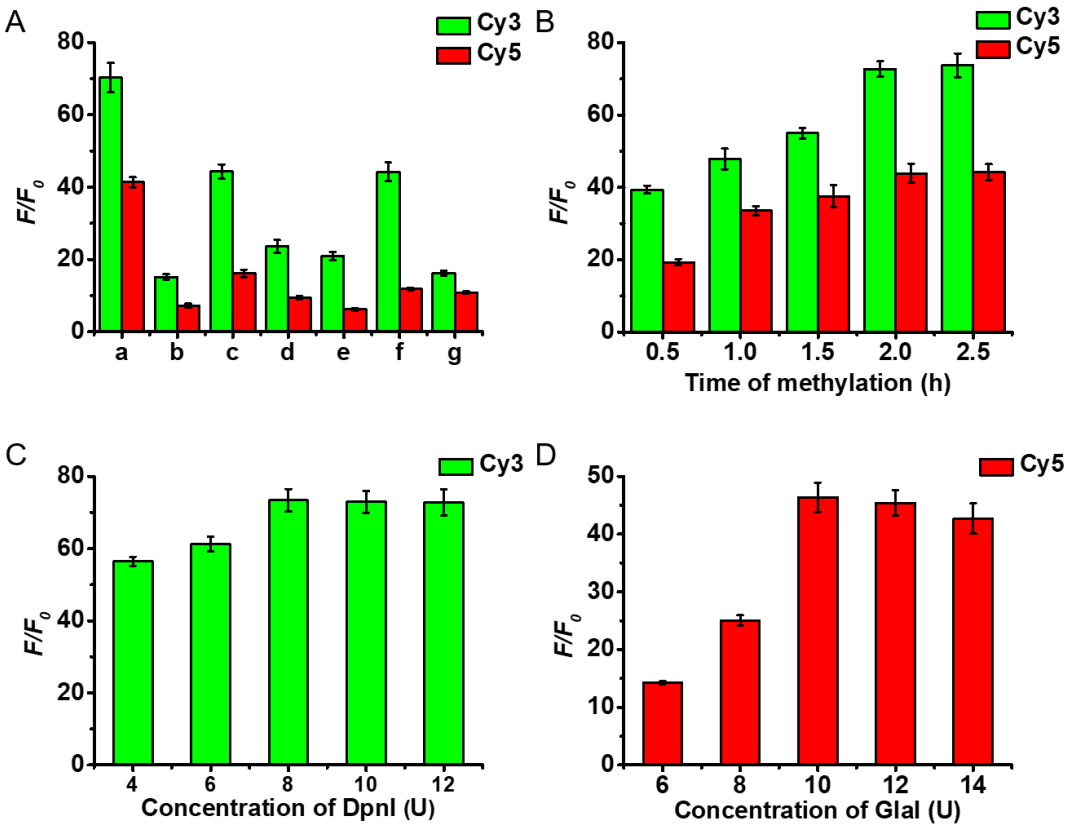

Fig. S1 (A) Variance of F/F_0 value with different buffers. a, 1× SEBuffer Y; b, 1× Dam buffer; c, 1× NEBuffer 2; d, 1× NEBuffer 2 + 1× SEBuffer Y; e, 1× NEBuffer 2 + 1× Dam buffer; f, 1× Dam buffer + 1× SEBuffer Y; g, 1× Dam buffer + 1× NEBuffer 2 + 1× SEBuffer Y. (B) Variance of F/F_0 value with the methylation time. (C) Variance of F/F_0 value with different concentrations of DpnI. (D) Variance of F/F_0 value with different concentrations of GlaI. F and F_0 are the fluorescence signals in the presence and absence of Dam and M.SssI, respectively. The Cy3 signal is indicated by green, and the Cy5 signal is indicated by red. The 40 U/mL Dam and 40 U/mL M.SssI are used in the experiments. Error bars show the standard deviation of three experiments.

Optimization of RCA reaction. To obtain the best performance of RCA reaction, we optimized the concentrations of circular templates. As shown in Fig. S2A, the F/F_0 value of Cy3 enhances with the increasing concentration of circular template 1 and reaches the highest value at the concentration of 30 nM, followed by the decrease beyond the concentration of 30 nM. Similarly, the F/F_0 value of Cy5 enhances with the increasing concentration of circular template 2 and reaches the highest value at the concentration of 30 nM, followed by the decrease beyond the concentration of 30 nM (Fig. S2B). Therefore, the optimal concentration is determined to be 30 nM for circular templates 1 and 2, respectively. The close cooperation of DNA polymerase and nicking enzyme is crucial for

efficient nucleic acid amplification.[1] The concentration of Vent (exo-) DNA polymerase influences the efficiency of RCA reaction. As shown in the Fig. S2C, the F/F_0 value of Cy3 improves with the increasing amount of Vent (exo-) DNA polymerase from 0.2 U to 1 U, followed by the decrease beyond the amount of 1 U. Similarly, the F/F_0 value of Cy5 improves with the increasing amount of Vent (exo-) DNA polymerase from 0.2 U to 1 U, followed by the decrease beyond the amount of 1 U. Thus, 1 U of Vent (exo-) DNA polymerase is used in the subsequent experiments. We further investigated the influence of Nb. BtsI amount upon the F/F_0 value at a fixed amount of Vent (exo-) DNA polymerase (1 U). As shown in the Fig. S2D, the F/F_0 value of Cy3 improves with the increasing amount of Nb. BtsI from 6 U to 10 U, followed by the decrease beyond the amount of 10 U. Similarly, the F/F_0 value of Cy5 improves with the increasing amount of Nb. BtsI from 6 to 10 U, followed by the decrease beyond the amount of 10 U. Thus, 10 U of Nb. BtsI is used in the subsequent experiments. We optimized the influence of dNTP concentration upon the fluorescence signal as well. As shown in the Fig. S2E, the F/F_0 value of Cy3 improves with the increasing concentration of dNTP from 200 μM to 400 μM, followed by the decrease beyond the concentration of 400 μM. Similarly, the F/F_0 value of Cy5 improves with the increasing concentration of dNTP from 200 to 400 μM, followed by decrease beyond the concentration of 400 μM (Fig. S2E). Thus, 400 μM dNTP is used in the subsequent experiments.

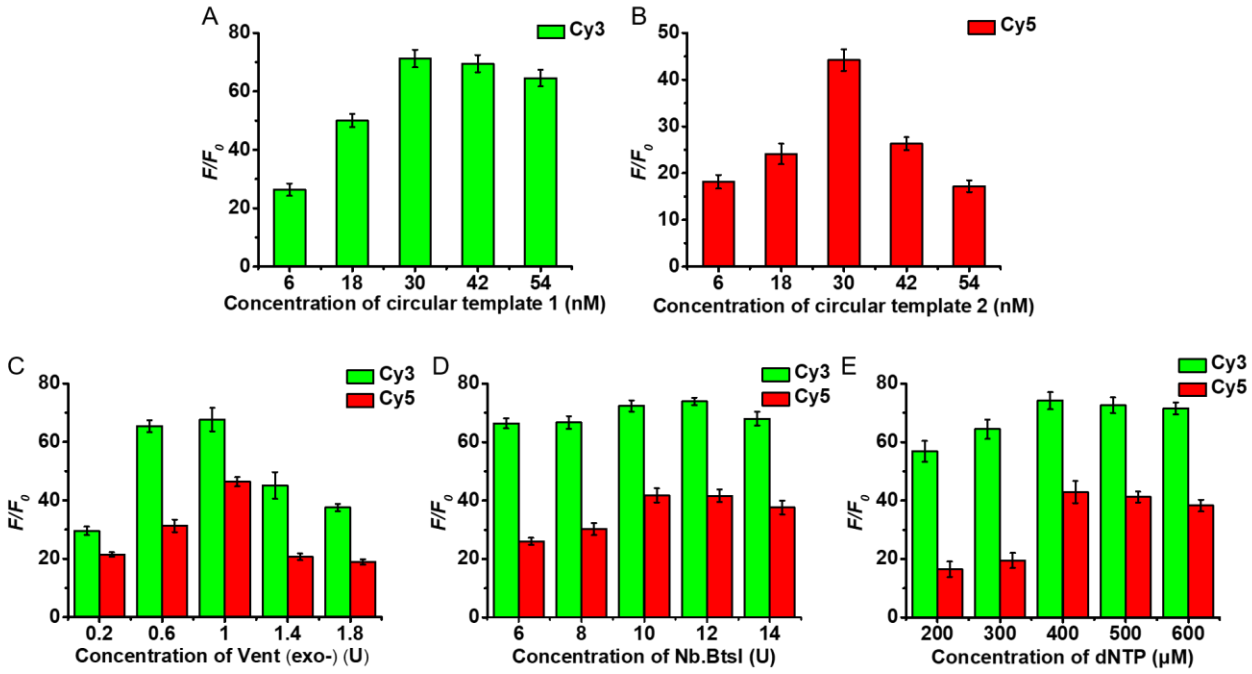

Fig. S2 (A) Variance of F/F_0 value of Cy3 with different concentrations of circular template 1. (B) Variance of F/F_0 value of Cy5 with different concentrations of circular template 2. (C) Variance of F/F_0 value with different concentrations of Vent (exo-). (D) Variance of F/F_0 value with different concentrations of Nb.BtsI. (E) Variance of

F/F_0 value with different concentrations of dNTP. F and F_0 are the fluorescence intensity in the presence and absence of Dam and M.SssI, respectively. The Cy3 signal is indicated by green, and the Cy5 signal is indicated by red. The 40 U/mL Dam and 40 U/mL M.SssI are used in the experiments. Error bars show the standard deviation of three experiments.

Optimization of Endo IV-assisted amplification reaction. We investigated the effect of the concentrations of signal probes and the amount of Endo IV upon the assay performance. The F/F_0 value of Cy3 enhances with the increasing amounts of Endo IV from 5 U to 10 U, followed by the decrease beyond the amount of 10 U (Fig. S3A). Similarly, the F/F_0 value of Cy5 improves with the increasing amounts of Endo IV from 5 U to 10 U, followed by the decrease beyond the amount of 10 U. Therefore, 10 U of Endo IV is used in the subsequent experiments. We studied the effect of the concentrations of signal probes upon the fluorescence signal with the amount of Endo IV being fixed at 10 U. The F/F_0 value of Cy3 enhances with the increasing concentration of signal probe 1 from 200 nM to 500 nM, followed by the decrease beyond the concentration of 500 nM (Fig. S3B). Similarly, the F/F_0 value of Cy5 improves with the increasing concentration of signal probe 2 from 200 to 500 nM, followed by the decrease beyond the concentration of 500 nM (Fig. S3C). Therefore, 500 nM signal probe 1 and 500 nM signal probe 2 are used in the subsequent experiments.

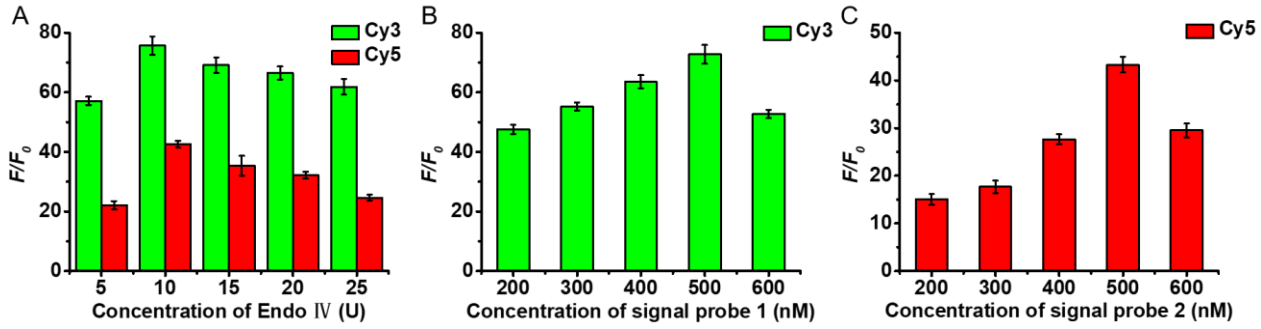

Fig. S3 (A) Variance of F/F_0 value with the different amounts of Endo IV. (B) Variance of F/F_0 value with different concentrations of signal probe 1. (C) Variance of F/F_0 value with different concentrations of signal probe 2. F and F_0 are the fluorescence intensity in the presence and absence of Dam and M.SssI, respectively. The Cy3 signal is indicated by green, and the Cy5 signal is indicated by red. The 40 U/mL Dam and 40 U/mL M.SssI are used in the experiments. Error bars show the standard deviation of three experiments.

Detection selectivity. To evaluate the selectivity of the proposed method, we used bovine serum albumin (BSA), immunoglobulin G (IgG), uracil DNA glycosylase (UDG), and T4 polynucleotide kinase (PNK) as the negative controls. As shown in the Fig. S4, a high Cy3 fluorescence signal is detected only in response to Dam, and a high Cy5 fluorescence signal is detected only in response to M.SssI. Moreover, both high Cy3 and Cy5 fluorescence signals can be detected simultaneously when both Dam and M.SssI are present. In contrast, neither Cy3 nor Cy5 fluorescence signal can be observed in the presence of BSA, IgG, UDG and PNK. This may be explained by the fact that the 5'-G-A-T-C-3' in the dumbbell probe can only be methylated by Dam and the 5'-G-mC-G-C-3' in the dumbbell probe can only be methylated by M.SssI. These results demonstrate the good selectivity of the proposed method toward Dam and M.SssI.

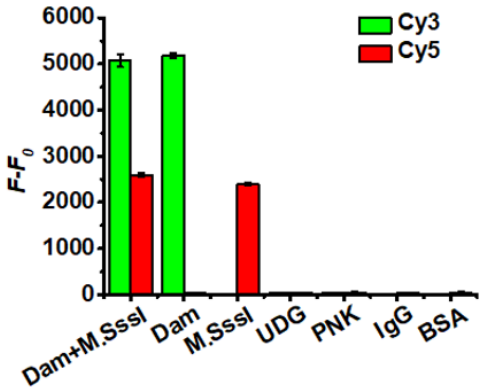

Fig. S4 Variance of the $F\text{-}F_0$ value of Cy3 (green column) and Cy5 (red column) in response to 40 U/mL Dam + 40 U/mL M.SssI, 40 U/mL Dam, 40 U/mL M.SssI, 40 U/mL UDG, 40 U/mL PNK, 10 μg/mL IgG ,10 μg/mL BSA , respectively. Error bars show the standard deviation of three experiments.

Measurement of MTases activity in complex biological samples. To investigate the feasibility of the proposed method for real sample analysis, we assessed the recovery of Dam by adding varying amounts of Dam (0.001–5 U/mL) to the human serum samples. As shown in Table S2, the recovery ratio is calculated to be 98.2% – 105.4% with a relative standard deviation (RSD) of 2.5% – 4.0 %. In addition, we assessed the recovery of M.SssI by

adding varying amounts of M.SssI (0.001 – 5 U/mL) to the human serum samples. As shown in Table S3, the recovery ratio is calculated to be 101.0% – 102.2% with a relative standard deviation (RSD) of 0.79% – 1.26%. Moreover, this method can be used to detect DNA MTases in cell lysates extracted from A549 lung cancer cells. As shown in Fig. S5, the fluorescence intensities of both Cy3 and Cy5 in the cell lysates and human serum are consistent with those obtained in reaction buffer, indicating that the proposed method can specifically detect Dam and M.SssI activities in real samples. Moreover, we measured the recovery of DNA MTases by spiking different concentrations of DNA MTases to the A549 cancer cell extracts (Tables S4 – S5), which are consistent with the results obtained by using the spiked human serum samples (Tables S2 and S3). We assessed the recovery of Dam by adding different amounts of Dam (0.001 U/mL – 5 U/mL) to the cell extracts. As shown in Table S4, the recovery ratio is calculated to be 92.0% –101.0 % with a relative standard deviation (RSD) of 1.4% – 2.2%. In addition, we assessed the recovery of M.SssI by adding different amounts of M.SssI (0.001 U/mL – 5 U/mL) into the cell extracts. As shown in Table S5, the recovery ratio is calculated to be 97.8% – 99.1% with a relative standard deviation (RSD) of 1.3% – 1.7%. These results demonstrate that the proposed method may be used for the sensitive detection of DNA MTase activity in complex biological samples. Those results demonstrate that the proposed method can be used for sensitive detection of multiple DNA MTases in complex biological samples.

Table S2. Recovery studies of Dam in the spiked serum samples.

sample	added (U/mL)	measured (U/mL)	recovery (%)	RSD (%)
1	5.0	5.27	105.4	2.5
2	1.0	0.96	95.7	3.9
3	0.1	1.06×10^{-1}	105.9	4.1
4	0.01	1.08×10^{-2}	107.7	5.5
5	0.001	9.82×10^{-4}	98.2	4.0

Table S3. Recovery studies of M.SssI in the spiked serum sample.

sample	added (U/mL)	measured (U/mL)	recovery (%)	RSD (%)
1	5.0	5.11	102.2	1.26
2	1.0	0.98	98.4	0.45
3	0.1	9.96×10^{-2}	99.6	0.69
4	0.01	1.03×10^{-2}	103.0	3.56
5	0.001	1.01×10^{-3}	101.0	0.79

Table S4. Recovery studies of Dam in the spiked A549 cell extracts.

sample	added (U/mL)	measured (U/mL)	recovery (%)	RSD (%)
1	5.0	4.6	92.0	1.4
2	1.0	1.08	107.9	3.5
3	0.1	1.09×10^{-1}	109.2	3.7
4	0.01	1.04×10^{-2}	104.1	4.8
5	0.001	1.01×10^{-3}	101.0	2.2

Table S5. Recovery studies of M.SssI in the spiked A549 cell extracts.

sample	added (U/mL)	measured (U/mL)	recovery (%)	RSD (%)
1	5.0	4.89	97.8	1.3
2	1.0	1.06	106.4	3.3
3	0.1	9.72×10^{-1}	97.2	2.5
4	0.01	9.73×10^{-3}	97.3	2.0
5	0.001	99.1×10^{-4}	99.1	1.7

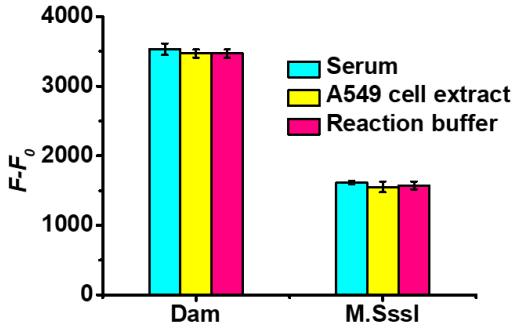

Fig. S5 Measurement of the F-F_0 value in response to serum (cyan column), A549 cell extract (yellow column), and reaction buffer (pink column) spiked with Dam and M.SssI, respectively. The 5 U/mL Dam and 5 U/mL M.SssI are used in the experiments. Error bars show the standard deviation of three experiments.

Table S6. Raw data for Fig. S1A, variance of Cy3 and Cy5 fluorescence intensity values with different buffers.[a]

Buffer	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
a	5720	77.31	2246	55.62
	5439	76.01	2217	53.65
	5334	77.80	2449	57.31
b	1214	75.64	398.8	53.31
	1231	78.21	409.7	56.82
	1250	77.80	462.3	57.79
c	3479	78.91	873.7	54.6
	3374	79.52	860.9	55,72
	3420	80.12	890.4	59.30
d	1869	73.58	506.3	55.64
	1781	75.40	546.1	58.21
	1800	82.56	597.8	57.60
e	1631	77.89	350.8	60.48
	1773	80.12	321.4	51.83
	1611	81.30	374.6	56.79
f	3434	82.15	652.5	56.73
	3406	77.00	628.5	53.28
	3476	74.31	694.8	57.90
g	1249	78.25	573.5	51.66

	1257	77.21	597.4	55.05
	1268	79.25	628.3	59.84

[a] a, 1× SEBuffer Y; b, 1× Dam buffer; c, 1× NEBuffer 2; d, 1× NEBuffer 2 + 1× SEBuffer Y; e, 1× NEBuffer 2 + 1× Dam buffer; f, 1× Dam buffer + 1× SEBuffer Y; g, 1× Dam buffer + 1× NEBuffer 2 + 1× SEBuffer Y. F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S7. Raw data for Fig. S1B, variance of Cy3 and Cy5 fluorescence intensity values with the methylation time.[a]

methylation time	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
0.5 h	3040	77.37	1079	54.19
	3053	79.71	1013	54.93
	3260	80.53	1098	56.89
1.0 h	3751	74.13	1827	56.44
	3656	76.48	1812	53.97
	3501	77.80	1964	56.50
1.5 h	4174	75.15	2035	58.98
	4335	78.92	2073	55.28
	4328	76.83	2280	56.30
2.0 h	5795	82.16	2403	58.31
	5451	75.01	2352	53.61
	5802	77.56	2610	56.11
2.5 h	5526	78.41	2413	57.45
	5861	79.50	2387	54.01
	5883	72.51	2576	55.52

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S8. Raw data for Fig. S1C, variance of Cy3 fluorescence intensity value with different concentrations of DpnI. [a]

Concentration of DpnI	Cy3 fluorescence intensity	
	F	F_0
4 U	4432	80.30
	4295	76.09
	4253	73.73
6 U	4738	80.11
	4729	77.24
	4873	76.99
8 U	5894	83.92
	5451	74.29
	5565	73.92
10 U	5448	77.89
	5337	73.15
	5682	74.81
12 U	5418	78.40
	5343	74.66
	5858	76.62

[a] F is the fluorescence intensity in the presence of Dam, and F_0 is the fluorescence intensity in the absence of Dam.

Table S9. Raw data for Fig. S1D, variance of Cy5 fluorescence intensity value with different concentrations of GlaI.[a]

Concentration of GlaI	Cy5 fluorescence intensity	
	F	F_0
6 U	785.6	56.11
	757.4	53.22
	785.7	54.19
8 U	1373	56.70
	1343	53.72
	1389	53.84
10 U	2486	56.50
	2543	54.80
	2783	56.80
12 U	2861	66.23
	2841	62.57
	2728	57.32
14 U	2275	56.78
	2323	54.40
	2639	59.77

[a] F is the fluorescence intensity in the presence of M. SssI, and F_0 is the fluorescence intensity in the absence of M. SssI.

Table S10. Raw data for Fig. S2A, variance of Cy3 fluorescence intensity value with different concentrations of circular template 1. [a]

Concentration of circular template 1	Cy3 fluorescence intensity	
	F	F_0
6 nM	1497	61.73
	1506	59.18
	1708	59.95
18 nM	3710	74.17
	3656	76.43
	3359	64.35
30 nM	5227	76.56
	5298	74.33
	5483	73.83
42 nM	4493	67.47
	4457	64.09
	4914	67.88
54 nM	4750	76.99
	4754	73.67
	4960	73.63

[a] F is the fluorescence intensity in the presence of Dam, F_0 is the fluorescence intensity in the absence of Dam.

Table S11. Raw data for Fig. S2B, variance of Cy5 fluorescence intensity value with different concentrations of circular template 2. [a]

Concentration of circular template 2	Cy5 fluorescence intensity	
	F	F_0
6 nM	1189	60.60
	1061	58.29
	957.2	56.31
18 nM	1350	61.55
	1352	56.05
	1381	52.65
30 nM	2502	59.52
	2400	54.29
	2605	56.04
42 nM	1399	56.16
	1370	52.11
	1593	59.94
54 nM	1132	70.88
	1112	64.57
	1203	65.15

[a] F is the fluorescence intensity in the presence of M. SssI, and F_0 is the fluorescence intensity in the absence of M. SssI.

Table S12. Raw data for Fig. S2C, variance of Cy3 and Cy5 fluorescence intensity values with different concentrations of Vent (exo-).[a]

Concentration of Vent (exo-)	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
0.2 U	1902	67.51	1193	57.63
	2306	77.91	1135	52.86
	2069	66.54	1232	55.79
0.6 U	5032	79.40	1826	62.53
	4921	75.33	1815	57.99
	5130	76.09	1942	58.16
1.0 U	5257	82.55	2486	55.44
	5216	77.06	2543	54.80
	5546	77.38	2598	54.19
1.4 U	5096	127.4	2135	109.2
	5105	113.2	2117	105.3
	5541	113.1	2240	102.9
1.8 U	4756	130.7	2131	118.3
	4797	127.6	2087	110.1
	4857	128.1	2203	111.3

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S13. Raw data for Fig. S2D, variance of Cy3 and Cy5 fluorescence intensity values with different concentrations of Nb.BtsI. [a]

Concentration of Nb.BtsI	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
6 U	4810	74.36	1452	58.36
	4636	69.80	1439	55.26
	4815	71.11	1509	55.49
8 U	4752	73.65	1675	59.48
	4734	70.98	1681	55.60
	5094	73.99	1831	56.70
10 U	5239	74.39	2335	59.27
	5047	69.79	2303	55.10
	5616	75.68	2395	54.22
12 U	5193	71.46	2307	58.33
	5139	69.53	2314	55.58
	5696	75.79	2553	58.42
14 U	4815	73.40	2074	58.80
	4853	71.39	2101	55.86
	5101	72.52	2293	57.40

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S14. Raw data for Fig. S2E, variance of Cy3 and Cy5 fluorescence intensity values with different concentrations of dNTP. [a]

Concentration of dNTP	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
200 μM	3913	73.48	1052	67.35
	3967	65.08	998.7	71.84
	4161	69.36	1186	62.13
300 μM	4484	73.41	1234	63.44
	4451	69.10	1184	70.01
	4371	67.52	1320	63.45
400 μM	5239	73.59	2634	67.24
	5047	68.07	2702	62.92
	5245	68.06	2865	63.20
500 μM	5548	79.41	2660	67.58
	5975	80.35	2559	61.67
	6096	81.92	2635	61.30
600 μM	5575	80.30	2597	71.62
	6023	84.07	2458	62.24
	6094	82.88	2760	69.52

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S15. Raw data for Fig. S3A, variance of Cy3 and Cy5 fluorescence intensity values with different amounts of Endo IV. [a]

Concentration of Endo IV	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
5 U	4556	81.70	1402	67.66
	4502	78.18	1461	66.04
	4489	76.71	1480	62.92
10 U	5389	74.24	2601	62.47
	5401	71.35	2697	63.26
	5557	71.27	2710	62.13
15 U	5130	77.11	2634	82.15
	5066	73.30	2602	73.58
	5283	73.69	2861	74.02
20 U	5152	80.09	2216	71.53
	5184	77.94	2234	69.46
	5261	76.58	2306	69.17
25 U	5088	85.91	1784	75.68
	5164	83.47	1783	72.56
	5305	82.26	1889	73.89

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S16. Raw data for Fig. S3B, variance of Cy3 fluorescence intensity value with different concentrations of signal probe 1. [a]

Concentration of signal probe 1	Cy3 fluorescence intensity	
	F	F_0
200 nM	2486	54.04
	2445	51.41
	2545	51.85
300 nM	3255	60.55
	3143	56.97
	3277	57.99
400 nM	4010	65.34
	4026	63.36
	4156	63.23
500 nM	5795	83.27
	5451	75.46
	5865	77.31
600 nM	4895	95.21
	5074	96.13
	5110	94.43

[a] F is the fluorescence intensity in the presence of Dam, and F_0 is the fluorescence intensity in the absence of Dam.

Table S17. Raw data for Fig. S3C, variance of Cy5 fluorescence intensity value with different concentrations of signal probe 2. [a]

Concentration of signal probe 2	Cy5 fluorescence intensity	
	F	F_0
200 nM	907.8	65.29
	923.9	61.36
	1037	64.10
300 nM	1109	67.87
	1180	66.77
	1176	61.92
400 nM	1581	60.87
	1668	60.32
	1780	63.32
500 nM	2675	64.21
	2738	63.24
	2768	61.63
600 nM	2557	91.13
	2591	87.80
	2691	86.92

[a] F is the fluorescence intensity in the presence of M.SssI, and F_0 is the fluorescence intensity in the absence of M.SssI.

Table S18. Raw data for Fig. S4, variance of Cy3 and Cy5 fluorescence intensity values in response to 40 U/mL Dam + 40 U/mL M.SssI, 40 U/mL Dam, 40 U/mL M.SssI, 40 U/mL UDG, 40 U/mL PNK, 10 μg/mL IgG ,10 μg/mL BSA , respectively. [a]

Sample	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
Dam + M.SssI	5239	77.56	2634	70.30
	5047	79.89	2702	68.32
	5146	79.61	2630	76.01
Dam	5227	74.57	73.02	61.23
	5298	77.89	79.14	59.65
	5255	73.23	70.44	63.13
M.SssI	78.42	74.23	2486	75.60
	75.95	69.56	2453	77.32
	77.56	72.50	2493	73.26
UDG	75.44	68.30	82.97	63.94
	86.54	66.22	89.51	73.19
	78.23	67.94	85.55	68.31
PNK	86.88	63.13	119.6	70.12
	86.89	65.12	121.6	68.65
	85.22	62.12	115.2	69.50
IgG	80.55	60.52	96.54	72.33
	79.63	61.69	90.17	70.26

	79.66	58.59	98.44	68.57
	99.94	58.66	82.64	56.60
BSA	86.89	63.32	80.10	60.87
	87.01	57.21	79.17	54.93

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

Table S19. Raw data for Fig. S5, variance of Cy3 and Cy5 fluorescence intensity values in response to serum, A549 cell extract, and reaction buffer spiked with Dam and M.SssI, respectively. [a]

Sample	Cy3 fluorescence intensity		Cy5 fluorescence intensity	
	F	F_0	F	F_0
Serum	3549	75.84	1670	69.30
	3667	74.74	1702	72.55
	3491	78.35	1653	70.79
A549	3505	78.66	1676	78.99
	3587	79.81	1567	71.30
	3500	80.21	1687	75.42
reaction buffer	3483	79.30	1638	68.92
	3567	80.45	1695	74.13
	3565	81.99	1615	69.56

[a] F is the fluorescence intensity in the presence of DNA MTase, and F_0 is the fluorescence intensity in the absence of DNA MTase.

References

1. (a) H. Jia, Z. Li, C. Liu and Y. Cheng, *Angew Chem Int Ed Engl*, 2010, **49**, 5498-5501; (b) J. Van Ness, L. K. Van Ness and D. J. Galas, *Proc Natl Acad Sci U S A*, 2003, **100**, 4504-4509.