Electronic Supplementary Information

Sensitive Detection of DNA Methyltransferase Activity by Transcription-Mediated Duplex-Specific Nuclease-Assisted Cyclic Signal Amplification

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EXPERIMENTAL SECTION

Materials. Dam Methyltransferase, CpG Methyltransferase (M.SssI), Hhal Methyltransferase, DpnI, T7 RNA polymerase, and Ribonucleotide Solution Mix (NTPs) were purchased from New England Biolabs (Ipswich, MA, USA). The Duplex-specific nuclease (DSN) and the corresponding buffer were obtained from Evrogen Joint Stock Company (Russia). Recombinant RNase inhibitor (RRI) and all oligonucleotides (Table S1) were purchased form TaKaRa Biotechnology Co. Ltd. (Dalian, China). SYBR Gold nucleic acid gel stain was obtained from life

technology (Carlsbad, CA, USA). Fast Silver Stain Kit was purchased from Beijing Tiandz Gene Technology Co. Ltd. (Beijing, China). Gentamycin and other chemicals with analytical grade were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Ultrapure water obtained from a Millipore filtration system was used throughout in this research.

Table S1. Sequences of the Oligonucleotides[α]

note	sequence (5′→3′)
hairpin substrate	ACT TAT CAG CTT AAG **GAT C**CT*T AAT ACG ACT CAC TAT A*A**G ATC** CTT AAG CTG ATA AGT
hairpin template-1	CAT GCA CCT CAA CGC GGT CTG TCT TAT AGT GAG TCG TAT TAA GAG ACT CAC
hairpin template-2	CAT GCA CCT CAA CGC GGT CTG TCT TAT AGT GAG TCG TAT TAA GAG ACT CAC T
hairpin template-3	CAT GCA CCT CAA CGC GGT CTG TCT TAT AGT GAG TCG TAT TAA GAG ACT CAC TA
T7 primer	TCT TAA TAC GAC TCA CTA TA A GA
QF probe-1 (13 nt)	FAM-TCA ACG CGG TCT G-BHQ
QF probe-2 (14 nt)	FAM-CTC AAC GCG GTCTG-BHQ
QF probe-3 (15 nt)	FAM-CTC AAC GCG GTC TGT-BHQ
QF probe-4 (16 nt)	FAM-ACC TCA ACG CGG TCT G-BHQ
QF probe-5 (17 nt)	FAM-CAC CTC AAC GCG GTC TG-BHQ
QF probe-6 (18 nt)	FAM-GCA CCT CAA CGC GGT CTG-BHQ

[a] In the hairpin probe, the binding sequence of hairpin template is shown in underline, the T7

promote sequence is shown in italic, and the recognition site of Dam is shown in boldface. In the hairpin template, the binding sequence of the cleaved hairpin probe is shown in underline.

Methylation and Cleavage of Hairpin Probe. A series of standard Dam MTase solutions were prepared from 0.1 unit to 10 unit. In order to preserve the activity of Dam MTase, they were prepared under 4 °C and stored under -20 °C. The methylation and cleavage of hairpin probe were performed in 200 μL of reaction mixture which consisted of 0.5 μM hairpin probe, 1× Dam buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT, pH 7.5), 160 μM SAM, 10 U DpnI and various amounts of Dam MTase. The experiment was conducted at 37℃ for 2 hours, followed by inactivation at 80℃ for 20 min.

DNA Transcription and Fluorescence Measurement. The 4 μL of digestion products were added into 16 μL of reaction mixture which contained 1× T7 RNA polymerase buffer (40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, and 2 mM spermidine, pH 7.9), 10 U T7 RNA polymerase, 2 mM NTP mixture, 20 U RNase inhibitor, and 50 nM hairpin template. The RNA amplification was performed at 37℃ for 90 min. Then 20 μL of RNA transcription products were incubated with a solution containing 1× DSN master buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, and 1 mM DTT, pH 8.0), 0.1 U DSN enzyme and 400 nM QF probe in a final volume of 50 μL at 50℃ for 30 min, followed by cooling to room temperature. The fluorescence spectra were recorded by a Hitachi F-4500 fluorometer (Tokyo, Japan) equipped with a xenon lamp as the excitation source. The excitation wavelength was 490 nm, and the emission spectra were recorded from 510 nm to 580 nm. The slit widths of excitation and emission were set for 5 nm and 5 nm, respectively. The fluorescence intensity at the emission wavelength of 520 nm was used for data analysis.

Selectivity and Inhibition Assay. The M.SssI MTase and HhaI MTase were selected as the interference enzymes to investigate the selectivity of the proposed method. The experiment was performed with 20 U interfere enzyme using same approach as the Dam assay. The fluorescence intensities of QF probes were recorded using the same approach described above.

To investigate the inhibition effect of gentamycin, various concentrations of gentamycin were incubated with the mixture of 1× Dam buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT, pH 7.5), and 0.5 μM hairpin probe at 37℃ for 15 min, followed by the addition of 160 μM SAM, 8 U Dam MTase and 10 U DpnI into the solution and incubation at 37℃ for 2 h and at 80℃ for 20 min. The fluorescence intensities of QF probes were used for calculating the relative activity of Dam MTase in the presence of inhibitors.

Gel Electrophoresis. The DpnI cleavage products and RNA amplification products were analyzed by non-denaturating polyacrylamide gel electrophoresis (PAGE). The electrophoresis was carried out in 0.5× TBE (4.5 mM Tris-HCl, 4.5 mM boric acid, 0.1 mM EDTA, pH 7.9) at 110 V for 45 min, and the gels were sliver-stained according to the manufacturer's procedure.

SUPPLEMENTARY RESULTS

Optimization of experimental condition. The cleavage of hairpin substrate by DpnI may generate a T7 primer. In this research, we first used a synthetic T7 primer to optimize the experimental conditions including the length and the concentration of QF probe, the amount of DSN and the incubation time. An ideal QF probe should not only generate a low background fluorescence signal but also keep the RNA/QF probe heteroduplexes stable under the reaction condition, thus the length of QF probe should be optimized carefully. Fig. S1A shows the variance

of F/F_0 value with the length of QF probe, where F and F_0 are the fluorescence intensity in the presence and in the absence of T7 primer, respectively. The F/F_0 value increases with the increase of QF probe length from 13 nt to 15 nt, followed by the decrease beyond the length of 15 nt. Therefore, the QF probe with a length of 15 nt is used in the subsequent experiments.

In theory, the higher the QF probe concentration, the higher the hybridization efficiency between the RNA products and the QF probes, and consequently the more the QF probes being cleaved by DSN and the greater the fluorescence signal. However, the high-concentration QF probes may result in a high background signal due to the presence of more unquenched fluorophores. On the other hand, although the low-concentration QF probes may reduce the background signal, it may adversely cause low hybridization efficiency and eventually a low fluorescence signal. Therefore, the concentration of QF probe should be optimized as well. Fig. S1B shows the variance of the F/F_0 value with the concentration of QF probe, where F and F_0 are the fluorescence intensity in the presence and in the absence of T7 primer, respectively. The F/F_0 value increases with the increase of QF probe concentration from 100 nM to 400 nM, followed by the decrease beyond the concentration of 400 nM. Thus, 400 nM QF probe is used in the subsequent research.

We further optimized the amount of DSN and the incubation time. As shown in Fig. S1C, the F/F_0 value increases with the increasing amount of DSN from 0.025 U to 0.1 U, followed by the decrease beyond the amount of 0.1 U (F and F_0 are the fluorescence intensity in the presence and in the absence of T7 primer, respectively). Although the high amounts of DSN enzyme may result in a high fluorescence signal, the background signal increases correspondingly. Based on Fig. S1B, we used 0.1 U as the optimal amount of DSN. Fig. S1D shows the F/F_0 value increases with the

increase of incubation time from 10 min to 30 min, and then levels off at 30 min (F and F_0 are the fluorescence intensity in the presence and in the absence of T7 primer, respectively). Thus, the incubation time of 30 min is used in the subsequent research.

In the T7 RNA polymerase-mediated transcription amplification, the ssDNA released from the hairpin substrate may hybridize with the hairpin template, enabling the open of stem structure and the formation of a double-stranded T7 promoter. The resultant double-stranded T7 promoter may be recognized by T7 RNA polymerase and subsequently transcribes large amounts of RNA strands. It should be noted that the stem length of hairpin template is crucial to the transcription reaction. When the stem length is too long, the stem of hairpin template is extremely stable and it cannot be opened by the released ssDNA. When the stem length is too short, the stem of hairpin template is unstable and it may induce nonspecific amplification. Therefore, the stem length of hairpin template should be optimized as well. To select the proper hairpin template, we investigated three hairpin templates with 7-bp stem (template-1), 8-bp stem (template-2), and 9-bp stem (template-3), respectively. As shown in Fig. S1E, the hairpin template-2 with 8-bp stem offers much higher F/F_0 value than the template-1 and the template-3, where F and F_0 are the fluorescence intensity in the presence and in the absence of T7 primer, respectively. Thus, the hairpin template-2 is used in the subsequent experiments.

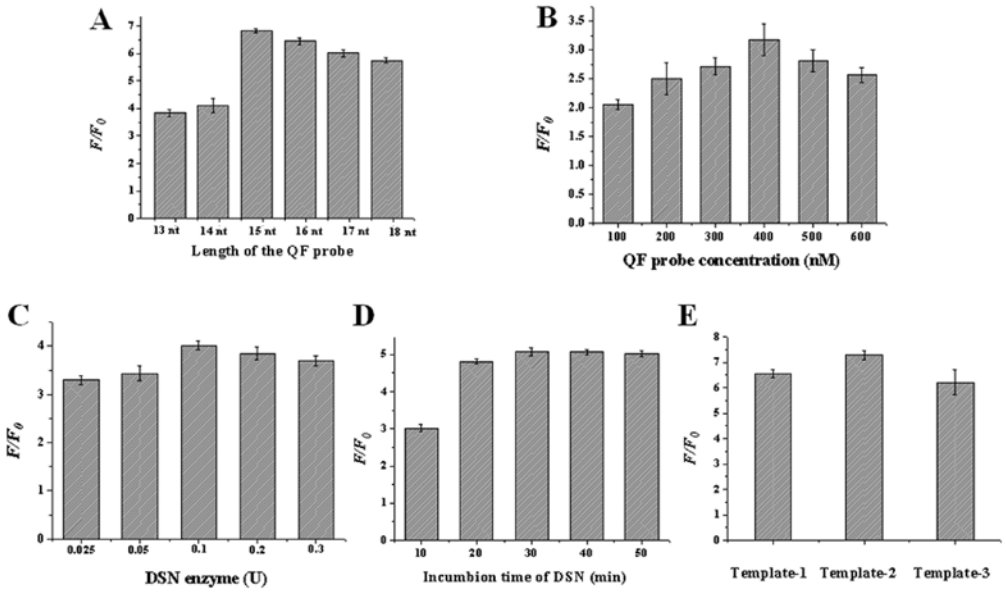

Fig. S1 (A) Variance of the F/F_0 value with the length of QF probe. (B) Variance of the F/F_0 value with the concentration of QF probe. (C) Variance of the F/F_0 value with the amount of DSN enzyme. (D) Variance of the F/F_0 value with the incubation time. (E) Variance of the F/F_0 value with the hairpin template which contains different stem length. F and F_0 are the fluorescence intensity in the presence and in the absence of 100 pM T7 primer, respectively. Error bars show the standard deviation of three experiments.

Effect of gentamycin on T7 RNA polymerase activity.

We performed gel electrophoresis analysis to investigate the effect of gentamycin on the activity of T7 RNA polymerase using a synthetic T7 primer (Fig. S2). In the inhibition assay, gentamycin was added in the T7 RNA polymerase-mediated transcription system. The RNA amplification reaction was performed in the mixture (20 μL) consisting of 1× T7 RNA polymerase buffer (40 mM Tis-HCl, 6 mM $MgCl_2$, 1 mM DTT, and 2 mM spermidine, pH 7.9), 10 U T7 RNA polymerase, 2 mM NTP mixture, 20 U RNase inhibitor, 50 μM gentamycin, 100 pM T7 primer and 50 nM hairpin template at 37 ℃ for 90 min. The transcription amplification reaction was

performed at 37°C for 90 min. The RNA amplification products were separated by non-denaturating polyacrylamide gel electrophoresis (PAGE). The electrophoresis was carried out in 0.5× TBE (4.5 mM Tris-HCl, 4.5 mM boric acid, 0.1 mM EDTA, pH 7.9) at 110V for 45min, and the gel was sliver-stained according to the manufacturer's procedure. As shown in Figure S1, a distinct band of transcription product (24 nt) is observed in lane 1 without the treatment of gentamycin and in lane 2 with the treatment of 50 μM gentamycin, respectively. These results show that gentamycin has no obvious effect on the activity of T7 RNA polymerase.

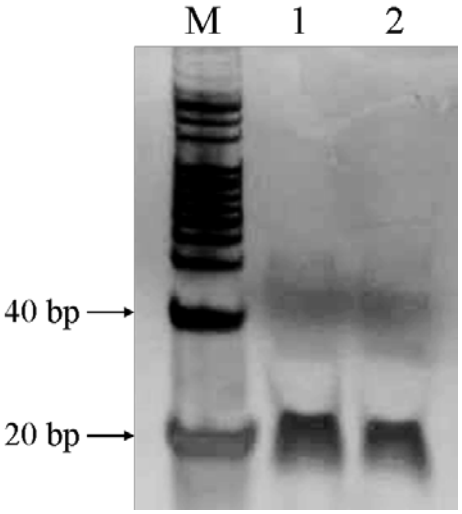

Fig. S2 Gel electrophoresis analysis of transcription amplification products by T7 RNA polymerase and the effect of gentamycin upon T7 RNA polymerase activity. M, marker; lane 1, T7 primer (100 pM) + hairpin template (50 nM) + T7 RNA polymerase (10 U); lane 2, T7 primer (100 pM) + hairpin template (50 nM) + T7 RNA polymerase (10 U) + gentamycin (50 μM).

Effect of gentamycin on DSN enzyme activity.

We performed fluorescence analysis to investigate the effect of gentamycin on the activity of DSN enzyme. The 20 μL of RNA transcription products was incubated with a solution containing 1× DSN master buffer (50 mM Tis-HCl, 5 mM $MgCl_2$, and 1 mM DTT, pH 8.0), 50 μM gentamycin,

0.1 U DSN enzyme and 400 nM QF probe in a final volume of 50 μL at 50℃ for 30 min, followed by cooling to room temperature. The fluorescence spectra were recorded by a Hitachi F-4500 fluorometer (Tokyo, Japan) equipped with a xenon lamp as the excitation source. As shown in Fig. S3, the fluorescence signal in the presence of 50 μM gentamycin shows no obvious difference from that in the absence of gentamycin. These results demonstrate that gentamycin exhibits no obvious effect on the activity of DSN enzyme.

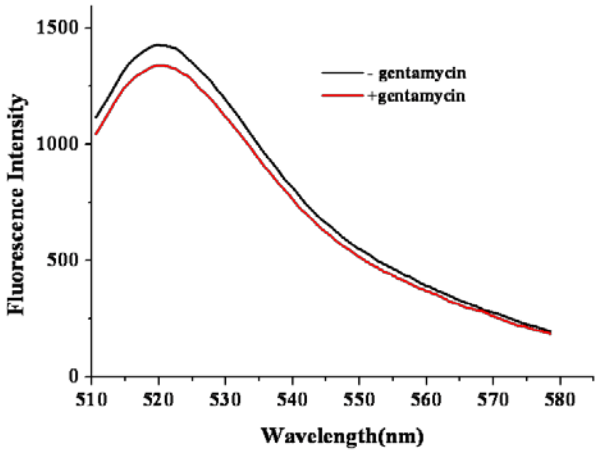

Fig. S3 The fluorescence spectra in the presence of 50 μM gentamycin (red line) and in the absence of gentamycin (black line).