Label-free Molecular Beacons-Based Quadratic Isothermal exponential Amplification: A simple and Sensitive One-Pot Method to Detection DNA Methyltransferase Activity

Qingwang Xue,[a,b] Lei Wang,[a*] Wei Jiang,[c*]

[a] *School of Pharmacy, Shandong University, Jinan 250012,P.R. China.*

[b] *Department of Chemistry, Liaocheng University, Liaocheng, 252059, Shandong, China.*

[c] *School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, P.R. China.*

Corresponding author:

Tel: +86-531-88363888; fax: +86-531-88564464.

Email: wangl-sdu@sdu.edu.cn; wjiang@sdu.edu.cn

Experimental

Chemicals

All the HPLC-purified oligonucleotide sequences were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and listed in Table 1: Protoporphyrin IX zinc (II) (ZnPPIX) was purchased from Sigma-Aldrich and used without further purification. Dam MTase, M.Sss I methyltransferase, HhaI methyltransferase and S-adenosylmethionine (SAM) were obtained from New England Biolabs Inc. Nicking endonuclease, Nb.BbvCI, and buffer were also purchased from New England BioLabs. Gentamycin, 5-fluorouracil and benzylpenicillin were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). The Bradford Protein Assay Kit was bought from Sangon Biotechnology Co., Ltd. (Shanghai, China). Other chemicals (analytical grade) were obtained from standard reagent suppliers. Water (≥ 18.2 MΩ) was used and sterilized throughout the experiments.

Table 1. Sequences of hairpin recognition probe(HRP), label-free molecular beacons(LFMB) and trigger signal primer(TSP) Used in the Experiment[a]

note	sequence (5'-3')
HRP_1	5'-T GG GA**G ATC** AAG GTC TGA C*TT TTT* GTC AGA CCT T**GA TC**T CCC A AC CTC AGC TAG C-3'
HRP_2	5'-GG TT GG GA**G ATC** AAG GTC TGA C*TT TTT* GTC AGA CCT T**GA TC**T CCC AAC CTC AGC TAG C-3'
HRP_3	5'-TGA GG TT GG GA**G ATC** AAG GTC TGA C*TT TTT* GTC AGA CCT T**GA TC**T CCC AAC CTC AGC TAG C-3'
LFMB	5'-**TCT CCC AAC CTC AGC TA***G CTG AGG* TTG GGT TGG GCG GGA TGG G-3'
TSP	5'- TCT CCC AAC CTC AGC TAG C-3'
N-TSP	**5'- TCT CCC AAC CTC AGC TA***G C***-3'**

[a] In the hairpin recognition probe (HRP), the recognition sites (GATC) of DAM and Dpn I is shown in bold, and the trigger signal primer (TSP) is shown in underline. In the label-free molecular beacons (LFMB), the nicking site is shown in the green portion, and the G-quadruplex sequence and the new trigger signal primer (N-TSP) is shown in the blue and boldface. The N-TSP is same as trigger signal primer (TSP).

Apparatus

All the fluorescence measurements were performed on a Hitachi F-7000 spectrofluorimeter (Hitachi, Japan). The excitation wavelength was 418 nm, and the spectra are recorded between 580 and 655 nm. The fluorescence emission intensity was measured at 592 nm.

Assay of DAM Activity

All of these standard solutions were prepared under 4 °C and stored at -20°C. The experiments were performed in 50 μL 1 × NEB buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) containing 50 nM HRP, 100 nM LFMB, 160 μM SAM, 8 U of Dpn I, 10 U of Nb.BbvCI, 20 μM ZnPPIX. After the addition of various concentrations of Dam MTase, the reaction buffer was performed at 37°C for 60 min prior to the fluorescence measurement. The fluorescence intensity of the mixture solution was measured in a 100 μL quartz cuvette at room temperature. The fluorescent spectra were measured using a spectrofluorophotometer. The excitation wavelength was 418 nm, and the spectra are recorded between 570 and 650 nm. The fluorescence emission intensity was measured at 592 nm. The control experiments were carried out under the same condition without adding Dam MTase. To achieve the best performance, the incubation time of Dam MTase was optimized.

Gel electrophoresis

Gel electrophoresis was used to confirm the feasibility of the methylation process of Dam MTase. Samples for gel electrophoresis assays were prepared as follows: (1) hairpin substrate and Dam MTase; (2) hairpin substrate and Dpn I; (3) hairpin substrate, Dam MTase, and Dpn I. The samples were put on a 12 % nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1×TBE (9 mM Tris-HCl (pH 7.9), 9 mM boric acid, 0.2 mM EDTA) with ethidum bromide (EB) staining. The gel was visualized under UV light and finally photographed with a digital camera.

Selectivity of the DAM Assay

To investigate the selectivity of the proposed Dam assay, two other methyltransferases, such as M.Sss I and HhaI, were selected as the potential interfering enzymes. The selectivity experiments were implemented with 5 and 50 U/mL of interfering enzymes in the same way as the Dam activity detection procedure.

Dam MTase inhibition evaluation

To further extend the potential application of this assay in the inhibition assay, the influence of drugs on Dam MTase activity was investigated. First, the influence of inhibitor, these drugs, on the activity of Dpn I and Nb.BbvCI was investigated. The methylation process was carried out in 25 μL 1 × NEBuffer 2 containing 100 nM HRP, 200 nM LFMB, 320 μM SAM, 40 μM ZnPPIX and 0.25 units Dam MTase at 37 °C for 60 min to ensure the absolute methylation. This 25 μL resultant mixture was added to another 25 μL 1 × NEB buffer 2 containing 8 units of Dpn I, 10 units of Nb.BbvCI and 2 μM different inhibitors. The 50 μL reaction mixture was performed at 37 °C for 60 min prior to the fluorescence measurement. Subsequently, the influence of drugs on the activity of Dam MTase was evaluated. All the inhibition experiments were carried out in conditions similar to those of Dam MTase activity assay except for 1 μM concentrations of different inhibitors in the samples. Briefly, before the addition of 0.25 unit of Dam MTase, different inhibitors were introduced into each sample. The solution was incubated at 37 °C for 60 min prior to the fluorescence measurement. To investigate the relationship between the concentration of 5-fluorouracil and the inhibition ratio, different 5-fluorouracil concentrations were added into these samples. The following procedures were similar as above.

Culture of Bacterial Cells

The DH5a (DAM positive) and JM110 (DAM negative) *E. coli cells* were cultured according to the procedure from the literature with slight modifications. Briefly, a colony was inoculated into 3 mL of liquid medium (5 g/L yeast extract, 10 g/L Trypton, 10 g/L NaCl) and incubated at 37 °C in a shaker (250 rpm) for 12 h.

Then, 500 μL of the cell suspension was subsequently added into 50 mL of medium and incubated for 2.5 h or 12 h. Subsequently, 3 mL of the cell suspension was centrifuged at 13000 rpm for 30 s to obtain a cell pellet followed by washing twice with Milli-Q water. The resulting *E. coli cells* were lysed using lysis buffer. The protein contents were determined using the Bradford Protein Assay Kit according to the manufacturer's recommended protocol.

Results and discussion

Optimization of assay conditions

One of the major challenges for the accurate detection of Dam MTase is the unspecific background noise. Thus, the stabilization of LFMB was also further investigated by gel electrophoresis experiments. As shown in **Fig. S1**, there is only two band of the original probe when only both label-free molecular beacons (LFMB) and hairpin recognition probe (HRP) is present (**Fig. S1, lane 4**), indicating that no hybridization event occurs. In other word, no nonspecific signal leakage occurs. The result was in agreement with the reported confirmatory experiment by fluorescence emission spectrum (**Fig. S1, cave a**). As a comparison, when only both trigger signal primer (TSP) and label-free molecular beacons (LFMB) are present, the anticipated high molecular weight of hybridization product can be observed (**Fig. S1, lane 3**), suggesting that a hybridization reaction happens. The above results demonstrated label-free molecular beacons (LFMB) is fairly stable and further improve the accuracy and sensitivity of detection.

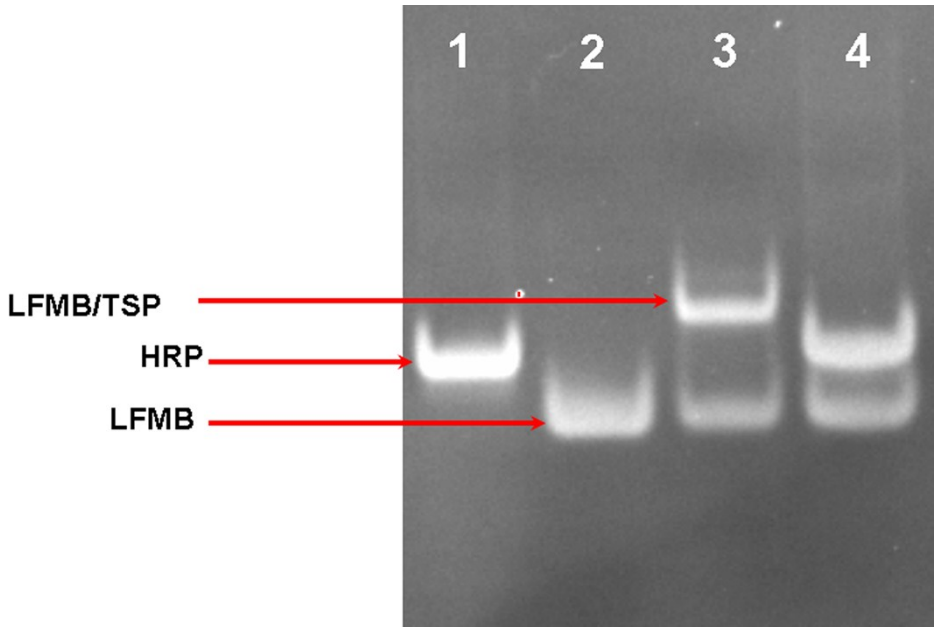


Fig. S1 Gel electrophoresis analysis of the stability of LFMB in the presence of (1) HRP, (2) LFMB, (3) TSP + LFMB, and (4) HRP + LFMB

Sequence Optimization of Hairpin Recognition Probe (HRP)

The key point of our proposed strategy was that, only when the hairpin recognition probe (HRP) was methylated by Dam MTase, it could be recognized and cleaved by Dpn I, which allows the release of a single-stranded oligodeoxynucleotide (ssODN). The ssODN as a trigger signal primer (TSP) then hybridizes to label-free molecular beacons (LFMB), subsequently triggering further LFMB-QIEA reaction. Therefore, the HRP was optimized in order to make sure that the TSP was preferentially released after the occurrence of methylation-induced cleavage, and the TSP hybridized to the LFMB only in the presence of the Dam MTase to specifically trigger the reaction. We compared fluorescence characteristics of three different HRPs by changing the length of the stem for selecting a proper probe to initiate further reaction. As shown in **Fig. S2**, the Dam MTase samples represented the fluorescence signals in the concentration of 5 U/mL Dam MTase. The control group contained all the components in the sample group except Dam MTase. The signal-to-background ratio was used to evaluate the assay performance; the highest signal-to-background ratio was observed for HRP_2. For this group, TSP of HRP perfectly matched the stem of HRP with 10 base-pairs, while HRP_3 matched with 7 base-pairs and HRP_1 matched

with 13 base-pairs. When the matched base-pairs of TSP in HRP were too long, the TSP is difficult to be separated into a independent single-stranded oligodeoxynucleotide after the methylation-induced cleavage reaction, impeding further reaction. when the matched base-pairs of TSP in HRP were only 7 base-pairs, the TSP could partially form double-stranded DNA with LFMB even without DAM methylation and Dpn I digestion which will cause high background signal. Thus, we chose HRP_2 as the substrate for further experiments.

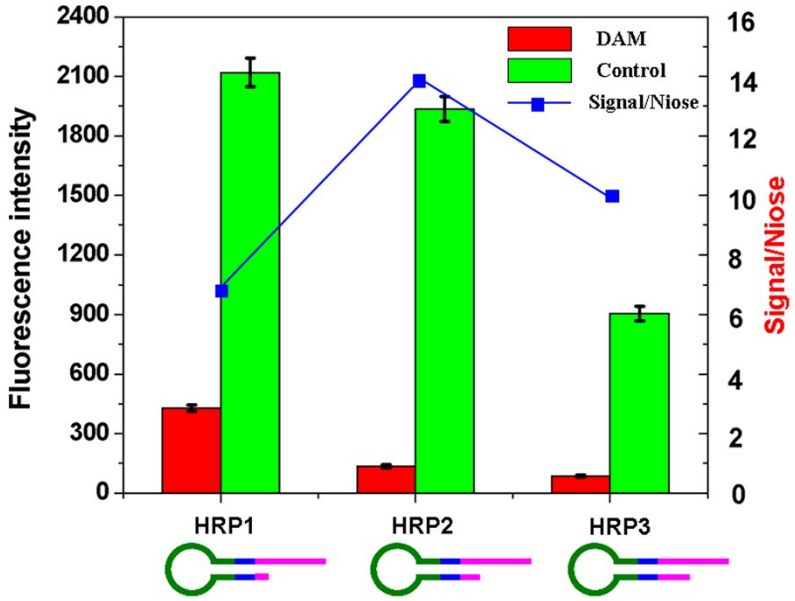


Fig. S2 Sequence optimization of hairpin recognition probe (HRP). The Dam MTase in the control groups was absent, but all the other compositions and reaction steps were the same as in the detection of DAM MTase sample. The concentration of the DAM MTase sample is 5 U/mL. The error bar was calculated from three independent experiments.

Optimization of the quadratic reaction time

One of the key factors that affects the assay performance of the proposed strategy (LFMB-QIEA) is the quadratic reaction time. In order to achieve optimal assay conditions, the quadratic reaction time was optimized. For this purpose, the effect of the reaction time on the signal output of the proposed method was investigated by monitoring the fluorescence intensity of the probe solution with the presence of Dam (5 U/mL), Nb.BbvCI (10 U), and Dpn I (8 U) at a time interval of 20

min from 0 to 100 min. As displayed in **Fig. S3A**, the fluorescence intensity of the mixture increases rapidly with increasing reaction time in the range from 0 to 60 min and reaches a plateau thereafter. The continuously increasing signal indicates that the designed method (LFMB-QIEA) was indeed taking place, while the signal saturation at 60 min suggests the quadruplex-forming oligomers were almost liberated from the HSP probe for the formation of fluorescent ZnPPIX/G-quadruplex supramolecular complex. To ensure complete quadratic reactions, the reaction time of 60 min was selected.

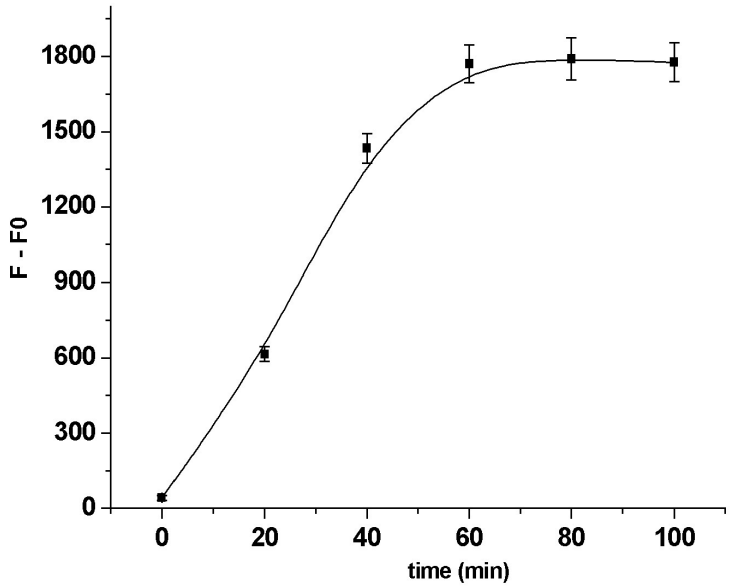
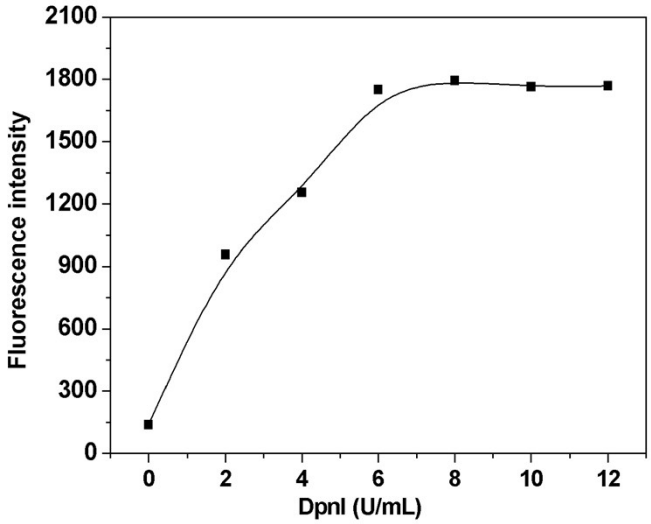


Fig. S3 Effect of the quadratic reaction time on the fluorescence intensity of the proposed quadratic amplification method for DAM detection. Reactions were performed at 37 °C.

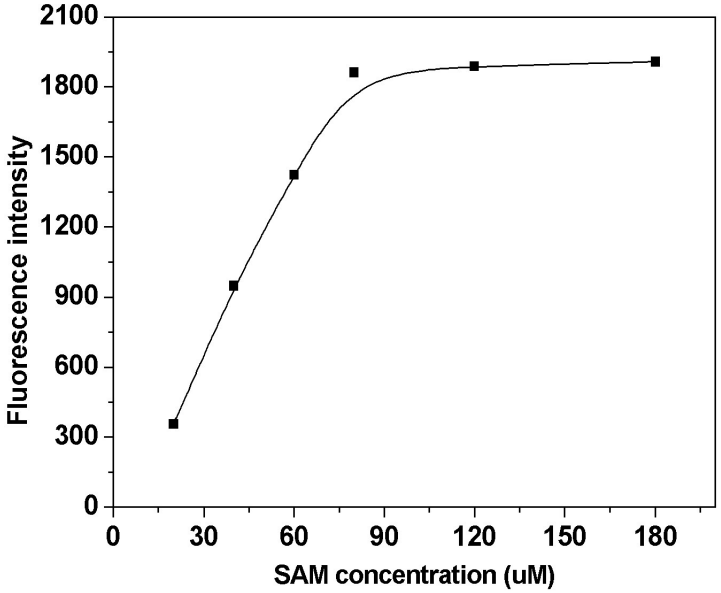
Effect of DpnI and SAM concentration

In the methylation and cleavage reation of hairpin recognition probe (HRP), the effects of the concentrations of DpnI and SAM were investigated, respectively. Dpn I endonuclease can only cut the sequence of 5'-G-Am-T-C-3' when the internal adenine is methylated. Therefore, it is important to investigate the effect of Dpn I concentration on the assay. As can be seen from **Fig. S4A**, with the increasing concentrations of DpnI, the fluorescence intensity increased and tended to a maximum at 8 unit. Thus, 8 unit of DpnI was chosen for the following experiments.

As the donor of methyl group, SAM is the critical factors in DNA methylation process catalyzed by Dam MTase. Therefore, it was necessary to optimize the concentration of SAM. **Fig. S4B** shows the effects of the concentration of SAM on the fluorescence signal. It could be seen that the fluorescence signal increased gradually with an increase in the concentration of SAM concentration, and then reached an equilibrium value at the concentration of 80 mM. However, considering that SAM is unstable in vitro experiments, a higher concentration of 160 mM was employed for the sensing system.



(A)

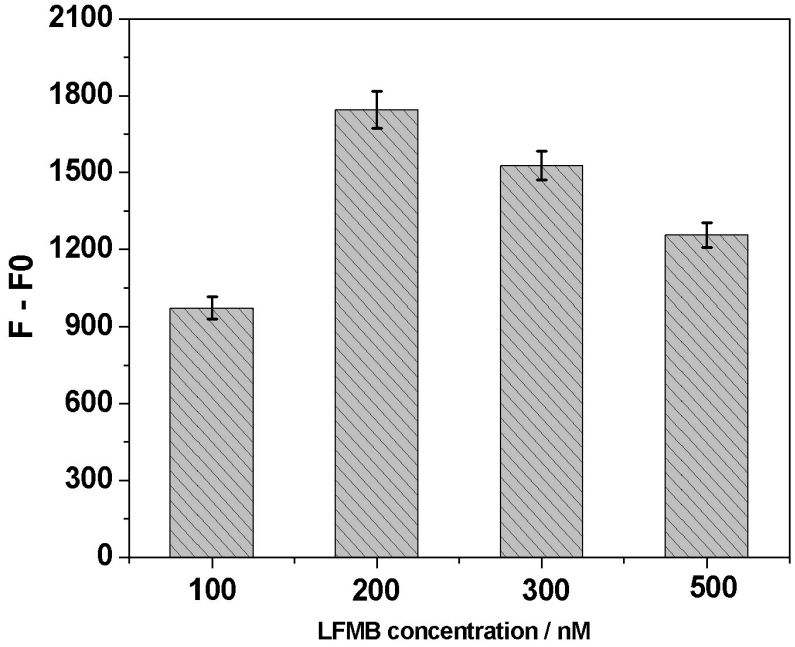


(B)

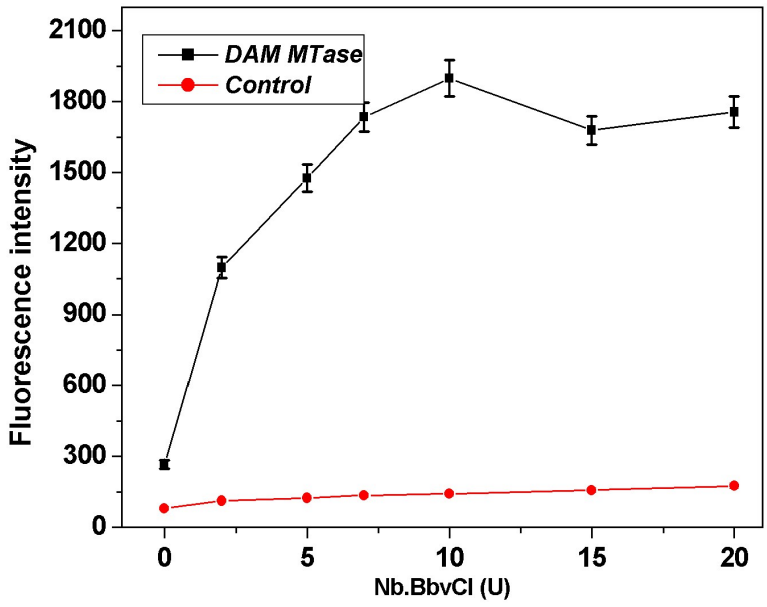
Fig. S4 The effect of (A) DpnI concentration and (B) SAM concentration on the fluorescence response of the sensing system.

Effect of the concentration of HSP and the amounts of Nb.BbvCI

In the sensing system, the cleaved HSP were used as the signal reporters. Detection signal was amplified through nicking enzyme cleavage with a exponential amplification. Therefore, the concentration of LFMB and the amount of Nb.BbvCI would have an important effect on the performance of the biosensing system. To obtain the best performance of the biosensing system, the concentration of LFMB and the amount of Nb.BbvCI were optimized in the presence of 5 U/mL Dam MTase, respectively. As shown in **Fig. S5A**, the fluorescence intensity increased with the increase HSP. When the concentration of LFMB reached 200 nM, the maximum signal was achieved. Thereafter, the fluorescence response exhibited a gradual decrease with a further increase LFMB. This was probably because a large excess of LFMB disturbed their hybridization with TSP and the LFMB-QIEA. As a result, 200 nM LFMB was selected for further investigation. We then evaluated the amount of Nb.BbvCI to the LFMB performance. As depicted in **Fig. S5B**, the fluorescence signal increased gradually with the increase of Nb.BbvCI amount as we expected. Unfortunately, the background fluorescence also slightly increased in the control groups. This unexpected phenomenon perhaps resulted from the competing hybridization between the LFMB and the HRP, which may cause the cleavage of LFMB in the absence of target. As we know, the rate of spontaneous interaction between hairpins can be reduced by increasing the stem length or decreasing the loop length. Thus, the HRP was elaborately designed with a very long stem and a short loop to reduce the background fluorescence. As can be seen, though the Dam MTase-induced fluorescence signal still followed an increasing trend with further increase of nicking enzyme, maximum net signal F - F0 value was observed for 10 units, where F and F0 are the fluorescence intensities of biosensing system in the presence and absence of Dam MTase, respectively. Thus, we chose 10 units of Nb.BbvCI for the following experiments.



(A)



(B)

Fig. S5 (A) Effect of the hairpin probe concentration on the fluorescence intensity of the proposed quadratic amplification method for Dam MTase detection. (B) Effect of the Nb.BbvCI amount on the fluorescence intensity of the proposed quadratic amplification method for DAM detection.

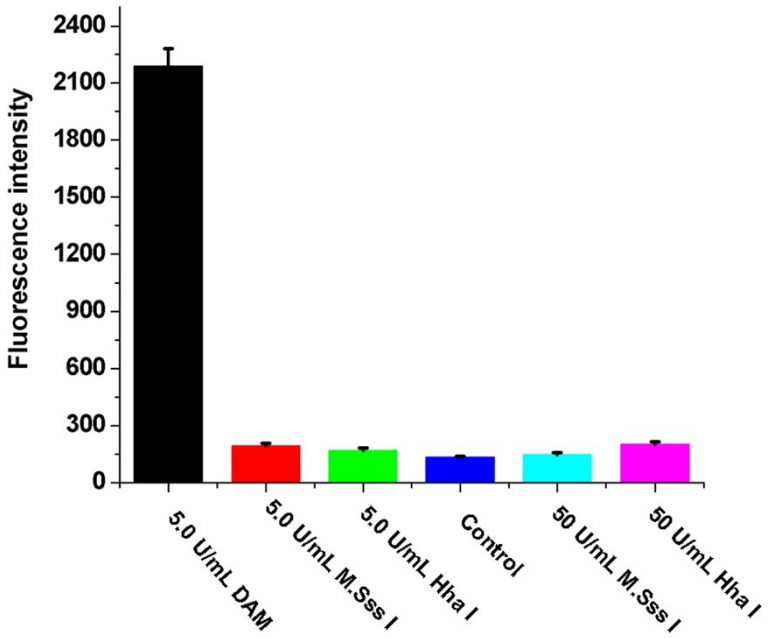
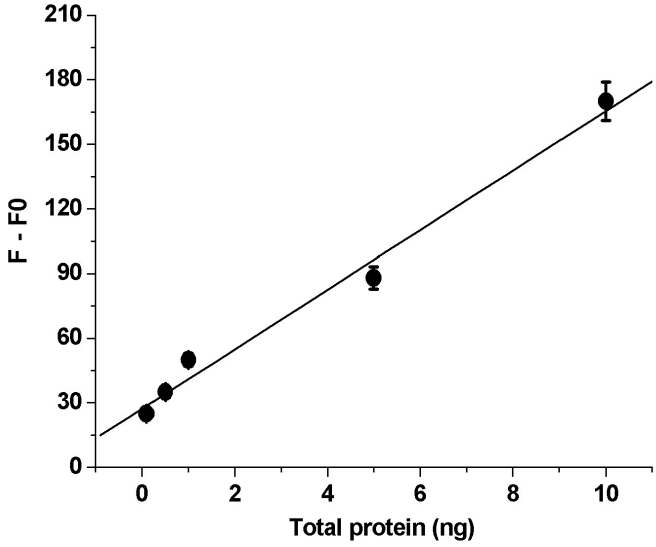
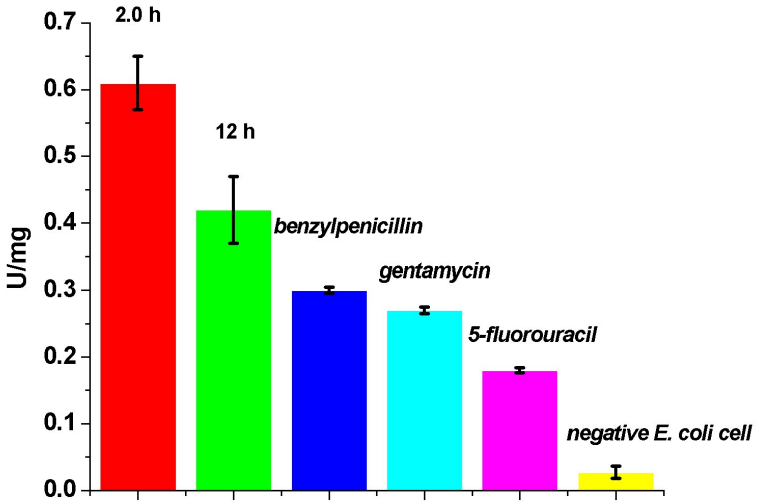


Fig. S6. Selectivity of the sensing system. Error bars show the standard deviation of three experiments.



(A)



(B)

Fig. S7 (A) Measurement of the activity of DAM in E. Coli cells *DH5a*. (B) The activity of Dam MTase at different growth stages of E. Coli cells *DH5a* and in DAM negative E. Coli cells *JM110*, and the inhibition of Dam MTase activity by gentamycin, benzylpenicillin and 5-fluorouracil. The concentration of each inhibitor is 1.0 μM.

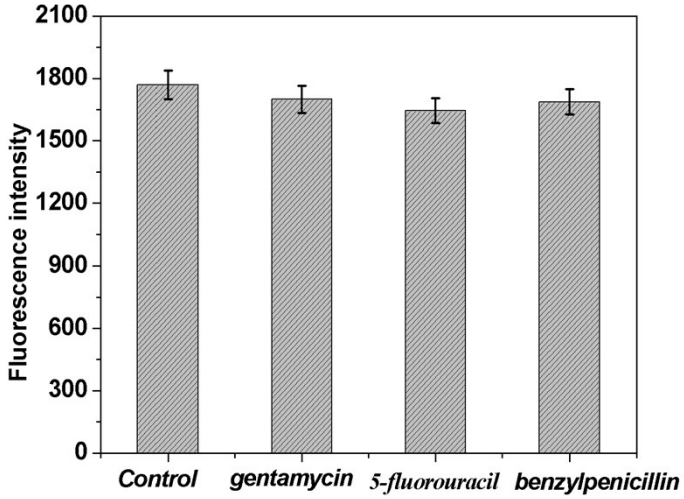
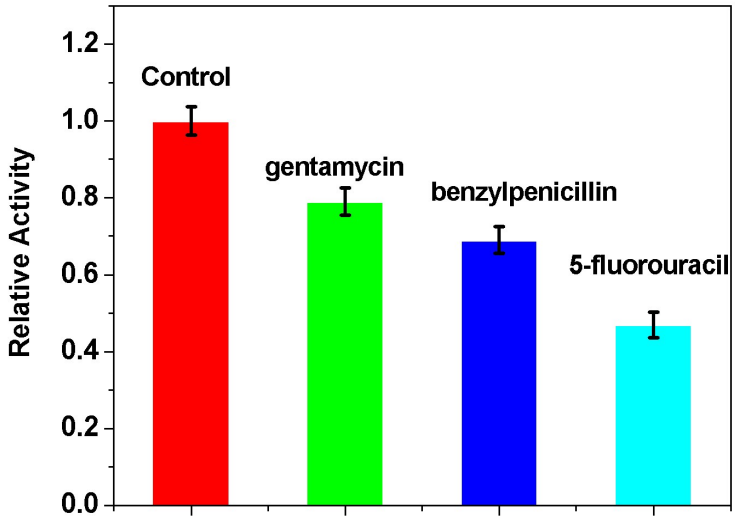
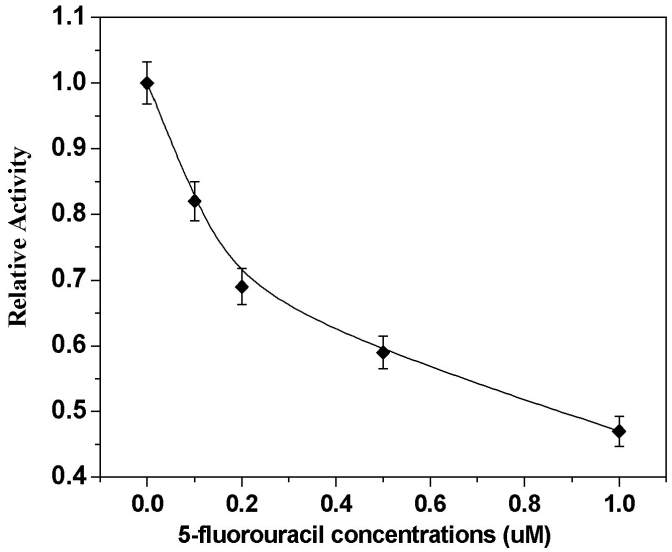


Fig. S8 Effect of different inhibitors on the activity of Dam MTase. the concentration of each inhibitor was 1.0 μM.



（A）



（B）

Fig. 9 (A) Effect of different inhibitors on the activity of Dam MTase. The concentrations of all these drugs are 1.0 μM. (B) The inhibitory effect of different concentrations of 5-fluorouracil on Dam MTase activity. The concentration of Dam MTase is 5 U/mL.

Table S2. Comparison of Different Signal Amplification-Based Dam MTase Assays

Method	*system*	*detection limit (U/mL)*	*detection time（min）*	**Biological Samples analyzed**
chemiluminescence	HCR-BRCA[1]	0.52	1895	NO
chemiluminescence	PG-RCA[2]	0.000129	120	NO
colorimetric	cross-linking Au nanoparticle aggregation[3]	2.5	1600	NO
electrochemical	AuNPs signal amplification[4]	0.12	1120	NO
electrochemical	SWCNTs signal amplification[5]	0.04	1340	NO
fluorescence	PG-EXPA[6]	0.000086	210	Human serum spiked with Dam
fluorescence	hairpin-structured fluorescent probe coupled with enzyme-linked reactions[7]	0.8	30	NO
fluorescence	Nicking enzyme-assisted signal amplification[8]	0.06	60	Human serum spiked with Dam
fluorescence	Exonuclease-Mediated Target Recycling[9]	0.01	60	E. coli cells
fluorescence polarization	Carbon nanotube signal amplification[10]	0.0001	125	Human serum spiked with Dam
electrochemical	Graphene Oxide signal amplification[11]	0.05 ± 0.02	2060	NO
colorimetric	SDA-assisted DNAzyme-based signal amplification[12]	0.25	155	NO
chemiluminescence resonance energy transfer (CRET)	exonuclease III assisted signal amplification[13]	0.007	1200	NO
fluorescence resonance energy transfer (FRET)	fluorescence quenching of graphene oxide with site-specific cleavage of a restriction endonuclease[14]	0.03 ± 0.01	420	NO
electrochemical	exonuclease III assisted cycling signal amplification[15]	0.004	400	NO
fluorescence	Hairpin Fluorescence Switch-Based Quadratic Isothermal Amplification (this work)	0.00015	60	E. coli cells

References

(1) Bi, S.; Zhao, T.; Luo, B.; Zhu, J. J. *Chem Commun*, 2031, *49*, 6906-6908.

(2) Zeng, Y. P.; Hu, J.; Long, Y.; Zhang, C. Y. *Anal Chem*, 2013, *85*, 6143-6150.

(3) Song, G.; Chen, C.; Ren, J.; Qu, X. ACS Nano 2009, 3, 1183-1189

(4) He, X.; Su, J.; Wang, Y.; Wang, K.; Ni, X.; Chen, Z. *Biosens Bioelectron*, 2011, *28*, 298-303.

(5) Wang, Y.; He, X.; Wang, K.; Su, J.; Chen, Z.; Yan, G.; Du, Y. *Biosens Bioelectron*, 2013, *41*, 238-243.

(6) Xue, Q.; Lv, Y.; Xu, S.; Zhang, Y.; Wang, L.; Li, R.; Yue, Q.; Li, H.; Gu, X.; Zhang, S.; Liu, J. *Biosens. Bioelectron.* 2015, 66, 547-553.

(7) Li, J.; Yan, H.; Wang, K.; Tan, W.; Zhou, X. *Anal. Chem* 2007, 79, 1050-1056.

(8) Zhao, Y.; Chen, F.; Wu, Y.; Dong, Y.; Fan, C. *Biosens. Bioelectron.* 2013, 42, 56-61.

(9) Xing, X. W.; Tang, F.; Wu, J.; Chu, J. M.; Feng, Y. Q.; Zhou, X.; Yuan, B. F. *Anal. Chem.* 2014, 86, 11269-11274.

(10) Huang, Y.; Shi, M.; Zhao, L. M.; Zhao, S. L.; Hu, K.; Chen, Z. F.; Chen, J.; Liang, H.; *Biosens. Bioelectron.* 2014, 54, 285-291.

(11) Li, W.; Wu, P.; Zhang, H.; Cai, C. X *Anal. Chem.* 2012, 84, 7583-7590.

(12) Li, W.; Liu, Z.; Lin, H.; Nie, Z.; Chen, J.; Xu, X.; Yao, S. *Anal. Chem.* 2010, 82, 1935-1941.

(13) Chen, C.; Li, B. *Biosens. Bioelectron.* 2013, 54, 48-54.

(14) Ji, L. J.; Cai, Z. W.; Qian, Y. D.; Wu, P.; Zhang, H.; Cai, C. X. *Chem Commun*, 2014, *50*, 1691-1694.

(15) Li, W.; Liu, X.; Hou, T.; Li, H.; Li, F. *Biosens. Bioelectron.* 2015, 70, 304-309.