



Highly sensitive fluorescence assay of DNA methyltransferase activity via methylation-sensitive cleavage coupled with nicking enzyme-assisted signal amplification



Yongxi Zhao^{a,*}, Feng Chen^a, Yayan Wu^a, Yanhua Dong^a, Chunhai Fan^b

^a Key Laboratory of Biomedical Information Engineering of Education Ministry, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shaanxi 710049, PR China

^b Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, PR China

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ABSTRACT

Herein, using DNA adenine methylation (Dam) methyltransferase (MTase) as a model analyte, a simple, rapid, and highly sensitive fluorescence sensing platform for monitoring the activity and inhibition of DNA MTase was developed on the basis of methylation-sensitive cleavage and nicking enzyme-assisted signal amplification. In the presence of Dam MTase, an elaborately designed hairpin probe was methylated. With the help of methylation-sensitive restriction endonuclease DpnI, the methylated hairpin probe could be cleaved to release a single-stranded DNA (ssDNA). Subsequently, this released ssDNA would hybridize with the molecular beacon (MB) to open its hairpin structure, resulting in the restoration of fluorescence signal as well as formation of the double-stranded recognition site for nicking enzyme Nt.BbvCI. Eventually, an amplified fluorescence signal was observed through the enzymatic recycling cleavage of MBs. Based on this unique strategy, a very low detection limit down to 0.06 U/mL was achieved within a short assay time (60 min) in one step, which is superior to those of most existing approaches. Owing to the specific site recognition of MTase toward its substrate, the proposed sensing system was able to readily discriminate Dam MTase from other MTase such as M.SssI and even detect the target in complex biological matrix. Furthermore, the application of the proposed sensing strategy for screening Dam MTase inhibitors was also demonstrated with satisfactory results. This novel method not only provides a promising platform for monitoring activity and inhibition of DNA MTases, but also shows great potentials in biological process researches, drugs discovery and clinical diagnostics.

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1. Introduction

DNA methylation, a major epigenetic modification, plays a significant regulatory role in both prokaryotes and eukaryotes (Heithoff et al., 1999; Robertson, 2005). In bacteria, DNA methylation affects a variety of cell functions such as gene expression, DNA replication and repair, chromosome segregation and the control of cell cycle (Garcia-Del Portillo et al., 1999; Heithoff et al., 1999; Low et al., 2001; Reisenauer et al., 1999). It has been well documented that the DNA methylation process is catalyzed by DNA methyltransferase (MTase) which can transfer a methyl group from S-adenosyl methionine (SAM) to target adenine or cytosine residues in the recognition sequences (Cheng and Roberts, 2001). Numerous studies have proved that the level of DNA MTase in bacteria is closely associated with restriction-modification systems, DNA

mutation rates, cell invasion, cytotoxicity, protective immune response and bacterial diseases such as salmonellosis (Boye et al., 1992; Duegera et al., 2003; Erova et al., 2006; Garcia-Del Portillo et al., 1999; Julio et al., 2001). Therefore, the development of novel approaches for screening DNA MTase activity and its inhibitors is not only valuable for fundamental biochemical research, but also of great significance in drug discovery.

So far, several traditional methods, including radioactive labeling, gel electrophoresis, high performance liquid chromatography and immune reaction (Adams et al., 1991; Bergerat et al., 1991; Boye et al., 1992; Messer and Noyer-Weidner, 1988), have been reported for the estimation of DNA MTase activity. Unfortunately, these approaches are time-intensive, DNA-consuming, laborious, complicated, inefficient, costly or radioactive substance-required. These limitations prevent the wide applications of these methods. In recent years, a lot of efforts have been focused on DNA MTase activity assay to overcome the above drawbacks. For example, using methylation-responsive DNA-gold nanoparticles (AuNPs) assembly and methylation-triggered

* Corresponding author. Tel./fax: +86 29 82663454.
E-mail address: yxzha@mail.xjtu.edu.cn (Y. Zhao).

DNAzyme-based strand displacement amplification (SDA), the colorimetric approaches have been developed to assay DNA MTase activity (Li et al., 2010; Liu et al., 2010; Song et al., 2009). However, in addition to relatively high detection limit, the sensing platforms also suffer from time-consuming DNA conjugation on AuNPs or polymerization process. Very recently, the electrochemical and electrogenerated chemiluminescence (ECL) biosensors have been described for DNA MTase activity assay with improved detection limits even down to 0.02 U/mL level (He et al., 2011; Li et al., 2012; Liu et al., 2011; Su et al., 2012; Wu et al., 2012). In addition, a novel bioluminescence assay based on protein expression in vitro has been reported for monitoring DNA MTase activity (Jiang et al., 2012). Despite the attractive sensitivity and other advantages, their performances have been compromised by cumbersome multistep processes and lengthy assay times (over 173 min). Owing to the advantages of simplicity, real-time detection, high-throughput ability and ease of automation, a fluorescence sensing system has been constructed, which enables the detection of DNA MTase activity with a poor detection limit within 30 min (Li et al., 2007). In order to further improve the sensitivity, Wang's group and Yang's group have proposed the fluorescence assays to detect DNA MTase activity using cationic conjugated polymer/DNA complexes and a DNA intercalator dye coupled with an endonuclease and carbon nanomaterials (CNPs), respectively (Feng et al., 2007; Ouyang et al., 2012). Unfortunately, the lengthy assay time, and/or complicated synthesis processes of CNPs and conjugated polymer limits the practical applications of the highly sensitive approaches. Therefore, it is still a continuous demand to develop a simple, rapid, and highly sensitive fluorescence sensing statatey for the detection of DNA MTase activity.

As a specific type of endonuclease that recognize a particular sequence in double-stranded DNA (dsDNA) and cleave only one strand at the specific site (Chan et al., 2011; Higgins et al., 2001; Morgan et al., 2000), DNA nicking enzyme have attracted considerable research efforts due to their excellent capability in mediating signal amplification in recent years. And some novel methods based on nicking enzyme-assisted amplification strategy have been successfully applied for the highly sensitive detection of nucleic acids (Gerasimova et al., 2010; Kiesling et al., 2007; Li et al., 2008; Xu et al., 2009; Xu et al., 2011; Yan et al., 2011), potassium (Zhu et al., 2011), proteins (Xue et al., 2012) and small molecules (Li et al., 2012). The whole reaction is carried out in the isothermal condition without the requirement of specialized instruments, which makes it more favorable for point-of-care analysis. Moreover, in comparison with polymerase-based isothermal amplification approaches such as SDA and rolling circle amplification (RCA) (Cheng et al., 2010; He et al., 2010; Lee et al., 2009; Li et al., 2010; Zhao et al., 2012; Zhou et al., 2010), which suffer from complex operation processes, high cost, and the false-positive results caused by the cross-contamination from amplicons, the nicking enzyme-assisted amplification strategy may provide a simpler, faster and cost-effective platform for constructing the highly sensitive biosensor. However, as far as we know, there are no reports available for screening enzyme activity and its inhibitors by this novel amplification strategy.

Herein, using DNA adenine methylation (Dam) MTase, a representative enzyme in bacteria, as a model target, we developed a simple method for rapid, and highly sensitive screening of its activity and inhibitors on the basis of methylation-sensitive cleavage coupled with nicking enzyme-assisted signal amplification. The analysis of Dam MTase activity was performed in a one-step reaction, and the detection limit was significantly improved to be 0.06 U/mL within a 60 min assay time. The selective discrimination of Dam from other MTase such as M.SssI and its application in Luria–Bertani (LB) medium were also

demonstrated. Furthermore, the proposed method was successfully extended to study the influence of some drugs on the activity of Dam MTase.

2. Experimental

2.1. Reagents and materials

The Dam MTase (*Escherichia coli*), DpnI, M.SssI, SAM, Nt.BbvCI, and the corresponding buffer solution were purchased from New England Biolabs Ltd (Beijing, China). All other chemicals were of analytical grade and were used without further purification. Deionized water was obtained through a Milli-Q system (Millipore, Bedford, MA, USA).

The synthetic oligonucleotides were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of oligonucleotides used in this work were as follows:

hairpin probe:

5'-CACGCTGAGGACGATCCAAGAGTTTTCTCTGGATCGTCTCA-3';

molecular beacon:

5'-(FAM)-CCACGAGTCAGTGTCCTCAGCGTGG-(DABCYL)-3'.

The underlined letters in both hairpin probe and molecular beacon indicate the specific recognition sequence for Nt.BbvCI; the green portion in hairpin probe is the methylation sequence for Dam MTase.

2.2. Apparatus

All fluorescence measurements of samples were carried out on FluoroMax-4 fluorescence spectrometer (Horiba Jobin Yvon). The emission spectra were collected from 510 nm to 600 nm with an excitation wavelength of 495 nm at room temperature. The fluorescence intensity at 518 nm was chosen as the optimal experimental conditions to evaluate the performance of the proposed sensing system. Both the excitation and emission slit widths were set at 5 nm.

2.3. Assay of Dam MTase activity

The experiments were performed in 20 μ L 1 \times NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol) containing 50 nM hairpin probe, 250 nM MB, 160 μ M SAM, 1 unit of DpnI and 1.5 units of Nt.BbvCI. After the addition of various concentrations of Dam MTase, the solutions were incubated at 37 °C for 60 min prior to the fluorescence measurement.

LB medium samples containing different concentrations of Dam MTase were assayed by this sensing system to demonstrate the application of this method in complex biological sample. Additionally, we examined the selectivity of the proposed assay by employing M.SssI MTase as an interference enzyme.

2.4. Influence of some drugs on Dam MTase activity

Except Dam MTase, there were another two enzymes involved in this reaction system. Thus, it was of great demand to investigate the influence of drugs on both DpnI and Nt.BbvCI. First, the methylation process was carried out in 10 μ L 1 \times NEBuffer 2 containing 100 nM hairpin probe, 500 nM MB, 160 μ M SAM and 0.8 unit of Dam MTase at 37 °C for 30 min, and then at 65 °C for 20 min to inactivate Dam MTase. This 10 μ L resultant mixture was added to another 10 μ L 1 \times NEBuffer 2 containing 1 unit of DpnI endonuclease, 1.5 units of Nt.BbvCI and 2 μ M different

inhibitors. The 20 μL reaction mixture was performed at 37 $^{\circ}\text{C}$ for 60 min before fluorescence was measured.

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 0.8 unit of Dam MTase, different inhibitors were introduced into each sample. The solution was incubated at 37 $^{\circ}\text{C}$ 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.

3. Results and discussion

3.1. Methylation-dependent sensing mechanism

The mechanism of our sensing system is depicted in Scheme 1. As can be seen, the hairpin probe with a long stem is designed to include two main components (Scheme 1a). The green portion is the 5'-G-A-T-C-3' methylation sequence for Dam MTase. And the red portion, containing the nicking sequence for Nt.BbvCI, is a 12-mer fragment, which is complementary to a part of the MB. In order to inhibit the competing hybridization, the 5' overhang in the 12-mer fragment is designed to partially hybridize with the stem of MB instead of the loop, leading to a low fluorescence background. In the presence of Dam MTase, the methylation reaction is catalyzed to generate the methylated duplex DNA (5'-G-Am-T-C-3'), acting as the substrate for DpnI. Subsequently, the cleavage reaction mediated by DpnI is initiated to cut this methylated substrate into two portions (Scheme 1b). One of them is a new short hairpin DNA (the blue and green portion). The other one is a 9-bp hybrid duplex with a 5' overhang. However, this duplex is unstable at the reaction temperature (37 $^{\circ}\text{C}$) due to its low T_m value about 28 $^{\circ}\text{C}$. Therefore, this duplex will be separated into two independent ssDNA fragments, one of which is a 14-mer ssDNA consisting of the 12-mer fragment and the other two nucleotides (the red and green portion). Subsequently, this separated 14-mer ssDNA can hybridize with the MB, resulting in the restoration of the fluorescence signal and formation of the double-stranded recognition site for Nt.BbvCI. Once the MB is

nicked by Nt.BbvCI, it will be dissociated from the sensing system, and the fluorescence signal is further enhanced because of the spontaneous separation of FAM and DABCYL. The released 14-mer ssDNA then hybridizes with another MB and triggers the second cycle of cleavage. Eventually, per Dam MTase-mediated methylation event can result in the recycling cleavage of many MBs, affording a dramatically amplified fluorescence signal.

Oppositely, in the absence of Dam MTase, there is no methylation at the specific sequence 5'-G-A-T-C-3', and the hairpin probe cannot be cut off by DpnI. Due to their own strong intramolecular hybridization, the intact hairpin probe cannot hybridize with the MB. Thereby, no remarkable fluorescence signal is observed.

3.2. Feasibility of the amplified sensing system

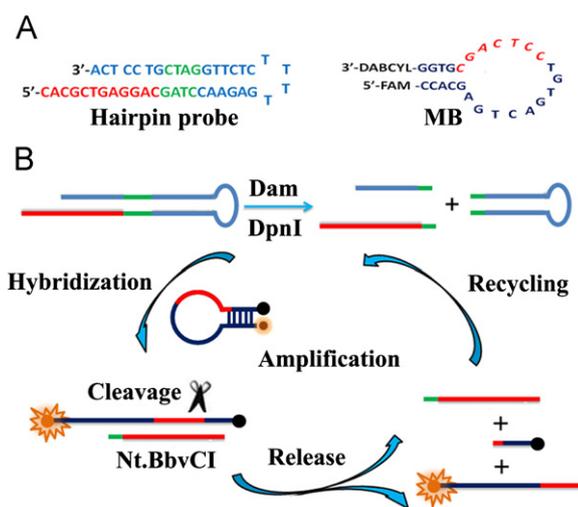
In order to estimate the amplification efficiency of the proposed sensing system, Dam MTase-triggered fluorescence response in the presence and absence of Nt.BbvCI were recorded, respectively. As shown in Fig. 1, a slightly increased background fluorescence in the presence of Nt.BbvCI was observed (curve c), which was attributed to the partially hybridization of hairpin probe with MB as well as subsequent cleavage of some MBs in the absence of Dam MTase. However, the methylation-triggered signal response (curve a) was much larger than the background fluorescence, assuring a high sensitivity for the detection of Dam MTase activity. By introducing nicking enzyme, a $(483 \pm 22)\%$ signal increase upon addition of 10 U/mL Dam MTase was achieved. In contrast, in the absence of Nt.BbvCI, the corresponding signal enhancement was only $(110 \pm 10)\%$. All these results indicated that the proposed sensing strategy could provide a significantly amplified signal for the detection of Dam MTase activity by nicking enzyme-assisted signal amplification.

3.3. Optimization of assay conditions

3.3.1. Effect of DpnI and SAM concentration

To obtain the best analytical performance, the effects of the concentrations of DpnI and SAM were investigated, respectively. With the increasing concentrations of DpnI, the fluorescence intensity increased and tended to a maximum at 1 unit (Fig. S1A). Thus, 1 unit of DpnI was chosen for the following experiments.

As the donor of methyl group, SAM plays an important role in DNA methylation process catalyzed by Dam MTase. Its concentration was optimized and the result is shown in Fig. S1B. It could be seen that the fluorescence signal increased gradually as the SAM concentration increased, and then reached an equilibrium value at the concentration of 80 μM . However, considering that



Scheme 1. (A) The sequences of the designed hairpin probe and MB labeled FAM and DABCYL and (B) Schematic illustration of methylation-dependent sensing system for the detection of Dam MTase activity by using methylation-sensitive cleavage coupled with nicking enzyme-assisted signal amplification.

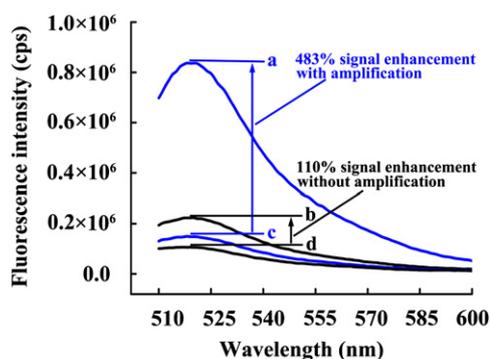


Fig. 1. The fluorescence responses of the sensing system to 10 U/mL of Dam MTase in the presence (a) and absence (b) of nicking enzyme, with corresponding backgrounds (c and d), respectively.

SAM is unstable in vitro experiments, a higher concentration of 160 μM was employed for the sensing system.

3.3.2. Effect of nicking enzyme concentration and molar ratio of hairpin probe to MB

Performance of the proposed sensing system is still strongly influenced by other assay conditions such as nicking enzyme concentration and the molar ratio of hairpin probe to MB. Therefore, these conditions were subsequently optimized. As depicted in Fig. 2A, the fluorescence signal increased gradually with the increase of Nt.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 MB and the hairpin probe, which may cause the cleavage of MBs 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 hairpin probe 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, the highest F/F_0 value was observed for 1.5 units. Thus, we chose 1.5 units of Nt.BbvCI for the following experiments.

Due to the competing hybridization between the MB and hairpin probe as described above, the molar ratio of hairpin probe to MB could also significantly affect the F/F_0 value. Thus, the effect of molar ratio was further investigated with a fixed concentration of MB (250 nM) and various hairpin probe concentrations from 25 nM to 125 nM, the result of which is depicted in Fig. 2B. It was observed that the fluorescence response was quickly enhanced as the hairpin probe concentration increased. However, increasing concentrations of hairpin probe also resulted in the increase of background fluorescence intensity. Finally, owing to the highest F/F_0 value, the molar ratio of 1:5 was selected for the subsequent investigations.

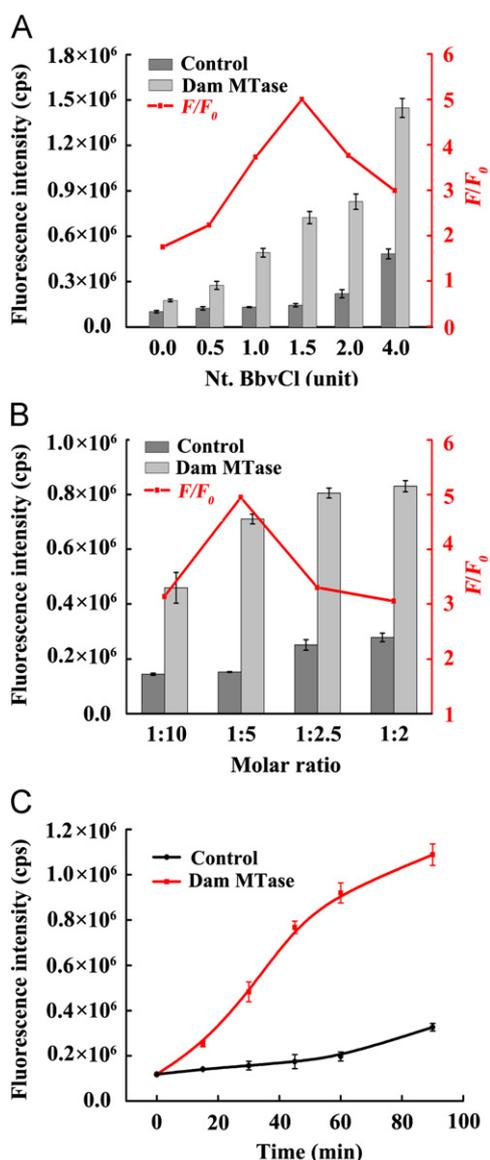


Fig. 2. Optimization of the experimental conditions. The effect of (A) the nicking enzyme concentration, (B) the molar ratio of hairpin probe to MB on the fluorescence response of the sensing system; (C) The time-dependent fluorescence response over background fluorescence with 10 U/mL Dam MTase. The error bars indicated the standard deviations of three independent experiments. F_0 and F are the fluorescence intensity in the absence and presence of target, respectively.

3.3.3. Effect of reaction time

The reaction time is another important factor for excellent sensing systems. In order to obtain optimized assay time, the fluorescence intensity of different reaction time in the presence and absence of Dam MTase were measured, respectively. As presented in Fig. 2C, when the reaction time was prolonged, the fluorescence response to Dam MTase increased quickly (the red line). Nevertheless, in the absence of Dam MTase, the control group also showed a slight increase in background fluorescence intensity (the black one). Although the fluorescence signal of 90 min was the highest, its noise was also much higher than those of other time. Finally, 60 min was selected as the optimized reaction time due to the maximum F/F_0 . As far as we know, the assay time of this method is very short compared with most existing approaches which are ranging from 120 min to 420 min (He et al., 2011; Jiang et al., 2012; Li et al., 2010; Ouyang et al., 2012; Su et al., 2012; Wu et al., 2012). And the details are listed in Table S1.

3.4. Analytical performance of the proposed sensing platform

Under the optimized conditions, the sensitivity of the proposed strategy was investigated by fluorimetric titration. Fig. 3A presents the fluorescence emission spectra of the sensing system upon the addition of different Dam MTase concentrations. As can be seen, the fluorescence signals gradually increased as the concentrations of Dam MTase varied from 0 to 40 U/mL, indicating that more and more MBs were cleaved by nicking enzyme. The corresponding calibration curve is depicted in Fig. 3B. The inset of Fig. 3B clearly shows that the fluorescence intensity was proportional to Dam MTase concentration in the range of 0.1–4 U/mL. This linear relationship can be described as $F = 1.127C + 1.504$ with a correlation coefficient of $R^2 = 0.994$, where C is the concentration of Dam MTase. And an extremely low detection limit of 0.06 U/mL was estimated in terms of the rule of 3 times standard deviation over the blank response. In contrast, experiments in the absence of Nt.BbvCI were also carried out under the same conditions, which obtained a detection limit of only 0.4 U/mL (Fig. S2). It is much poorer than that of the developed signal amplification strategy, indicating that the introduction of nicking enzyme could remarkably improve the sensitivity by the recycling cleavage of MBs. To the best of our knowledge, this detection limit of the proposed approach is much lower than those of most existing methods, and is even comparable to the lowest one (0.04 U/mL) of two electrochemical biosensors

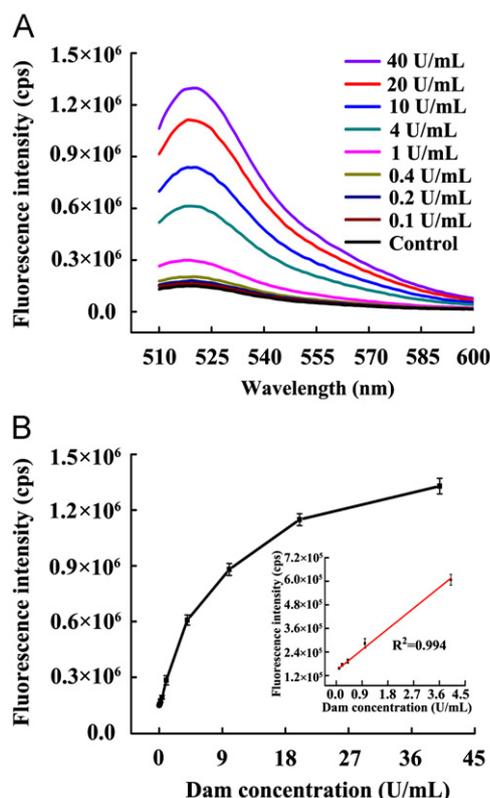


Fig. 3. Sensitivity of the amplified fluorescence sensing system for Dam MTase. (A) Fluorescence emission spectra in the presence of different concentrations of Dam MTase ranging from 0 to 40 U/mL. (B) The relationship of the fluorescence intensity with various Dam MTase concentrations. The inset displays the linear correlation between fluorescence intensity and low Dam MTase concentrations in the range of 0.1–4 U/mL.

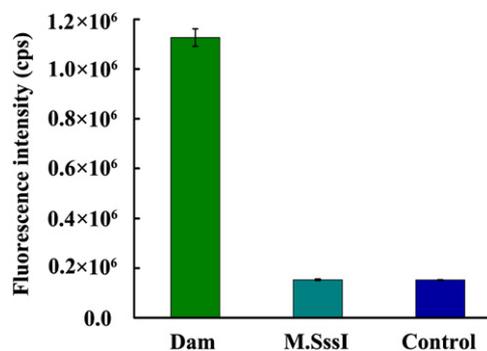


Fig. 4. Selectivity of the sensing system. Both the concentrations of Dam MTase and M.SssI are 20 U/mL.

(Wang et al., 2012; Wu et al., 2012). The details of the comparisons are summarized in Table S1.

Besides the sensing sensitivity, selectivity is another vital factor to assess the performance of a sensing system. Thus, M.SssI MTase, an enzyme methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5'...CG...3', was selected as an interference enzyme to estimate the selectivity of this proposed method (Fig. 4). As expected, there was no conspicuous fluorescence enhancement upon the addition of M.SssI. However, the same concentration of target Dam MTase induced a remarkable increase in fluorescence intensity. These results demonstrated that the amplified strategy for Dam MTase activity exhibited a good selectivity originating from the high specific sequence recognition between Dam MTase and the hairpin probe.

3.5. Analysis of complex biological samples

In order to further evaluate the practical applicability of the proposed sensing system, LB medium samples (diluted in 1:20 ratio with NEBuffer 2) spiked with 1 U/mL and 2.5 U/mL Dam MTase were analyzed under the optimized conditions and the result is given in Table S2. The recoveries were 91.67% and 98.53% for triplicate measurements, respectively. And the maximum value of relative standard deviation (RSD) was about 7%. These results indicated that the proposed strategy held great potential for the accurate quantification of Dam MTase in complex biological matrixes.

3.6. Dam MTase activity inhibition evaluation

Since Dam MTase is associated with the level of bacterial virulence and diseases, the inhibition of Dam MTase activity may contribute to important applications in antibiotic development. Therefore, the capacity of the proposed sensor to detect the inhibition of target was also investigated by using several antibiotics and anticancer drugs as model inhibitors. Considering that another two enzymes DpnI and Nt.BbvCI were involved in this sensing system, it was essential to ensure whether these drugs have influence on them. Control experiments were carried out as described in experimental procedures. The result indicated that all the inhibitors (gentamycin, 5-fluorouracil, benzylpenicillin and ampicillin) exhibited negligible influence on the activity of both DpnI and Nt.BbvCI when the concentration of each inhibitor was 1.0 μ M (Fig. S3). As a result, 1.0 μ M of different inhibitors was used to estimate the influence on Dam MTase activity. As presented in Fig. 5A, all drugs could inhibit the activity of Dam MTase. In comparison with other inhibitors, the 5-fluorouracil, a well-known broad spectrum anticancer drug, exhibited the strongest inhibitory effect at the same concentration, causing about 52% reduction of Dam MTase activity.

Moreover, we further investigated the concentration-dependant inhibitory effect of 5-fluorouracil on the activity of Dam MTase. It is observed in Fig. 5B that with the increase of

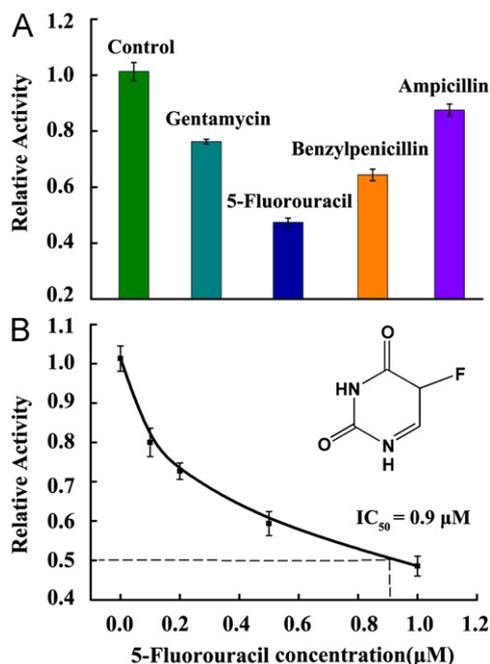


Fig. 5. (A) Influence of different inhibitors on the activity of Dam MTase. The concentrations of all these drugs are 1 μ M. (B) The inhibitory effect of different concentrations of 5-fluorouracil on Dam MTase activity.

inhibitor concentration, the inhibitory effect gradually enhanced. Finally, the IC_{50} value, half-maximal inhibitory concentration, was calculated as 0.9 μ M from the plot of relative activity of Dam MTase versus 5-fluorouracil concentration. These findings demonstrated that the proposed fluorescent sensor could be used as a promising platform to screen the inhibition of Dam MTase.

4. Conclusion

In summary, we developed a fluorescence sensing method for simple, rapid and highly sensitive detection of the activity and inhibition of Dam MTase by the combination of methylation-sensitive restriction endonuclease cleavage and nicking enzyme-assisted signal amplification. By taking advantage of the unique strategy, it enabled the assay of MTase activity with an impressive detection limit as low as 0.06 U/mL, much lower than those of most existing methods. Owing to the specific site recognition of MTases, this new approach could discriminate Dam MTase from other MTases such as M.SssI with high selectivity. The application in LB medium further demonstrated that the assay showed great potential for the detection of Dam MTase activity in complex biological samples. Furthermore, the inhibition effects of several antibiotics and anticancer drugs were also investigated, indicating the excellent performance of the developed method in DNA MTases inhibitor screening. With minor modification of the hairpin probe and the change of corresponding restriction endonuclease, the proposed sensing strategy might be used as a universal method for the detection of other DNA MTases such as cancer-related Dnmt1. In addition, it was worthwhile to point out that the whole reaction could be accomplished within relatively short time of 60 min in one step. Given attractive analytical characteristics, the sensing strategy might find many important applications in biomedical research, clinical diagnosis, and biomedicine.

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Appendix A. Supplementary materials

Supplementary materials associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.10.022>.

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