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RESEARCH PAPER

Highly sensitive detection of M.SssI DNA methyltransferase activity using a personal glucose meter

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Abstract A simple method for highly sensitive and selective detection of M.SssI CpG methyltransferase (M.SssI MTase) activity is developed, leveraging on the portability and ease of use of a personal glucose meter (PGM). Briefly, DNA-invertase conjugates are hybridized with their complementary DNA strands pre-immobilized on magnetic beads. The 5′-CCGG-3′ sequence present in the DNA duplexes serves as the recognition site for both Hpa II restriction enzyme and M.SssI MTase (5′-CG-3′). Hpa II restriction enzyme specifically cleaves at unmethylated 5′-CCGG-3′ sequence, and the invertase that remains on the methylated DNA catalyzes the hydrolysis of sucrose to glucose and fructose. It is found that the amount of glucose is proportional to the M.SssI MTase methylation activity in the range of 0.5 to 80 U/mL with a detection limit of 0.37 U/mL. Due to the specific recognition sequence present in the DNA strands, this method also shows high selectivity for M.SssI MTase. In addition, inhibition studies with 5′-azacytidine demonstrate the capability of inhibition screening using this method.

Keywords DNA Methyltransferase · Invertase · Electrochemical biosensor · M.SssI MTase

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Introduction

DNA methylation is one of the central regulating processes in the epigenetics of living organisms. DNA methylation plays a critical role in many biological processes such as regulating gene expression [1]. It has been shown that methylation in humans commonly occurs at the CpG islands of the promoter sequences of genes. Methylation at the CpG promoter sites blocks the recognition sites of transcription factors, leading to suppressed gene expression levels [2]. DNA methylation process is catalyzed by a family of enzymes known as DNA methyltransferases (MTases). These DNA MTases catalyze the transfer of the methyl group from *S*-adenosyl-L-methionine (SAM), to adenosine or cytosine nucleotides in specific DNA sequences according to their respective recognition sites [3]. Accumulated evidence has indicated that aberrant DNA MTase activity, which often leads to aberrant DNA methylation patterns, is closely associated with many types of genetic disorders and a large number of human malignancies [4, 5]. Other implications of aberrant methylation levels include X-chromosome inactivation, genomic imprinting, gene silencing, and other epigenetic processes [6]. As such, DNA MTases are potentially biomarkers for disease diagnosis [7], and the development of assays capable of selectively and sensitively detecting DNA MTase is of fundamentally and clinically interest.

As the first step toward improving human health, the activity of DNA MTase at cellular level has to be screened in order to gain an insight into the regulation of methylation and determine therapeutic strategy. Isotope-labeling is one of the earliest platforms widely used in the detection of the activity of DNA MTase and methylated DNA [8]. During methylation, isotope-labeled methyl moiety is grafted on DNA and thus granting the methylated DNA radioactivity. Unfortunately, the involvement of radioactive agents is the biggest drawback which has limited this assay in centralized laboratories. In the past few

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years, a variety of non-radioactive detection methods have been proposed to circumvent the use of radioisotopes. They include liquid chromatography coupled with mass spectrometry [9], capillary electrophoresis [10], colorimetry [11], chemiluminescence [12], fluorometry [13], bisulfite sequencing [14], and more recently, electrochemical methods [15]. Among them, bisulfite sequencing is considered as the gold standard in DNA methylation research. Bisulfite sequencing involves the use of bisulfite treatment for the deamination of unmethylated cytosine residues, and converting them to uracils, whereas methylated cytosine residues are not converted. Although some notable improvements have been achieved in the above-mentioned methods over radioactive labeling, eliminating the need of radioisotopes in particular, tedious procedures, the requirements of trained personnel, bulky and expensive equipment, and complexity in operation largely limit those methods in centralized settings. Hence, a simple and cost-effective method for detecting and quantifying DNA MTase activity is much desired.

Until very recently, the only analyte that the personal glucose meter (PGM) detects is glucose [16]. However, Xiang and Lu [17] have successfully overcome this limitation by proposing an approach using DNA-invertase conjugate as a signal producer for the detection of several non-glucose analytes. Other similar approaches include the detection of HIV-related DNA sequences [18], protein biomarkers [19], molecular toxins [20], and metal ions [21]. In addition, Yan and colleagues [22] suggested another approach using "sweet" hydrogel for such detection. It involves the use of a glucoamylase-trapped aptamer-cross-linked hydrogel which collapses when target analytes are introduced, releasing glucoamylase, which consequently hydrolyses amylose to glucose to be detected by the PGM.

Hence, motivated by the ease of use and portability of the PGM and the need for a simpler and less expensive device for the detection of DNA MTase activity, the possibility of utilizing the PGM to detect MTase activity was explored. Since the PGM can only detect glucose, M.SssI CpG methyltransferase (M.SssI MTase) activity was first correlated to glucose concentration with the use of invertase, which acts as a signal producer for the detection by the PGM. This simple detection method allowed for the quantification of DNA MTase and was also highly selective for the type of MTase (M.SssI MTase) to be detected due to the highly specific recognition sequences of different DNA MTases. Also, the method could be conveniently adapted to the detection of all other MTases by simply incorporating their recognition sequences in the DNA-Invertase conjugates. Satisfactory analytical performance was obtained with a detection limit down to 0.37 U/mL. Leveraging on the PGM's many advantages mentioned above, the proposed method offered some very attractive features in the pursuit of a simple, low-cost, and portable DNA MTase activity detection platform.

Experimental section

Oligonucleotides used in this work were custom-made by 1st Base Pte Ltd. (Singapore). The sequences of the oligonucleotides were Thiol-modified DNA (Thio-DNA) was 5′-/5ThioMC6-days/AAA AAA AAA ATC TTG ACA TAA ATA CCG GAA TCT AAC ATT AGT TC-3′ and the complementary Biotin-modified DNA was 5′-/5Biosg/AAA AAA AAA GAA CTA ATG TTA GAT TCC GGT ATT TAT GTC AAG AT-3′. The two oligonucleotides were dissolved in ultrapure water to 1.0 mM and stored at −20 °C. The PGM used in this work was from Nipro Diagnostics Inc. The streptavidin-coated magnetic beads (Dyanobeads M-280) were purchased from Thermo Fisher Scientific Inc.

The use of sulfo-SMCC linker for the conjugation of the thiol-DNA with invertase was carried out according to a previously published procedure with slight modifications [23]. To a solution containing 40 μL of 1 mM, the thiol-DNA, 3 μL of pH 5.5 PBS buffer, and 3 μL of 30 mM TCEP were mixed in a centrifuge tube. The mixture was then kept at room temperature for 1 h, to allow the breakage of the disulfide bond that forms between two strands of the thiol-DNA. The DNA was then purified by washing three times with buffer A (0.1 M NaCl, 0.1 M sodium phosphate buffer, pH 7.3) using Amicon 3 K. The mixture containing 400 μL of 20 mg/mL invertase in buffer A and 1 mg of Sulfo-SMCC was placed on the shaker for 1 h after vortexing for 5 min. The excess of insoluble Sulfo-SMCC was removed by centrifugation, and the solution was mixed with the thiol-DNA and kept at room temperature for 72 h. This solution was washed thrice with Amicon 100 K using buffer A to remove unreacted thiol-DNA. An 8 % SDS-PAGE analysis was carried out to characterize the DNA-invertase conjugate synthesized.

A solution containing 40 μL of the biotin-modified DNA in 1.6 mL of buffer B (0.2 M NaCl, 0.1 M sodium phosphate buffer, pH 7.4) and 200 μL of DNA-invertase conjugate (from Section 2.2) was placed in a 38 °C water bath for 2.5 h. Thereafter, using Amicon 100 K, the solution was washed three times with buffer B. The resulting solution was made up to 200 μL, sealed and stored at 4 °C for subsequent experiments.

The Hpa II restriction enzyme digestion was optimized by carrying out a series of different Hpa II restriction enzyme digestion experiments at 38 °C in a water bath. The magnetic beads anchored with the methylated DNA strands were suspended in 100 μL of incubation solution containing 200 U/mL Hpa II restriction enzyme (2 μL of 10,000 U/mL of Hpa II restriction enzyme, 10 μL of 10× CutSmart Buffer, 88 μL of ultrapure water). The restriction enzyme digestion was carried out at 38 °C in the water bath for 5, 15, 30, 60, 90, and 120 min. After centrifugation and magnetic separation, the supernatant was discarded and the magnetic beads were washed twice with 100 μL of buffer A before transferring the magnetic beads to a centrifuge tube and suspending them in 10 μL of

freshly prepared 1.0 M sucrose in buffer A and placed on a shaker at room temperature for 50 min. The resulting solution was then centrifuged and tested for glucose using the PGM.

Results and discussion

Figure 1 illustrates the principal steps of the detection of M.SssI MTase activity using the PGM. The principle behind this method is based on the use of a pair of methylation specific enzymes, M.SssI MTase and Hpa II restriction enzyme, to quantify the methylation activity. This is indicated by the amount of invertase remaining on the magnetic beads, which produces glucose during the hydrolysis of sucrose for the PGM. The oligonucleotides chosen in this work contain 5′-CCGG-3′ sequences. These sequences serve as the recognition sites of both Hpa II restriction enzyme and M.SssI MTase, ensuring the high selectivity for the detection of M.SssI MTase methylation activity.

Hpa II restriction enzyme recognizes and cleaves unmethylated 5′-CCGG-3′ sequences [23], while M.SssI MTase methylates at the 5′-CG-3′ site. For the unmethylated DNA duplexes, Hpa II restriction enzyme recognizes and cleaves at those sites and the cleaved DNA carrying invertase is removed by a thorough washing. For those DNA duplexes methylated by M.SssI MTase, Hpa II restriction enzyme is no longer able to recognize and cleave at those sites. Hence, invertase remains on those uncleaved methylated duplexes and produces glucose during the hydrolysis of sucrose. A higher activity of M.SssI MTase would block more recognition sites of Hpa II restriction enzyme, leading to a larger amount of invertase remaining on the methylated duplexes, thereby, registering a higher signal at the PGM. Consequently, the M.SssI MTase activity can be conveniently monitored by the PGM.

A polyacrylamide gel electrophoretic (PAGE) experiment was first carried out to confirm the successful conjugation of invertase with the thiol-DNA. A discontinuous buffer system

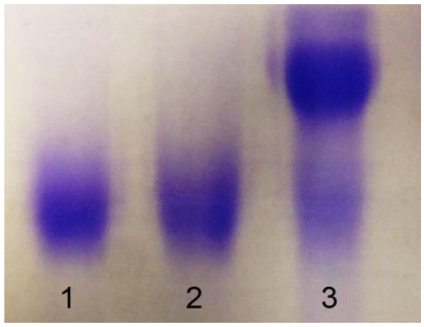

Fig. 2 Eight percent SDS-PAGE gel image of DNA-invertase conjugate and control. *Lane 1*: Control 1—free invertase without linker and the thiol-DNA, *lane 2*: Control 2—mixture of invertase and the thiol-DNA without linker, and *lane 3*: Sample—invertase-thiol-DNA conjugate

was used. From the gel image in Fig. 2, the migration of the DNA-invertase conjugate in lane 3 was less than that in lane 1. This is due to the conjugation of the thiol-DNA with invertase, which increased its molecular size and more importantly its negative charges. Hence, the DNA-invertase conjugate moved much slower than the free invertase (lane 1). Moreover, a negligible difference between lanes 1 and 2 was observed in terms of band positions. This is due to the fact that the thiol-DNA alone cannot be conjugated to the invertase in the mixture of invertase and thiol-DNA. Hence, invertase in lane 2 moved similarly as free invertase in lane 1, showing similar band positions. These results confirmed that invertase has successfully been conjugated to the thiol-DNA.

Feasibility studies were then carried out to confirm the feasibility of the proposed method. The results are shown in Fig. 3. As seen in Fig. 3, in control 1, when there was no methylation or digestion been carried out, the PGM signal showed "Hi," exceeding its working range. This suggested that a very large amount of the duplexes of the biotin-modified DNA and the DNA-invertase conjugates have been successfully captured onto the streptavidin-coated magnetic beads, and the invertase is able to catalyze the hydrolysis of sucrose to produce a very large amount of glucose. For control

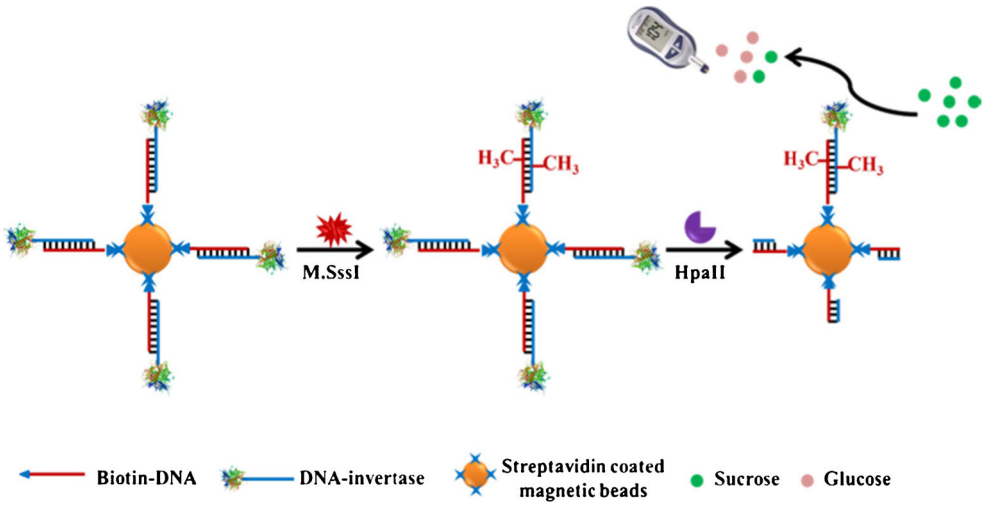

Fig. 1 Schematic illustration of the MTase activity assay using the PGM

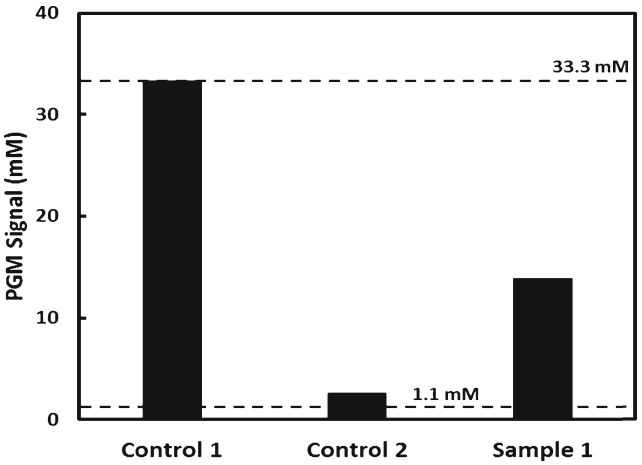

Fig. 3 PGM signals for Control 1, Control 2, and Sample 1. *Dotted lines* denote the working range of the PGM (1.1–33.3 mM), Control 1: methylation '−', digestion '−', Control 2: methylation '−', digestion '+', Sample 1: methylation '+', digestion '+'

2, methylation was not carried out, but Hpa II restriction enzyme digestion was carried out. The extremely low PGM signal indicated that 200 U/mL of Hpa II restriction enzyme is in sufficiently large excess to cleave all unmethylated sites of the DNA duplexes on the magnetic beads and that the washing step is effective to remove the cleaved invertase.

On the other hand, upon methylation, sample 1 showed a much higher reading than that of control 2, but much less than that of control 1. This indicated that the methylation step does interfere with Hpa II restriction enzyme activity, effectively blocking the recognition sites of Hpa II restriction enzyme. However, the M.SssI MTase concentration used was not sufficiently high to methylate all the DNA duplexes on the magnetic beads; some of the DNA duplexes were not methylated and hence cleaved off by Hpa II restriction enzyme. Consequently, in this case, sample 1 showed a lower signal than control 1. The above results also suggested that a wide range of MTase activity can be detected by the PGM. More importantly, the above tests unambiguously confirmed the feasibility of the proposed method and indicated that this method effectively maximized the usage of the entire working range of the PGM (1.1 to 33.3 mM). Hence, a wide range of M.SssI MTase activity could be detected using the PGM.

Before establishing the calibration curve, the experimental conditions such as the Hpa II restriction enzyme digestion time and the M.SssI MTase methylation time have been optimized to obtain better results. Under the optimized conditions, the relationship between the activity of M.SssI MTase and PGM signal was investigated. From Fig. 4, it was observed that increasing the activity of M.SssI MTase increases the PGM response in a linear fashion. The regression equation within the linear range of 0.5 to 80 U/mL is $y = 0.3863x + 1.9961$ ($R^2 = 0.99$), where y is the PGM signal (mM) and x is the activity of M.SssI MTase in U/mL. A detection limit of 0.37 U/mL was obtained using three times of the standard deviation of the control. In principle, a lower detection limit is obtainable if a longer period of incubation in the sucrose solution is performed. However, the upper limit of the calibration curve is largely determined by the working range of the PGM. The linear detection range is considerably wider than many other methods such as fluorometry (1–10 U/mL) [24]. The detection limit is slightly lower than the reported 0.4–0.8 U/mL [24–26].

The selectivity of this method for M.SssI MTase was tested by introducing other DNA MTases as potential interferants (Fig. 5). The interferants used were Alu I, Hae III, and dam DNA MTases, with identical concentrations at 40 U/mL each. M.SssI showed a much higher PGM signal than that of any other DNA MTases, while those interfering DNA MTases showed similar readings close to that of the control sample (no DNA MTase added). This high selectivity is attributed to

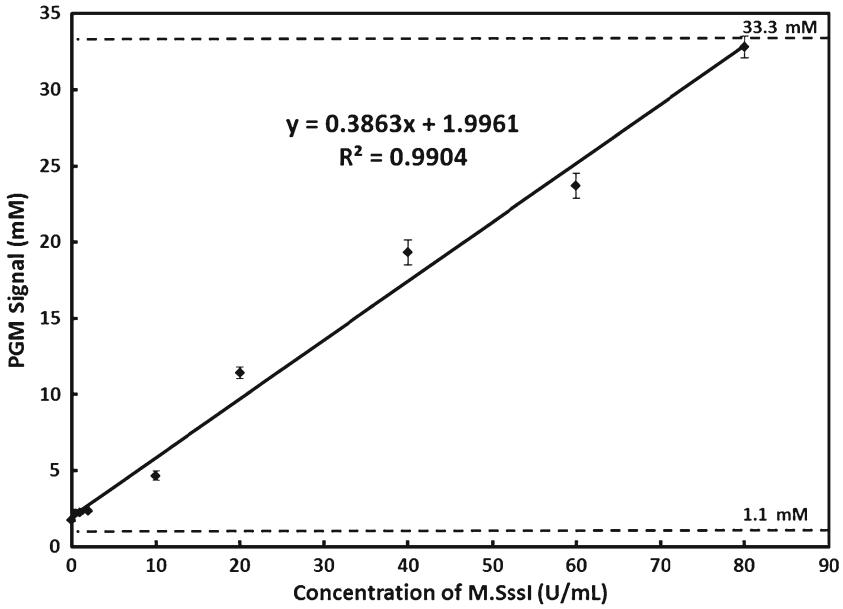

Fig. 4 The calibration plot of M.SssI MTase activity assay using the PGM

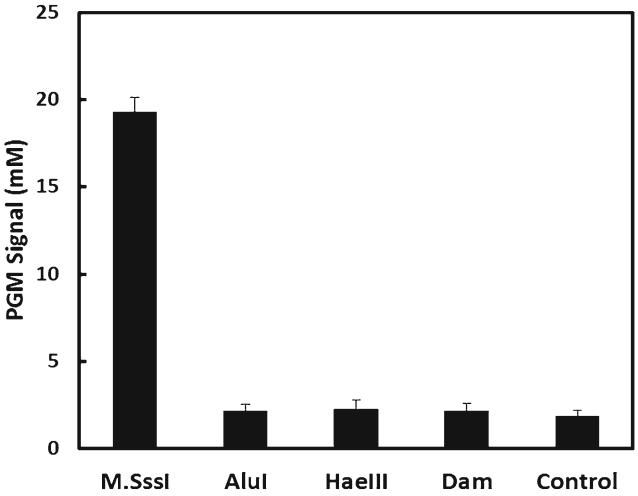

Fig. 5 Selectivity study of the PGM method for the detection of M.SssI MTase activity

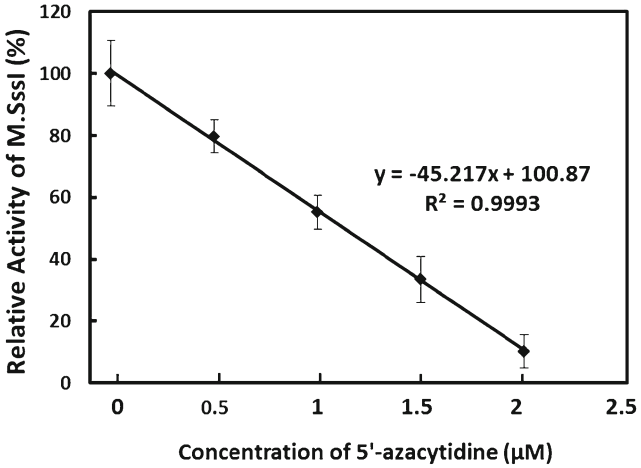

Fig. 6 Inhibition effect of 5′-azacytidine on M.SssI MTase activity

the unique recognition sequences of DNA MTases. Each of the interfering DNA MTases has a different recognition sequence from that of M.SssI (5′-CG-3′). The recognition sequences of each of the interfering DNA MTases are 5′-GATC-3′ (dam), 5′-AGCT-3′ (Alu I), and 5′-GGCC-3′ (Hae III). Hence, since the interfering DNA MTases have specific recognition sites which are not overlapping with the desired M.SssI MTase target, they do not methylate at the 5′-CCGG-3′ site. Since there is no methylation at this site, Hpa II restriction enzyme can recognize and digest at this site, thus producing the low PGM signals.

As the developed biosensor is highly selective to M.SssI DNA Mtase, attempts were made to analyze M.SssI DNA Mtase activity in cultured cancer cell lysates and serum (Table 1). These results clearly demonstrated that the proposed biosensor is capable of selectively detecting M.SssI DNA Mtase in biological matrices.

Studies have found that DNA MTase inhibitors have the potential to alter DNA methylation, reactivating genes that are silenced by aberrant methylation activity and hence, DNA MTase inhibitors are of great interest in cancer therapy [27]. For example, DNA MTase inhibitors could potentially reactivate silenced tumor suppressor genes due to DNA MTase methylation activities at the promoter regions [27]. One of the most popular DNA MTase inhibitors is 5′-azacytidine, which has been a widely used chemotherapeutic agent in the past 40 years [28, 29]. 5′-Azacytidine is an analogue of cytidine nucleoside. After activation to 5′-aza-2′-deoxycytidine-triphosphate, it acts as a substitute for cytidine nucleoside being taken up by the DNA MTase as its substrate. A nucleophilic attack occurs, as per the usual DNA methylation reaction, which forms a covalent bond between C6 of cytosine with the DNA MTase. In a usual DNA methylation reaction, this is followed by a β-elimination reaction across the C5-C6 bond, releasing the enzyme. However, in the case of the analogue, a nitrogen atom substitutes the C5 position in cytosine, blocking this reaction, and hence inhibiting DNA MTase. In addition, this reaction further prompts the degradation of trapped DNA MTases through damage signaling [30]. Herein, the effect of 5′-azacytidine on M.SssI MTase activity was investigated.

Table 1 Sample analysis

Sample	M.SssI Mtase activity (U/mL)	M.SssI Mtase added (U/mL)	Recovery (%)
Hela cell lysate	22	10	98
MCF-7 cell lysate	15	10	106
Human serum	0	10	95

The inhibition effect of 5′-azacytidine on M.SssI MTase activity is illustrated in Fig. 6. The activity of M.SssI MTase decreased linearly with increasing 5′-azacytidine concentration. The half maximal inhibitory concentration (IC_{50}) measured the drug concentration (inhibitor in this case) that inhibits the enzyme activity by 50 %. The IC_{50} value obtained from the plot in Fig. 6 was found to be 1.12 μM, which is in good agreement with those reported previously [31]. This inhibition study indicated that this method can be used as a tool for inhibitor screening in cancer therapy.

Conclusion

In summary, a simple, cost-effective and highly selective method for the detection of MTase activity using the PGM has been developed. The proposed method had a wide linear range of 0.5 to 80 U/mL for the detection of M.SssI MTase activity and showed good sensitivity. The specific recognition sequence of DNA MTase used allowed for the high selectivity and convenient adoption for the detection of other MTases by simply altering the oligonucleotide sequences used. Inhibition study conducted demonstrated the capability of this method in screening for MTase inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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