



## Short communication

## Real-time monitoring of DNA methyltransferase activity using a hemimethylated smart probe

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## ABSTRACT

A real-time assay for DNA methyltransferase (MTase) activity has been developed. A hemimethylated smart probe is used as the substrate for DNA MTase. Cleavage of the methylated product leads to separation of fluorophore from quencher, giving a proportional increase in fluorescence. The method permits real-time monitoring of DNA methylation process and makes it easy to characterize the activity of DNA MTase. It also has the potential to screen suitable inhibitor drugs for DNA MTase.

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DNA methylation is a crucial epigenetic modification both in eukaryotes and prokaryotes, and plays a pivotal role in gene expression, cellular differentiation, and pathogenesis of various human diseases [1]. The DNA methylation process is catalyzed by DNA methyltransferase (MTase) which can recognize specific sequences and transfer a methyl group from the donor S-adenosylmethionine (SAM) to target cytosine or adenine [2,3]. An abnormal level of DNA MTase leads to the aberrant DNA methylation which has been proved to be a potential target for anticancer therapy and drug screening [4]. Therefore, the development of novel approaches for simple, rapid and sensitive activity assay and inhibitor screening for MTases is crucial to clinical diagnostics, disease therapy and biomedical research.

The traditional methods for DNA MTase activity assay include gel electrophoresis, radioactive labeling, high performance liquid chromatography, and immune-based assays [5–7]. However, these methods have some drawbacks, including laborious and time-consuming operation, dangerous radio-labeled substrates, expensive equipment, or costly antibodies. To overcome the shortcomings of the traditional methods, alternative methods have been developed, such as fluorescence assays [8–10], colorimetric assay

[11,12], bioluminescence assay [13], photoelectrochemical methods [14–16], and electrochemical methods [17–20], in which fluorescence strategies attracted much attention due to their high sensitivity. For example, previously, we have developed a novel fluorescence assay for DNA MTase activity detection using a molecular beacon [21]. Recently, Roach et al. reported a real-time assay for CpG-specific cytosine-C5 methyltransferase activity using a hemimethylated molecular break light oligonucleotide probe [22]. However, design and synthesis of double labeled molecular beacon probes are difficult and costly. Very recently, Zhao et al. realized the detection of DNA MTase activity by using graphene oxide as a super quencher [23]. However, synthesis of graphene oxide is time-consuming and the quenching ability is unstable. Therefore, the development of simple, sensitive and cost-effective methods for monitoring of DNA MTase activity is of considerable interest.

Recently, singly labeled smart probes, which take advantage of the selective quenching of fluorophores by neighboring guanosine residues via photo-induced electron transfer (PIET) were introduced. In contrast to MBs, smart probes are relatively easy to synthesize (single labeling step), are cheaper, and have a free terminus for further modification [24–29]. They have been found to have wide applications in DNA and RNA detection [24–26], T4 polynucleotide kinase analysis [27], silver ion and lead ion detection [28,29]. In this paper, we report a novel simple and sensitive DNA MTase assay method using a hemimethylated smart probe.

The principle of application of smart probe to monitor DNA

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MTase activity is shown in Fig. 1. To improve the specificity of DNA MTase detection, we chose hairpin DNA as a recognition probe which is single-labeled with carboxyfluorescein at its 5' end. Four guanines at its 3' end were designed as fluorescence quencher based on the inherent quenching ability of guanine bases. The fluorescence of smart probe is effectively quenched by four guanines at the 5' end of its complementary DNA due to an effective PIET between deoxyguanosines and fluorophore. Dam MTase and DpnI restriction endonuclease are chosen as the model MTase and endonuclease, respectively. First, Dam MTase methylates the recognition sequence to generate the methylation duplex DNA (5'-G-mA-T-C-3'), acting as the substrate for DpnI. Then, DpnI cleaves the fully methylated probe into three short ssDNA fragments at the reaction temperature (37 °C) owing to their low melting temperature ( $T_m = 4\text{ °C} \times G:C\text{ pair} + 2\text{ °C} \times A:T\text{ pair} = 26\text{ °C}$ ) [21], which restores the quenched fluorescence of the smart probe due to the break of PIET. Therefore, taking advantage of the present strategy, we can easily detect Dam MTase activity.

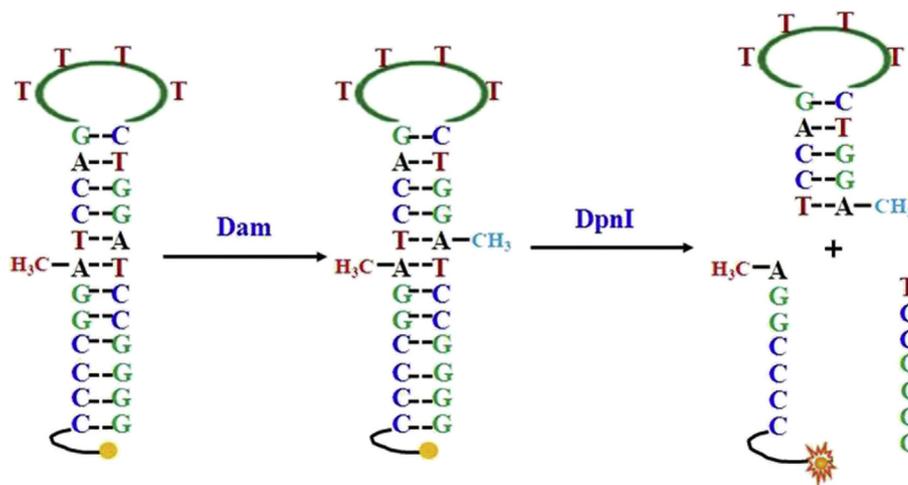
To demonstrate the feasibility of this method, we prepared three samples. Sample a contains smart probe (5'-FAM-CCCCAmTC-CAGTTTCTGGATCGGGG-3', the Am represents adenosine methylation) and Dam MTase. Sample b contains smart probe and Dpn I. Sample c contains smart probe, Dpn I and Dam MTase. The concentration of smart probe, Dpn I and Dam MTase was 200 nM, 100 units/mL and 40 units/mL, respectively. The buffer containing 70 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 10 mM Mg(Ac)<sub>2</sub>, 5.0 mM DTT and 80 μM SAM was used in all experiments. All samples were incubated at 37 °C and fluorescence intensity was obtained on an F2700 (Hitachi, Japan) with excitation at 490 nm and emission at 520 nm. The fluorescence intensity of these samples was monitored and the time courses were plotted in Fig. 2A.

As illustrated in Fig. 2A, after the addition of Dam MTase or Dpn I individually, there was no fluorescence change in curves a or b (corresponding to samples a or b). This result implied that the conformation of smart probe was not affected by Dam MTase or Dpn I. In sample c represented by curve c, after the addition of both Dpn I and Dam MTase, the fluorescence was enhanced rapidly. The smart probe was fully methylated by Dam MTase and the product was immediately cleaved by Dpn I, releasing short ssDNA fragments, followed by enhancement of fluorescence. Based upon the above data, we could monitor the activity of Dam MTase in real-time using this approach.

We investigated a series of reactions using various concentrations of Dam MTase at 0.5, 2, 10, 20 and 40 units/mL respectively. The result indicated that as the Dam MTase concentration was increased, the fluorescence enhancement rate was increased accordingly. With the enzyme ranging from 0.5 to 40 unit/ml, a good linear correlation ( $r^2 = 0.9940$ ) between initial velocity of fluorescence enhancement and Dam MTase concentration was observed (Fig. 2B). The detection limit for Dam MTase was determined to be 0.5 unit/mL. This assay is convenient, rapid and sensitive, which makes it a useful approach to analyze the activity of Dam MTase.

Our assay could also be used for the screening of potential Dam MTase inhibitors. Gentamycin, a well known DNA MTase inhibitor, was tested. A previous report suggested that gentamycin had no influence on the activity of Dpn I when the concentration of gentamycin was lower than 20 μM [23]. Thus, gentamycin at less than 20 μM concentration was used in our study. Fig. 2C shows that the relative activity of Dam MTase decreased gradually with increasing gentamycin concentration. It can be concluded from the figure that the activity of the DAM MTase was inhibited by 50% when the concentration of gentamycin was approximately 12 μM. With the gentamycin concentration higher than 12 μM, a slight decrease of Dam MTase activity was observed. The data determined using the assay is comparable to those in the literature [23]. These results demonstrated that the proposed DAM MTase activity assay can be successfully applied in DAM inhibitor screening and is a potentially useful tool for antibiotic drug discovery.

In conclusion, we have developed a new approach for the sensitive detection of DAM MTase activity. The assay uses a hemimethylated smart probe for signal transduction. Unlike traditional methods, this assay was done in real-time and could be used to monitor the dynamic process of DNA methylation. And because no labeling with a fluorophore-quencher pair was required, the method is fairly simple, fast, low cost and universal. Furthermore, this assay could be potentially used to screen for DAM MTase inhibitors. We envision that our novel fluorescent detection method for DAM MTase activity could be applied as a useful tool in biochemical and biomedical research such as drug screening and cancer diagnosis [30,31].



**Fig. 1.** Real-time monitoring of DNA methyltransferase activity. The fluorescence of the hemimethylated smart probe is effectively quenched by four guanines. It is a substrate for Dam MTase and upon methylation yields the fully methylated product, which is rapidly cleaved by Dpn I endonuclease, owing to their low melting temperature, the cleaved products are unstable at 37 °C, separating the fluorophore from the quencher and resulting in an increase in fluorescence.

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