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# The colorimetric assay of DNA methyltransferase activity based on strand displacement amplification

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ABSTRACT

In this paper, we present a colorimetric method for the assay of DNA methyltransferase (MTase) activity based on strand displacement amplification (SDA). In our study, a well-designed hairpin DNA I (HPI) containing the sequence of 5′-CCGG-3′ is specifically recognized by CpG methyltransferase (M.SssI) and HpaII endonuclease. The methylated HPI is able to coexist with all the DNA and enzymes in the solution while the unmethylated HPI can be cleaved into single-stranded DNA (ssDNA) fragments. The amplification can be triggered by the HpaII digestion products hybridization with another hairpin structure DNA II (HPII) to form a duplex, which would be replaced by probe DNA, leading to the aggregation of gold nanoparticles (AuNPs). Simultaneously, ssDNA fragments released from the duplex, and triggered the cycle anew. Varying concentrations of M.SssI in the solution therefore would lead to differences of absorption and color changed from red to pale. A linear response was obtained when the M.SssI concentration ranging from 0.2 to 50 U $mL^{-1}$ with a detection limit of 0.08 U $mL^{-1}$. In addition, the developed assay in this study can also be applied to screen the inhibitors of M.SssI.



## 1. Introduction

DNA methylation, a common gene protection approach, plays an important role in both prokaryotes and eukaryotes. It is extremely important to lots of normal cellular processes including development, transposon silencing, gene regulation, and X chromosome inactivation, among others [1,2]. Alterations of methyltransferases (MTases) activity may lead to aberrant DNA methylation patterns, which are associated with several genetic diseases and various types of cancer [3,4]. DNA MTases have become potential therapeutic targets and predictive biomarkers in a variety types of cancer of lung [5], gastric [6], colon [7], prostate [8] and so on. Moreover, abnormalities in MTase activity usually occur far before other signs of malignancy and could thus be used for early cancer diagnosis [9,10]. Thus, sensitive activity assay and inhibitor (anti-methylation drugs) screening for MTases represent a valuable strategy to both clinical diagnostics and therapeutics. In addition, it's also crucial in understanding how MTase activity contributes to cancer initiation and progression.

The conventional methods for DNA MTase activity determination include radioactive labeling [11,12] liquid chromatography/mass spectrometry [13], high performance capillary electrophoresis (HPCE) [14], fluorescence [15–18], colorimetric [19,20], electrochemical [21–23], electrochemiluminescence [24,25] and chemiluminescence [26]. In recent years, various advanced techniques have been proposed. For example, Cai et al., reported an approach by coupling the fluorescence quenching of graphene oxide with site-specific cleavage of a restriction endonuclease [17]. Gao and coworkers described an electrochemical assay employing a threading intercalator, *N*,*N*′-bis(3-propylimidazole)-1,4,5,8-naphthalene diimide (PIND) functionalized with electrocatalytic redox $Os(bpy)_2Cl^+$ moieties (PIND-Os), for signal amplification [27]. Nevertheless, these methods still carry obvious drawbacks, such as radioisotope labeled substrate, expensive antibodies, fluorescently labeled substrates, bulky detection equipment, time-consuming sample preparation, or inflexible detection schemes. Therefore, further efforts are needed in the development of simple and sensitive MTase activity assays.

Nowadays, colorimetric assay has been applied in detecting DNA MTase activity. Li et al. used DNA modified gold nanoparticles (AuNPs) coupled with enzyme-linkage reactions to detect the activity of methylases [20]. In a previous study, our group presented

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a label-free colorimetric method, using unmodified Au nanorods with enzyme-linkage reaction for the assay of DNA methylation [19]. The methods applying AuNPs with enzyme linkage reactions have emerged as the most important colorimetric reporters owing to their high extinction coefficients and strong size-dependent surface plasmon resonance (SPR) properties [28]. The color of the AuNPs solution can be changed from red to purple or pale, in response to the SPR absorption of dispersed and aggregated nanoparticles.

Although AuNPs based methods have accomplished substantial progress, relatively poor sensitivity is one of the common limitations, which is mainly due to the shortage of signal amplification [29]. It is extremely important to develop amplification strategies for AuNPs-based methods to address this drawback. Up until now, several signal amplification strategies have been reported including loop-mediated isothermal amplification (LAMP) [30], rolling circle amplification (RCA) [31], exponential amplification reaction (EXPAR) [32]. Motoi and coworkers designed a fluorescent-amplified adenosine aptasensors by toehold mediated click chemical ligation DNA strand displacement system [33]. A new approach of reverse DNA strand displacement by using functional nucleic acids as toeholds was reported by Yang's group [34]. Among these methods, strand displacement amplification (SDA) has attracted increasing attention due to its excellent property of homogeneous and lable-free. DNA displacement reactions between double-stranded (ds)-DNA with strands of unequal length and single-stranded (ss)-oligonucleotides using a toehold structure, as a trigger point, enables DNA rehybridization in a fast "base-by-base" programmable controlled manner [35,36]. This method, SDA, has the advantage that the route of strand displacement can be easily predicted due to the fact that the displacement is always known at the commencement. Furthermore, the reacting oligonucleotides sequence is known, considering all mismatches, such as deletions, insertions, or point mutations occurring during strand exchange, making it possible to forecast the kinetic, thermodynamic behavior and outcome of the displacement reaction [37]. Therefore, the SDA method has potention applications in many fields. Nevertheless, there are only a few amplified strategies available for development of colorimetric methods based on AuNPs.

Here we take advantage of strand displacement to design colorimetric SDA biosensor for CpG methyltransferase (M.SssI) detection. The M.SssI catalyzes the transferring of methyl to C-5 position of cytosine in the CpG region of double-stranded DNA from SAM and the HpaII restriction endonuclease can identify the duplex symmetrical sequence of 5′-CCGG-3′ and catalyze the digestion of double-stranded DNA between the unmethylated cytosines. According to the procedure, HpaII endonuclease catalyzed cleavage will be blocked once the CpG dinucleotide site in the 5′-CCGG-3′ sequence is methylated [38]. In this work, the amplification is triggered by the HpaII digestion products region I hybridization with a hairpin structure DNA, and then region I is replaced by DNA probes. With the use of AuNPs-based colorimetric assay, the developed strategy can create a convenient platform for visualized detection of enzyme activity as well as the screening of the inhibitors of M.SssI with high sensitivity and selectivity.

## 2. Experimental

### 2.1. Materials and apparatus

All oligonucleotides (Table S1 in Supplementary material.) were purchased from Invitrogen Co. Ltd (Shanghai, China). All the SH-DNA was treated with DTT and separated by a Nap-5 column before use. HPI and HPII were annealed by heating in a 90 °C water bath for 10 min and slowly cooled down to room temperature prior to use. Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4{\cdot}3H_2O$) and trisodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were obtained from Shanghai Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). S-Adenosylmethionine (SAM), *E. coli* CpG methyltransferase M.SssI, and E. coli restriction endonuclease HpaII and the corresponding buffer solution were purchased from New England Biolabs. Inc (Beijing, China). 20% TBE Gel was purchased from Invitrogen Co. Ltd (Shanghai, China). DL-Dithiothreitol (DTT, $C_4H_{10}O_2S_2$), Ethidium bromide solution, 5-azacytidine (5-Aza), and 5-aza-2′-deoxycytidine (5-Aza-dC) were supplied by Sigma-Aldrich (USA) and used as received. All other chemicals were of analytical grade and used without further purification. The ultrapure water with an electrical resistance larger than 18.2 MΩ was used for all the experiments. UV–vis absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). Transmission electron microscopy (TEM) measurements were conducted on a JEM-2010 transmission electron microscope (JEOL Ltd.).

### 2.2. Assay of M.SssI activity

The procedures of synthesis and modification of AuNPs were illustrated in Supporting information. The methylation experiment was performed in 20 μL of 1 × NEBuffer2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) containing 80 μM SAM, 40 nM HPI and various amounts of M.SssI. The reaction mixture was incubated at 37 °C for 5 h. Then the resulting mixture was heated at 65 °C for another 20 min to inactivate the M.SssI. Next, HpaII, final concentration was 40 U $mL^{-1}$, was added and incubated at 37 °C for 2 h to allow the cleavage reaction to take place. After that, HPII was added to the cleaved solution and then mixed with equal amount of DNA1-AuNPs and DNA1′-AuNPs. The resulting samples were incubated at 37 °C for 90 min to allow sufficiently hybridization and strand displacement reaction, then diluted to 350 μL with Tris-HCl and tested with an UV–vis spectrometer.

### 2.3. Polyacrylamide gel electrophoretic analysis

The experiment was performed in 10 μL reaction solution which contained 1 × NEBuffer2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), 1 μM HPI, 160 μM SAM, 120 U $mL^{-1}$ M.SssI, 40 U $mL^{-1}$ HpaII, 3 μM HPII, 3 μM probe DNA1 and 3 μM probe DNA1′. The incubation steps were the same as the assay of M.SssI activity. The samples were then put on a polyacrylamide gel (20% TBE gel) to separate the cleaved products from the substrate. The electrophoresis was carried in 1 × tris-borate-EDTA (TBE) (pH 8.0) at 110 V constant voltages for 4 h at room temperature. After EB staining, the gel was scanned using the Gel Image Analysis System (Bioshine GelX1650, Shanghai, China).

### 2.4. Selectivity and inhibition of M.SssI activity assay

Dam MTase was selected as the potential interfering enzymes. The selectivity experiments were conducted with 50 U $mL^{-1}$ Dam MTase in the same way as the M.SssI activity detection procedure, except for that M.SssI was replaced by Dam MTase in the methylation step.

The experiments of inhibitors influence on the activity of M. SssI MTase were similar to that noted above except for various concentrations of the inhibitors in the samples. The methylation of HPI was performed at 37 °C in 10 mM Tris-HCl, (50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) containing 80 μM SAM, 50 U $mL^{-1}$ M.SssI, and various concentrations of the inhibitors. After the HpaII digestion and incubation with HPII and DNA1/1′-AuNPs, the absorption