

## A nonradioactive DNA methyltransferase assay adaptable to high-throughput screening

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### Abstract

We have developed a nonradioactive assay method for DNA methyltransferases based on the ability to protect substrate DNA from restriction. DNA immobilized to a microplate well was treated sequentially with methyltransferase and an appropriate endonuclease. The amount of methylated DNA product is reflected by a proportional decrease in endonuclease cleavage, which is in turn reflected by increased retention of the end-labeled affinity probe. A single universal substrate was designed to assay multiple methyltransferases including those that do not have a cognate endonuclease. The methodology developed is suited to screen a large number of compounds for inhibitors of various methyltransferases.

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**Keywords:** DNA methyltransferase; Restriction/modification system; DNA immobilization

DNA methylation is an epigenetic process that occurs in both prokaryotes and eukaryotes. In bacterial systems, DNA methyltransferases (Mtases)<sup>2</sup> are mainly involved in restriction/modification systems to protect against foreign DNA. Some bacterial Mtases such as the cell-cycle-regulated DNA Mtase (CcrM) from *Caulobacter crescentus*, however, lack cognate restriction enzymes and instead play crucial roles in cell cycle progression and consequently in some cases cellular virulence [1–3]. Hence these CcrMs have attracted attention as potential targets for the development of novel antibiotics [4]. In eukaryotes, DNA methylation plays significant

roles in maintaining genomic stability, gene silencing, and gene expression [5]. Importantly, human cancer cells often show altered DNA methylation patterns, particularly at CpG islands [6]. Therefore, targeting DNA methyltransferases holds promise as a novel strategy for therapeutic intervention.

Reflecting the significance of studying DNA Mtases are several assay methods. Most of these methods involve the use of radiolabeled cofactor *S*-adenosyl methionine (SAM) in either a filter binding or a scintillation proximity assay format [7–9]. Nonradioactive methods are limited to restriction–protection assays visualized by gel electrophoresis and assays based on bisulfite modification [10,11]. While eliminating the use of radioactive material, these assays have limited throughput and are not suited to screen libraries of small molecules. Here we describe a robust DNA Mtase assay based on restriction–protection and detection by ELISA [12,13]. We have adapted the methodology to microtiter plates, thus providing a means for high-throughput screening for inhibitors of methyltransferases.

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<sup>2</sup> Abbreviations used: ELISA, enzyme-linked immunosorbent assay; SAM, *S*-adenosyl methionine; SAH, *S*-adenosyl homocystein; Mtase, methyltransferase; CcrM, cell-cycle-regulated methyltransferase; POD, peroxidase; TMB, tetramethylbenzidine; AP, alkaline phosphatase; pNPP, *p*-nitrophenylphosphate; DMSO, dimethyl sulfoxide; TBS, Tris-buffered saline.

## Materials and methods

Mtases (CcrM and *HhaI* Mtase) were cloned and overexpressed in *Escherichia coli* [14]. Endonucleases were purchased from New England BioLabs (Beverly, MA) and used as recommended by the manufacturer.

### Preparation of *HhaI* Mtase

The gene encoding *HhaI* Mtase was amplified from a *Haemophilus haemolyticus* DNA containing plasmid (ATCC) using a forward primer (5'-GCATGAATTCC ATATGATTGAAATAAAAGATAAACAGC-3') and a reverse primer (5'-GCATGTGGATCCTTAATATG GTTTGAAATTTAATGATG-3'). The PCR product was cloned into pET16b using the *NdeI* and *BamHI* restriction sites. After sequence verification, the *HhaI* Mtase was expressed as an N-terminal histidine tagged fusion from *E. coli* ER2566. NZCYM medium containing 75 mg/L ampicillin was used with 1 mM of isopropyl β-D-thiogalactoside for induction. The harvested cells were resuspended in a stock buffer (50 mM Tris, pH 7.7, 0.5 M NaCl, 10% glycerol), disrupted with lysozyme (1 h, 4°C), and sonicated. The crude protein sample thus prepared was purified by Ni-nitrilotriacetic acid chromatography.

### Preparation of cell-cycle-regulated methyltransferase

CcrM was prepared as previously described from *C. crescentus* [14].

### Substrate DNA preparation

A double-stranded DNA with multiple recognition sites was designed as shown in Fig. 1 and the corresponding single strands were purchased from a commercial source (Integrated DNA Technologies). The 3' end of one strand was amine-labeled (3'-amino-modifier C7 CPG: (1-dimethoxytrityloxy-3-fluorenylmethoxycarbonylamino-hexan-2-methylsuccinoyl)-long chain alkylamino-CPG, Glen Research) and the other strand was purchased as labeled with biotin at the 3' end. The amino end was derivatized with 3-amino-3-deoxydigoxigenin hemisuccinamide succinimidyl ester (Molecular Probes). To 1 ml of reaction mixture containing 650 μl of 210 μM amino-labeled DNA (0.137 μmol), 100 μl of 1 M borate, pH 8.5, 150 μl of DMSO, and 100 μl of 34 mM 3-amino-3-deoxydigoxigenin hemisuccinamide succinimidyl ester in DMSO (3.4 μmol) were added. This mixture was set on a rocking surface at room temperature for 2 days. An additional 50 μl of 34 mM 3-amino-3-deoxydigoxigenin hemisuccinamide succinimidyl ester in DMSO was added after the first day. The reaction was quenched by 10-fold dilution into 50 mM Tris, pH 7.7, and 1 mM EDTA. The free reagent was removed by anion exchange Hi-Trap Q after loading the reaction mixture

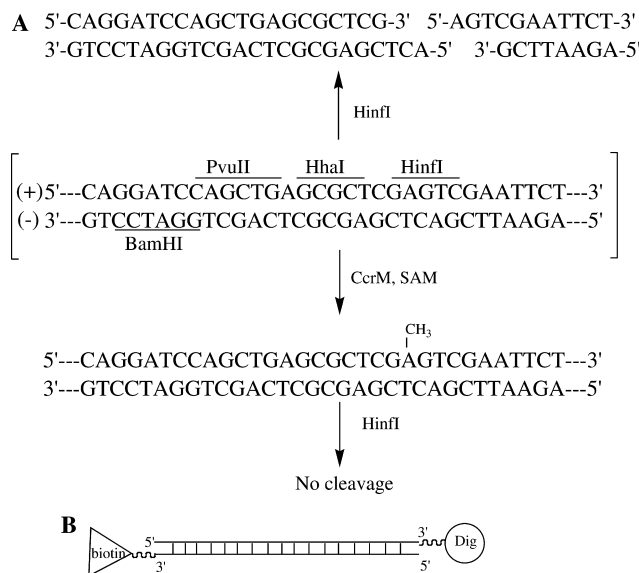


Fig. 1. DNA substrate. (A) Substrate DNA shown with selected recognition sites marked out of possible combinations of recognition sites. (B) Modified substrate, labeled with digoxigenin at one 3' end and biotin at the other 3' end. Dig, digoxigenin.

on the column with buffer A (50 mM Tris, pH 7.7, 1 mM EDTA), washing with 25% buffer B (50 mM Tris, pH 7.7, 1 mM EDTA, 1 M NaCl) and 75% buffer A, and eluting with a gradient of 25% buffer B to 100% buffer B (2 ml/min). The collected fractions (150 ml total) were monitored with UV absorbance. The fractions with DNA were pooled and purified by ethanol precipitation. The two labeled DNA strands were annealed by heating at 95°C for 15 min followed by cooling to room temperature over 12 h. The concentration of double-stranded DNA was determined by absorbance at 260 nm.

### DNA immobilization

DNA immobilization was performed following established protocols [13]. MaxiSorp plates (Nunc) were coated with 100 μl of a 5-μg/ml solution of streptavidin in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) by overnight incubation at 4°C. Each well was washed with 200 μl of TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) three times. Then 100 μl of 1-ng/ml DNA solution in TBS-T was added to each well and the plates were incubated at 37°C for 1 h followed by washing with 200 μl of TBS-T three times.

### Methyltransferase reaction

Each well with DNA was treated with a given methyltransferase in an appropriate reaction buffer (CcrM: 50 mM potassium phosphate (pH 7.5), 150 mM potassium acetate, 5 mM β-ME; *HhaI*: 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM β-ME) with 30 μM SAM and 250 nM CcrM or 300 nM *HhaI*, respectively, at 37°C for 1 h with or without inhibitors. The plate was then washed with 200 μl of TBS-T three times.

### Endonuclease reaction

Reactions were performed using the manufacturer recommended buffers and conditions followed by washing the wells with 200  $\mu$ l of TBS-T three times.

### Measuring restriction protection

The remaining substrate on the plate was probed at 37°C for 1 h with 0.075 U/well of Anti-Dig-Alkaline-phosphatase (Roche) in 100  $\mu$ l of TBS-T (1:1000 dilution). After washing the plate with 200  $\mu$ l of TBS-T five times, 100  $\mu$ l of substrate solution containing 10  $\mu$ l of 5 mg/ml *p*-nitrophenyl phosphate (pNPP; Sigma) and 90  $\mu$ l of alkaline phosphatase buffer (50 mM glycine-NaOH, pH 9.0, 0.5 mM MgCl<sub>2</sub>) was added to each well. The absorbance was measured at 405 nm after incubating the plate at 37°C for 30–45 min.

### Results and discussion

The substrate DNA was designed/engineered to permit site-specific methylation by multiple Mtases as shown in Fig. 1A. A carefully designed substrate allowed us to assay even the Mtases without cognate restriction enzymes. For example, both *Hinf*I and *Ccr*M have the same recognition site (5'-GANTC-3'); thus methylation of this site blocks *Hinf*I activity. Likewise, hDNMT1 (human DNA Mtase1) with a (5'-GCGC-3') recognition site can also be tested using the same DNA substrate and *Hha*I endonuclease. The design of the probe provides a positive selection in an inhibition of the methyltransferase since signal is lost by restriction endonuclease cleavage (Fig. 1A).

The 3' end of the (–) strand was labeled with biotin to permit immobilization onto the streptavidin-coated plate. The 3' end of the (+) strand, which contained an amino group, was derivatized with digoxigenin as a substrate for the later ELISA quantification (Fig. 1B). Our assay design is depicted in Fig. 2.

Maxisorp plates (Nunc) were selected after testing a series of different ways to immobilize DNA. Initially, the substrate was immobilized onto precoated maleimide plates (Pierce) through the 3' amino group. However, the ability of endonucleases to cleave the DNA was compromised. It is also likely that the commercially available precoated streptavidin plates have streptavidin at too high a density, thus preventing the enzyme from binding the DNA in a productive state to interact with the substrate. Different series of plates and magnetic beads were tested for the application, but none provided the desired level of restriction reaction. This difficulty was circumvented only with certain plates that we constructed to control the concentration of streptavidin on the plate and accordingly the concentration of DNA immobilized. It is crucial to maintain an optimal streptavidin density on the plate so that the DNA and enzyme can readily

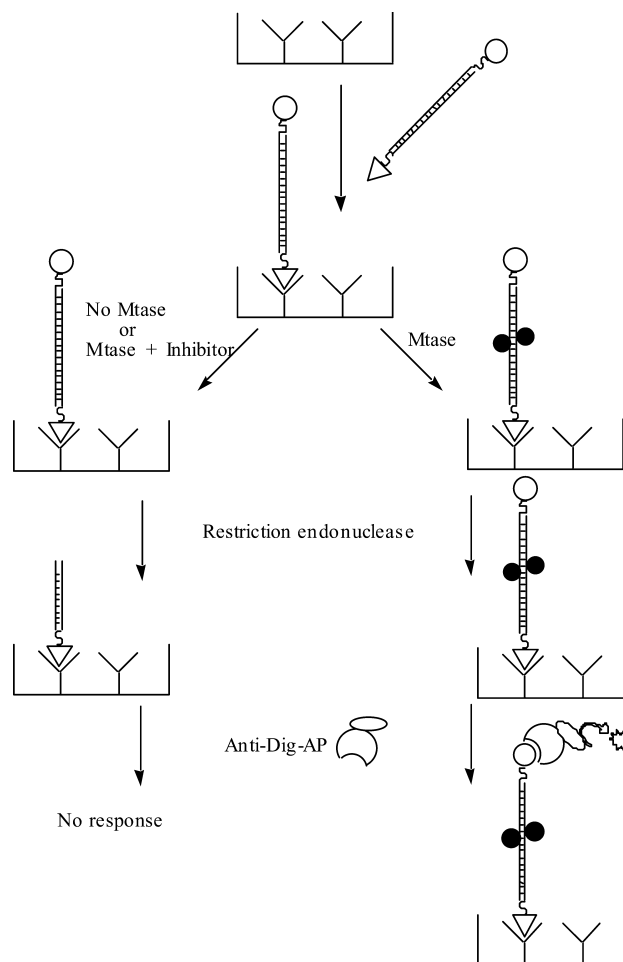


Fig. 2. Assay scheme where digoxigenin  $\circ$ , methylated base  $\bullet$ , biotin  $\Delta$ , and a DNA substrate is bound to streptavidin Y.

interact with each other and avoid steric hindrance to their association [13,15]. After applying streptavidin at a series of different concentrations onto the plate, 10 pmol/well of streptavidin gave the most desirable ELISA response.

Once the concentration of streptavidin was optimized, different concentrations of DNA were added to the plates, which were incubated at 37°C, washed, and probed with either Anti-Dig-POD (Roche, detection by TMB) or Anti-Dig-AP (Roche, detection by pNPP) to quantitate the amount of attached DNA. The requisites to be considered in selecting the method were (1) a baseline level of sensitivity to detect the remaining methylated DNA and (2) a large dynamic range of signal for a small change in substrate concentration. The response by TMB did not meet the second requirement; i.e., the difference between the background (a control reaction without Mtase but with endonuclease) and the positive control (a control reaction with both Mtase and endonuclease) was too small. On the other hand, pNPP provided a significant range of signal (ca. 10-fold difference between the background and the positive control).

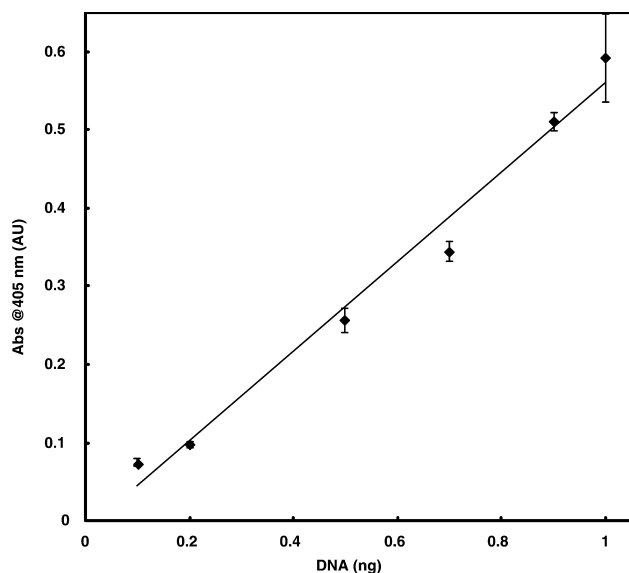


Fig. 3. Relationship between absorbance at 405 nm and DNA concentration ( $y = 0.57x - 0.018$ ,  $R = 0.99$ ). Error bars represent the standard deviation from the results performed in triplicate.

The response utilizing pNPP with different concentrations of DNA showed a linear correlation in a range of 0–1 ng of DNA (Fig. 3).

Despite a prolonged reaction time of 20 min to 2 h, endonucleases did not complete cleavage of the DNA substrates and a residual signal that originated from uncleaved DNA was noticed. We attribute this to the nature of endonucleases being tested and the difficulty of achieving complete cleavage causing the signal from a very small amount of the remaining substrate to be amplified. Therefore, control reactions were set up with endonuclease but without Mtase treatment to define a control baseline that varies as the extent of substrate cleavage changes from the different restriction enzymes. Correspondingly the dynamic range was set as follows: the upper level of signal from the reaction with Mtase only without treatment with the corresponding endonuclease was defined as 100% activity of the Mtases and the lower level of signal from the reaction with endonuclease but without the Mtase was defined as 0% activity.

S-adenosyl-homocysteine (SAH), a universal inhibitor for methyltransferases, was used as a standard inhibitor and a series of compounds developed in our laboratory were tested against two model DNA methyltransferases, *HhaI* and *CcrM*. *HhaI* is a very-well-studied example of a bacterial C5-cytosine Mtase, whereas *CcrM* is a N6-adenine Mtase without a cognate endonuclease. Fig. 4 shows the results of select inhibitors against both enzymes. Assays were repeated three times in triplicate for each concentration of inhibitor (two-tailed  $p$  value,  $0.82 \pm 0.08$ ;  $t$  value,  $0.22 \pm 0.06$ ; % error,  $7.5 \pm 5\%$ ). In Fig. 5 we show that the activity of *HhaI* Mtase changes in a concentration-dependent manner with a select inhibitor, AN0195 (details to be published else-

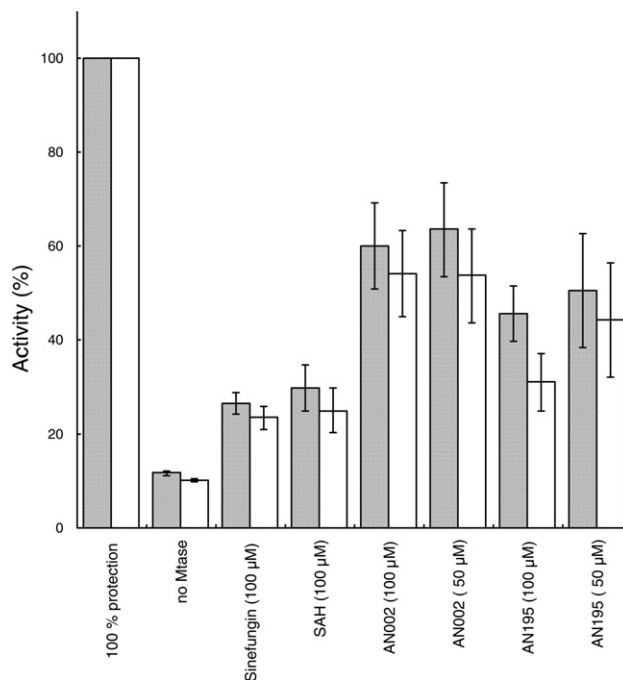


Fig. 4. Inhibition of Mtases by select compounds; gray bars indicate % *HhaI* Mtase activity; white bars indicate % *CcrM* activity. Error bars represent the standard deviation from three experiments in triplicate.

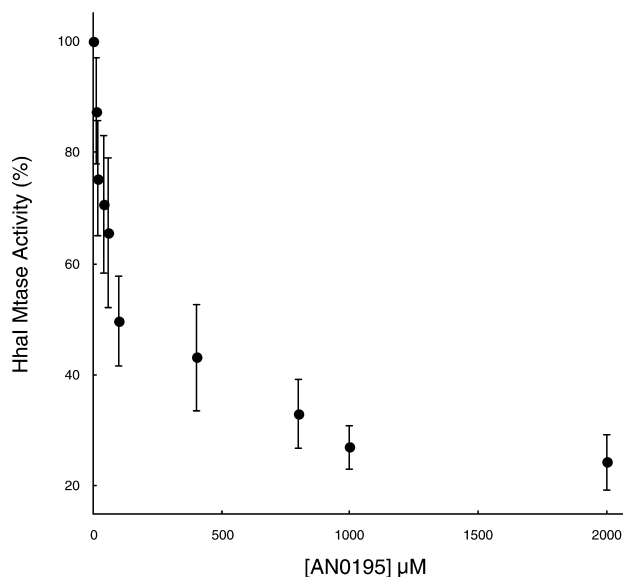


Fig. 5. *HhaI* Mtase activity related to concentration of inhibitor (AN0195). Error bars show the standard deviation from the experiments performed in triplicate.

where), which suggests that this method can also be applied to kinetic studies of inhibition.

A filter-binding assay utilizing [ $^3$ H]SAM and DE81 (Whatman) filter paper [14] was also performed with the same inhibitors in parallel. The compound AN002 (details to be published elsewhere) showed  $60 \pm 5\%$  inhibition of *CcrM* activity at 100  $\mu$ M from our filter-binding assay, which is close to the  $58 \pm 4\%$  inhibition obtained from this protocol.

This assay has a number of advantages: it requires no radiolabeled cofactor, it is robust and reproducible, it can be configured for high-throughput screening and it can be generalized to a broad spectrum of DNA methyltransferases that have no cognate restriction enzyme.

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