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Fluorescence-based high-throughput assay for human 5-methylcytosine DNA methyltransferase ¹

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Abstract

We have developed the first economical and rapid nonradioactive assay method that is suitable for high-throughput screening of the important pharmacological target human DNA (cytosine-5)-methyltransferase I (DNMT1). The method combines three key innovations: the use of a truncated form of the enzyme that is highly active on a 26 base pair hemimethylated DNA duplex substrate, the introduction of the methylation site into the recognition sequence of a restriction endonuclease, and the use of a fluorogenic readout method. The extent of DNMT1 methylation is reflected in the protection of the DNA substrate from endonuclease cleavage, which would otherwise result in a large fluorescence increase. The assay has been validated in a high-throughput format and trivial changes in the substrate sequence and endonuclease allow adaptation of the method to any bacterial or human DNA methyltransferase.

Keywords

human cytosine DNA methyltransferase 1; high-throughput assay; fluorescence detection

DNA (cytosine-5)-methyltransferases (DNMTs) play an important role in genome modification in both prokaryotic and eukaryotic cells. While bacterial methyltransferases are one component of a restriction/modification system used to distinguish self from foreign DNA [1], human DNA methyltransferases are key regulators in the epigenetic control of gene expression through the S-adenosyl methionine (SAM)-dependent methylation of the DNA base cytosine [2] (Figure 1). Two human DNMTs (DNMT1 and DNMT3) are involved in preferentially methylating CpG dinucleotides in the human genome [3,4]. While both enzymes show *de novo* methylation activities, DNMT1 reacts preferentially with hemimethylated DNA [5,6], and functions primarily to maintain methylation patterns after DNA replication [6,7]. DNMT1 has been implicated in the hypermethylation of CpG islands at the promoter regions of tumor suppressor genes, an epigenetic modification that occurs in nearly all cancers and often results in transcriptional silencing of the anticancer gene [8–11]. Unlike mutations that

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promote cancer, such epigenetic events are reversible and amenable to pharmacological intervention, making DNMT1 an attractive anticancer drug target [12,13].

Two FDA-approved drugs that target DNMTs (azacytidine and decitabine) have been found to provide treatment benefit as single agents for a specific indication (myelodysplasia) [14]. However, both of these are nucleoside prodrugs that must first be converted to their triphosphate forms and then incorporated into DNA before they act on their DNMT targets. Not surprisingly, these compounds show high toxicity [15–17], and despite their demonstrated therapeutic effects, it is desirable to have non-nucleoside inhibitors that act without being incorporated into genomic DNA. Thus, there is a great need for additional compounds that target this enzyme, and a robust and convenient high-throughput (HTP) activity assay would be highly desirable for such discovery efforts.

A nonradioactive HTP activity assay for human DNMT1 has been elusive partly because this is a large, complex, multidomain protein that shows complicated allosteric activation by hemimethylated DNA. In addition, the full length protein shows relatively slow turnover on nonmethylated DNA substrates as compared to the simpler *HhaI* bacterial methylase [18,19]. Although a nonradioactive HTP screening method for the *HhaI* enzyme has been developed [20], no such assays have been reported for DNMT1.

In this manuscript, using a truncated and highly active form of human DNMT1, and a carefully designed hemimethylated DNA substrate, we have fully developed the first robust, efficient and economical fluorescence assay suitable for in vitro HTP screening of DNMT1.

Material and methods

5'-Fluorescein labeled 26-mer oligonucleotide (5'-Fam-CATATCAGGATCGATGGCAGTTAGAA-3') and 3'-Dabsyl labeled 26-mer (5'-TTCTAACTGCCAT^{me}CGATCCTGATATG-Dab-3') oligonucleotide were purchased from Integrated DNA Technologies (IDT). The DNA substrate was annealed by heating at 95°C for 10 min followed by cooling slowly to room temperature over 16 hr. Restriction enzyme *Sau3AI* was purchased from New England Biolabs. A DNA sequence (5'-GTACATGXGCTCCAGA-3', X=DHC) containing the transition-state analogue nucleoside, 5,6-dihydro-deoxycytidine (DHC), was synthesized on a ABI 394 solid phase DNA synthesizer using commercially available nucleoside phosphoramidites (Glen Research). The resulting oligonucleotide and its complement strand were purified by anion exchange chromatography and were desalted using a reverse phase C18 HPLC column before hybridization.

Preparation of $\Delta 501$ DNMT1

The intein-chitin binding domain (intein-CBD) tag was obtained by digesting pTYB2 vector using *EcoRI* and *PstI* restriction enzymes. The tag was purified by 1% agarose gel and ligated into pFastBac plasmid using *EcoRI* and *PstI* restriction sites. The gene encoding $\Delta 501$ DNMT1 was amplified from the full-length DNMT1 gene (kindly provided by Dr. William Nelson) using forward and reverse primers (5'-ATATTTTCGGTCCGATGCAGGAGAAGATCTACATCAGCAAGATTGT-3') and (5'-ATATTTGAATTTCGTCCTTAGCAGCTTCCTCCTCCTTTATTTTAG-3'), respectively. The PCR product was inserted into pFastBac containing the intein-CBD tag using *RsrII* and *EcoRI* sites. To obtain Bacmid DNA suitable for transfection of insect cells, the recombinant plasmid containing the $\Delta 501$ DNMT1-intein-CBD insert was transformed into competent DH10Bac *E. coli* cells. The Bacmid DNA was purified using a Midiprep kit (Qiagen), and was used to transfect Sf9 insect cells using Cellfectin reagent to generate a P1 insect cell virus stock². The P1 virus stock was amplified twice with a multiplicity of infection (MOI) of 0.1. The resulting P3 virus (titer $\approx 1 \times 10^8$ /mL) was utilized to infect Sf9 insect cells (2×10^6 cells/

mL, 750 mL, MOI = 0.7) that were maintained in TNM-FH media supplemented with 10% (v/v) bovine calf serum and 50 µg/ml each of streptomycin and penicillin. The infected Sf9 cells were stirred at 70 rpm at room temperature for 72 hr.

The cells were spun down at 1000 g for 15 min, suspended in 25 mL buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA and one Roche Mini Tab protease inhibitor) and lysed with a Downs homogenizer. The lysed cells were passed through a chitin beads column and washed with buffer containing 25 mM HEPES, pH 7.5, 250 mM NaCl, and 1 mM EDTA to allow binding of the intein-CBD tagged $\Delta 501\text{DNMT1}$. The cleavage was induced by incubating the column overnight with 50 mM DTT, 50 mM HEPES, 250 mM NaCl and 1 mM EDTA. Next the column was flushed with 50 mL column buffer and the eluate, which contained $\Delta 501\text{DNMT1}$ and residual chitinase from the insect cells, was collected and concentrated 10-fold using an Amicon Ultra-15 concentrator. The concentrated solution was passed back over the chitin bead column to remove the chitinase ($\Delta 501\text{DNMT1}$ yield ~0.32 mg, purity $\geq 95\%$, see supplemental information). This amount of enzyme is sufficient for 2,000 screening reactions.

High-throughput activity assay for $\Delta 501\text{DNMT1}$

Fluorescence measurements were made using a Spex Fluoromax 3 instrument outfitted with a MicroMax 384 microwell-plate reader. To reduce interference by stray excitation light, a 510 nm cut-on filter was inserted before the emission detector. Assays were performed in 96-well microtiter plate format (Corning 3600, white) in two steps. First, methylation was performed by adding 2 µL of $\Delta 501\text{DNMT1}$ (10 nM final) to each well that contained 73 µL of the substrate mixture (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.002% Brij35, 5% glycerol, 4 µM SAM, and 125 nM labeled DNA substrate). The reactions were incubated at room temperature for 1 hr resulting in about 60% methylation of the substrate. Then in a second step, restriction endonuclease cleavage was performed by adding 80 µL of a solution consisting of 100 mM KCl, 11 mM MgCl₂ and SauA3I restriction enzyme (8 units) to each well. The endonuclease digestion was continued overnight at room temperature. The percentage of substrate methylation was calculated from measurements of the background corrected fluorescence signals ($\lambda_{\text{em}} = 521$ nm, 6 nm slit width, $\lambda_{\text{ex}} = 482$ nm, 1 nm slit width) of each methylase reaction (F) as compared to two reference reactions corresponding to 0% (F_{max}) and 100% (F_{∞}) methylation (eq 1).

$$\% = (F_{\text{max}} - F) / (F_{\text{max}} - F_{\infty}) \times 100 \quad (1)$$

The 0% reference reaction was identical to the screening reactions except that the methyltransferase was omitted. The 100% reference was identical to the screening reactions except that the SAM concentration was increased to 100 µM to drive complete methylation of the substrate. The Z-factor for the assay was calculated using eq 2, where F_0 and F_{max} are the initial final fluorescence readings of the reactions, and σ_{max}^2 and σ_0^2 are the standard deviations of the measured values of F_{max} and F_0 .

$$Z = 1 - \{3(\sigma_{\text{max}}^2 + \sigma_0^2) / (F_{\text{max}} - F_0)\} \quad (2)$$

Inhibition by Adenosine and DHC

The inhibition assays were performed by dissolving 0.1–10 mM adenosine and 4–200 nM DHC in reaction buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.002% Brij 35, 5%

²The plasmid expression constructs containing $\Delta 501\text{DNMT1}$ are available upon request.

glycerol, 4 μ M SAM) containing 125 nM DNA substrate. The methylation and endonuclease reaction steps were performed as described in the previous section. The percentage of inhibition was calculated using eq 1 and the observed inhibition values at each inhibitor concentration were fitted to eq 3,

$$\% \text{ inhibition} = 100 \times [I]/[I] + IC_{50} \quad (3)$$

where $[I]$ is the concentration of the inhibitor and IC_{50} is the apparent inhibition constant.

Results and discussion

Our fluorescence-based high-throughput assay is based on the idea that DNMT1 can protect a DNA site from restriction endonuclease cleavage through its methylation activity (Fig. 2). An ideal restriction endonuclease for this application is Sau3AI because the enzyme recognizes the palindromic sequence 5'-GATC-3' even when a ^5MeC base is adjacent to the 5' G of the consensus sequence (i.e. 5'- ^5MeC GATC-3'). Although the presence of a ^5MeC at this position has no effect on the Sau3AI activity, it generates a hemimethylated $^5\text{MeCpG}$ target site that is a preferred substrate for DNMT1. Thus in this hemimethylated sequence context, DNMT1 readily methylates the 3' C of the consensus sequence thereby inactivating the site for restriction cleavage. In order to accelerate the rate of DNMT1 with this hemimethylated substrate, the 501 amino acids at the N-terminus were deleted. Previous studies have demonstrated that the truncated DNMT1 ($\Delta 501\text{DNMT1}$) exhibits a four-fold increased k_{cat}/K_m as compared to the full-length enzyme and does not require allosteric activation by hemimethylated DNA[5].

The time-dependent methylation status of the restriction site is assessed using convenient molecular beacon technology (Fig. 3). The DNA substrate has low fluorescence because the 5'-Fam group is strongly quenched by the dabsyl group attached to the 3' end of the opposite strand. However, after complete digestion with Sau3AI, the DNA substrate is cut into two pieces and the two strands containing the Fam and dabsyl labels spontaneously dissociate at room temperature due to their limited base pairing (seven AT-rich base pairs). Separation of the Fam-dabsyl pair results in a six-fold increase in Fam fluorescence, providing a robust signal change to follow the methylation state of the site (Fig. 3A, compare spectra 1 and 4). Maximization of the signal difference between the digested and undigested substrate required careful optimization of the monovalent and divalent cation concentrations during the endonuclease readout step (Fig. 2). While low cation concentrations lead to incomplete digestion of the substrate, high monovalent or divalent cation concentrations stabilize the digested duplex and therefore prevent the fluorescein labeled 7-mer from disassociating. In both cases, the signal difference between intact and digested substrate is reduced. In our assay conditions, the salt concentration has been optimized to ensure the maximum sensitivity of the assay. Moreover, addition of 5.5 mM MgCl_2 to the methylation reaction inactivates $\Delta 501\text{DNMT1}$, providing a convenient quench, while also providing the essential divalent cation for the endonuclease read-out step.

As expected, complete methylation of the substrate DNA by $\Delta 501\text{DNMT1}$ protects the substrate from endonuclease digestion (Fig. 3, spectrum 2), and inclusion of the specific DNMT1 inhibitor during the reaction prevents methylation and results in full development of the expected fluorescence (Fig. 3, spectrum 3). The fully methylated DNA product has a 1.6-fold higher fluorescence than the hemimethylated substrate is possibly due to slight changes in the average environment of the FAM and dabsyl groups after methylation (Fig. 3, compare spectra 2 and 1).

We tested the linearity of the assay under a range of conditions (1 to 100 μ M SAM, 125 to 500 nM DNA)(Fig. 4). Using standard screening conditions, the rate of product formation was in

the linear range for about 2 hours (~40 % conversion). For screening purposes it is useful to allow methylation to proceed to approximately ~50 to 60%, which provides a robust signal from which to measure inhibition. In addition, the assay is quite flexible with respect to the substrate concentrations that may be used. For optimal detection of inhibitors that may be competitive with SAM, it is useful to use a cofactor concentration that is equal to or less than the $K_m^{\text{SAM}} \sim 4 \mu\text{M}$ [21], while uncompetitive and noncompetitive inhibitors can be easily detected using much higher concentrations of SAM. The standard DNA concentration of 125 nM is lower than the $K_m^{\text{DNA}} \sim 200 \text{ nM}$ [21], and thus allows for the detection of inhibitors that are competitive with respect to the DNA substrate. However, DNA concentrations in the range 125 to 500 nM have been validated in our studies.

The reproducibility of the assay under HTP conditions was validated using 24 replicate reactions (Fig. 5). The coefficient of variation is only 2.3 % for the change in fluorescence upon full conversion of 125 nM molecular beacon substrate to product by the restriction enzyme. Even a modest amount of MTase inhibition (30%) by the potent DHC DNA inhibitor, gives a statistically significant signal change as compared to the uninhibited reaction ($p < 0.0001$). The Z-factor (eq 2) calculated from these data is excellent (0.84). Assuming a typical screening concentration of 20 μM compound, these results indicate that the assay is sufficiently robust to detect weak micromolar inhibitors (K_i values $\leq 50 \mu\text{M}$).

To further validate the HTP assay for the detection of inhibitors, we characterized the binding of both a weak small-molecule inhibitor (adenosine) and a strong transition-state DNA inhibitor (DHC) of DNMT1 (Fig. 6). In order to allow adenosine to compete effectively with SAM cofactor binding, the SAM concentration was reduced to 1 μM in the experiment in Figure 4A, and the DNA concentration was 125 nM in both experiments. Inhibition by adenosine ($\text{IC}_{50} = 3 \text{ mM}$) and DHC ($\text{IC}_{50} = 50 \text{ nM}$) showed hyperbolic concentration dependences that extrapolated to complete inhibition at infinite inhibitor concentrations. Further studies where the SAM concentration was varied in the range 1 to 100 μM confirmed that adenosine and SAM bind competitively to the cofactor site (not shown). Thus, the adenosine fragment of SAM appears to be able to capture some of the interactions of the enzyme with the adenine base and 2' and 3' sugar hydroxyl groups as observed in the crystal structure of bacterial *Hha1* MTase in presence of the bound SAM cofactor [22]. DHC containing DNA is competitive with the substrate, with the DHC moiety mimicking the transition-state geometry of the substrate. Thus, the assay is useful for detection of both weak and strong inhibitors of either substrate of the DNMT1 reaction.

Conclusions

We have validated an economical and rapid HTP assay for human DNMT1 that does not require radioactive substrate. The assay is easily implemented using a standard fluorescence spectrometer using a microtiter plate format. Using manual multichannel pipetting, the assay is suitable for screening about five hundred compounds in a single day (one compound per well). With modern robotic instrumentation, the method is easily scalable to allow screening of large libraries containing $>10^5$ compounds. In addition, with simple modifications to the substrate and read-out restriction endonuclease, the method is easily adapted for the screening of other cytosine and adenine DNA methyl transferases. For instance, the bacterial *Hha1* cytosine- C^5 -DNA methyltransferase may be assayed using a 24 basepair DNA substrate containing the sequence GCGC, using *Hha1* endonuclease as the read-out restriction enzyme. The adenine- N^6 -DNA methyltransferase *dam* may be assayed using a duplex containing the sequence GATC, using *Mbo1* as the read-out restriction endonuclease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TNM-FH	Trichoplusia ni medium-formulation Hink
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid

Fig. 1.
Enzymatic mechanism of DNMTs. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine.

Fig. 2.

Two-step fluorescence-based high-throughput activity assay for human DNMT1. The Sau3AI restriction site is shown by arrows. Fam: 6-carboxyfluorescein (green). Dab: Dabsyl quench (yellow). The cytosine 5-methyl group is colored in red.

Fig. 3.

Fluorescence emission spectra of reactant and product states ($\lambda_{\text{ex}} = 484 \text{ nm}$). Spectra were obtained for the following conditions: (1) substrate DNA only; (2) substrate DNA, $\Delta 501\text{DNMT1}$ and RE; (3) substrate DNA, DHC-quench duplex, $\Delta 501\text{DNMT1}$, and RE; (4) substrate DNA + RE. RE, Restriction enzyme. All reactions contained $[\text{SAM}] = 100 \mu\text{M}$, $[\text{DNA}] = 125 \text{ nM}$, and $[\Delta 501\text{DNMT1}] = 10 \text{ nM}$.

Fig. 4.

Time course for cytosine methylation by $\Delta 501\text{DNMT1}$ as determined by the fluorescence assay. Conditions were: $[\text{SAM}] = 1 \mu\text{M}$, $[\text{DNA}] = 125 \text{ nM}$, $[\Delta 501\text{DNMT1}] = 10 \text{ nM}$. The % methylation was calculated from the fluorescence signals using eq 1.

Fig. 5.

Reproducibility and statistics of methylation assay in a microtiter plate format. The values from twenty-four replicate measurements are shown as dots, with mean values and standard deviations shown by lines ($\lambda_{em} = 521$ nm, $\lambda_{ex} = 484$ nm). Measurements were made using the following conditions (1) DNA substrate only (S), (2) S after complete digestion by restriction enzyme (RE), (3) S and $\Delta 501$ DNMT1 (MT) sufficient to fully methylate and protect the DNA from subsequent endonuclease digestion, and (4) S, MT and sufficient DHC duplex inhibitor (I) to result in 30 % inhibition of methylation.

Fig. 6. Inhibition of DNMT1 by adenosine nucleoside (A) and a 25-mer duplex DNA containing 5-aza-5,6-dihydrocytosine (DHC) (B). The experiments were performed in triplicate and the standard deviations are indicated. Conditions were [SAM] = 1 μ M, [DNA] = 125 nM, and [Δ 501DNMT1] = 10 nM. Abbreviations: (A), [adenosine] = 0.1, 0.5, 1, 4, 10 mM; [DHC] = 4, 10, 20, 50, 100, 200 nM.