Development of a Non-Radioactive, No-Wash Biochemical Assay for High-Throughput Screening of Small Molecule Modulators of DNA Methyltransferases

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1 Abstract

Covalent modification of DNA through methylation is catalyzed by specific DNA methyltransferases (DNMTs). DNMT1, 3a and 3b are the best characterized mammalian enzymes of this class, with DNMT1 thought to be responsible for maintenance of the methylated state, and DNMT3a and 3b responsible for de novo DNA methylation. Changes in DNA methylation patterns alter gene expression that may lead to human diseases. Until recently, DNA methylation was thought to be an irreversible process that was active only in mitotic cells; however recent findings suggest that the reverse process, through active demethylation, may occur in both mitotic and postmitotic cells. This opens the door to the possibility of using DNMT inhibitors to modulate aberrant gene expression in a variety of quiescent cell types. We describe here the development of a high-throughput, non-radioactive bead-based assay for DNA methylation that is suitable for screening applications to identify new DNMT inhibitors. For this purpose, biotinylated oligonucleotide is incubated with the enzyme in the presence of the cofactor SAM, following which 5-methyl-cytosine residues are quantified in a homogenous bridging assay format. The activity of different DNMT preparations was compared using this approach. Purified DNMT1 showed higher activity than 3a and 3b for the methylation of the oligonucleotide substrate, however we noted significant differences between DNMT1 preparations from different commercial vendors. A signal-to-background ratio (S/B) of approximately 40 was obtained using as little as 10 nM of DNMT1 with intra-assay variation of 7%. Signal generation was dependent on the presence of SAM, and decreased using known DNMT inhibitors, demonstrating the specificity of the reaction. Overall, the results presented will demonstrate that this novel homogenous and non-radioactive DNMT assay could represent a powerful alternative to established assay technologies for measuring methyltransferase activity.

2 Materials and Methods

Materials

DNMT1 was obtained from Abcam and the different oligonucleotides were synthesized by Integrated DNA Technologies, Inc. S-adenosylmethionine (SAM) was purchased from Zymo Research. White OptiPlate™-384, TopSeal-A™, EnVision® Multilabel Plate Reader, AlphaLISA® Acceptor beads, and Streptavidin Donor Beads were from PerkinElmer Inc. SAH and sinefungin were purchased from Sigma-Aldrich, Inc. The target DNA sequence contained 10 CpG methylation sites sites on each strand (shown in blue below), as described before[1].

biotin-A_{12}- GATCCGACGACGACGCGCGCGCGACGACGAGATC
CTAGGCTGCTGCTGCGCGCGCGCTGCTGCTCTAG-A_{12}-biotin

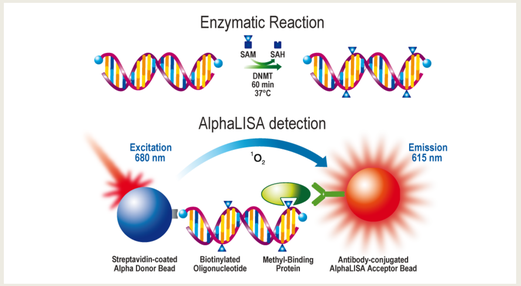
DNA Methylation Protocol

The typical methylation reaction contained 5 µL of DNMT1 mixed with SAM to obtain reaction concentrations of 10 nM and 600 µM, respectively. 5 µL of oligonucleotide was added (10 nM). The enzymatic reaction was incubated at 37ºC for one hour. The enzymatic reaction was performed in 20 mM Tris-HCl, pH7.4, 0.5 mM EDTA, 0.2 mM DTT, 5% glycerol. The negative control contained equivalent reagents, but lacked cofactor S-adenosylmethionine.

AlphaLISA Protocol

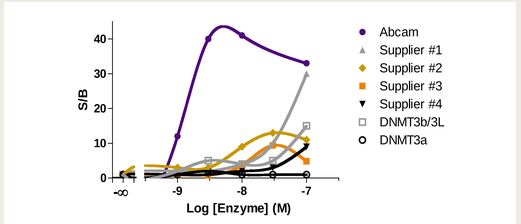
Immediately upon the methylation reaction, 15 µL of AlphaLISA anti-tag Acceptor beads were added (10 µg/mL final) along with tagged-methyl binding protein (MeBP) followed by an incubation time of 60 min at 23ºC. Finally, 25 µL of AlphaScreen® Streptavidin-coated Donor beads was added at a final concentration of 40 µg/mL. All the detection reagents were diluted in PBS, 0.1% BSA and 0.1% Tween-20. The detection reaction was then incubated for 30 minutes at 23ºC and read with an Envision Multilabel Plate Reader. The final assay volume is 50 µL.

3 DNMT Assay Principle



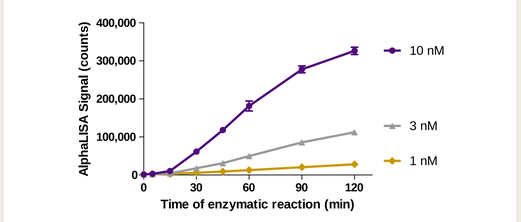
Following the enzymatic reaction, methylated product is simultaneously captured by the streptavidin Donor beads and anti-tag Acceptor beads that interact with the tagged MeBP. An Alpha signal is generated once Donor beads are excited at 680 nm which provokes the release of singlet oxygen molecules that activates the Acceptor beads in close proximity to emit a sharp peak of light light at 615 nm.

4 DNMT1 Enzyme Selection



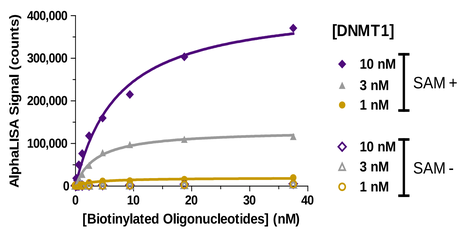
Different DNMT1 suppliers were tested at multiple concentrations. The S/B were calculated as the ratio of counts generated with and without SAM. For DNMT1 high variation between providers was observed. DNMT3a and 3b generated lower signals on the non-methylated substrate. The enzyme from Abcam was selected for future experiments.

5 Enzyme Reaction Progress Curves



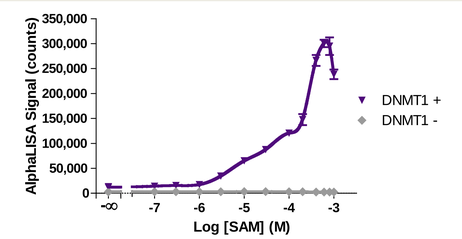
Three concentrations of enzymes were used in a time-course experiment for up to 2 hours of enzymatic reaction at 37°C. Optimalconditions were determined as 10 nM of enzyme with an incubation period of 60 minutes. There is 15 minutes lag before the reaction becomes linear, this could represent the time needed for the microplate to heat up to 37°C.

6 Oligonucleotide Titration



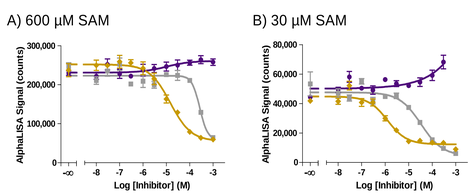
Substrate (biotinylated oligonucleotide) was titrated with 1, 3 and 10 nM of enzyme. Using 10 nM of enzyme a $K_{m\ app}$ of 7.43 nM for the oligonucleotide was calculated. Therefore, 10 nM of substrate was determined as the optimal condition.

7 SAM Titration



A SAM titration curve was performed. Surprisingly, a biphasic curve was obtained. The purple arrows indicate the selected concentrations used for the inhibitors assay and the Z' study (30 and 600 µM).

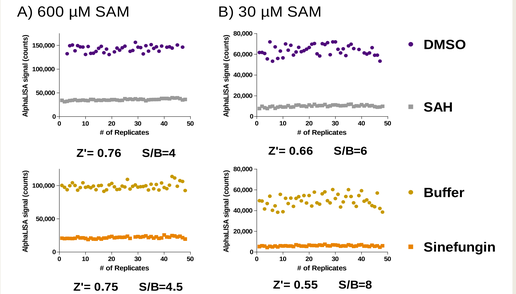
8 Inhibition of DNMT1 Activity



	[SAM] (µM)	Sinefungin	SAH
IC_{50} (µM)	30	33.5	1.3
	600	268.0	14.8

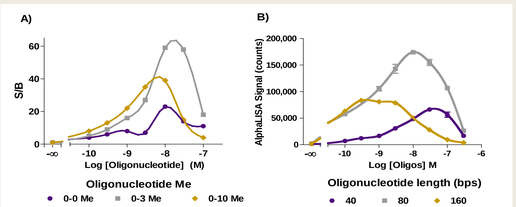
Known DNMT1 competitive inhibitors were tested at both 600 µM (A) and 30 µM (B) of SAM. As demonstrated previously, DMSO was shown to have an activator effect on DNMT1[2].

9 Z' study of DNMT1 Assay



DNMT1 assay robustness was evaluated in a Z'-factor analysis with two concentrations of the cofactor SAM. Sinefungin and SAH were used at a concentration inhibiting all enzymatic activity (1 mM). Using 600 µM of SAM, Z'-factor was ≥0.75 while 30 µM of SAM resulted in Z'-factor ≥ 0.55.

10 Oligonucleotide Optimization



Different methylation levels of the 40 bps oligonucleotide were tested (A) along with the length of the oligonucleotide (B). The results indicate that the assay window can be increased using an oligonucleotide that is already partially methylated on one of its strands. Increasing the length of the oligonucleotide from 40 bps to 80 bps also resulted in higher counts.

11 Summary

- **These results demonstrate the successful development of a non-rad DNMT1 assay using the Alpha technology.**
- **The characteristic sensitivity and reproducibility of Alpha technology makes this assay a fast, robust, and easily HTS-amenable alternative for screening of DNMT modulators..**
- **Results obtained using different substrate configurations indicate that assay performance can be further improved and different substrates might be optimized for different class of DNMT.**

References
1.Yokochi, T. et al. J Biol Chem. 2002 Apr 5;277(14):11735-45.
2.Yokochi, T. et al. Bioorg Chem. 2004 Aug;32(4):234-43.

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