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 DNMT3b 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 oligonucleotides is incubated with the enzyme in the presence of the cofactor SAM, following which 5-methylcytosine residues are quantified in a homogenous bridging assay format. The activity of different DNMT preparations was compared using this approach. Purified DNMTs 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. Inter-assay variation of 7%. Signal generation was dependent on the OptiPlateTM-384, TopSeal-ATM, EnVision® Multilabel Plate Reader, adenosylmethionine (SAM) was purchased from Zymo Research. White AlphaLISA® Acceptor beads, and Streptavidin Donor Beads were from PerkinElmer Inc. SAM was purchased from Sigma-Aldrich Inc., Inc. The data collected on 10 ng oligonucleotide sites on each strand (shown in blue below), as described before1.

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 AlphaLISA® Acceptor beads, and Streptavidin Donor Beads were from PerkinElmer Inc. SAM was purchased from Sigma-Aldrich Inc., Inc. The data collected on 10 ng oligonucleotide sites on each strand (shown in blue below), as described before1.

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 37°C for one hour. The enzymatic reaction was performed in 20 mM Tris-HCl, pH 7.4, 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 (MBP) followed by an incubation time of 60 minutes. The conjugate from AlphaLISA reaction was added to 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 23°C 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 MBP. 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. 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 23°C and read with an Envision Multilabel Plate Reader. The final assay volume is 50 µL.

4 DNMT1 Enzyme Selection

Different DNMT1 suppliers were tested at multiple concentrations. The S/B was calculated as the ratio of counts generated with and without SAM. For DNMT1 high saturation (between yellow lines) 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. Optimization/dilutions were determined as 10 nM (A) and 30 µM (B) of SAM. As demonstrated previously, DMSO was shown to have an activator effect on DNMT1.

6 Oligonucleotide Titration

Substrate (biotinylated oligonucleotide) was titrated with 1, 3 and 10 nM of enzyme. Using 10 nM of enzyme a Km 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 curve indicates that the selected concentrations used for the inhibitors assay and the Z’ study (10 and 600 µM).

8 Inhibition of DNMT1 Activity

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.

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 of 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-radioactive DNMT 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 DNMTs.

References


PerkinElmer, Inc., 940 Winter Street, Waltham, MA USA (800) 762-4000 or (+1) 203 925-4662 www.perkinelmer.com