Automation of a Generic Fluorescent Methyltransferase Activity Assay

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Epigenetic processes are attracting considerable attention in drug discovery as their fundamental roles in controlling normal cell development and contributions to disease states become more clearly defined. Methylation is a known ubiquitous covalent modification involved in regulation of a diverse range of biomolecules. As histone methylation is linked to certain disease states, including many cancer types, Histone methyltransferases (HMTs) are of particular interest as drug targets. A high-throughput screening (HTS)-ready, universal methyltransferase activity assay was recently developed based on competitive fluorescent polarization immunodetection of adenosine monophosphate (AMP), formed from the methyltransferase (MT) reaction product S-adenosylhomocysteine (SAH) in a dual enzyme coupling step. Here we demonstrate automation of the assay in a 384-well microplate format suitable for HTS.

A combination 8-channel and single channel liquid handling instrument was used to automate histone methyltransferase (HMT) G9a titration and transfer to the assay plate in quadruplicate. Automated serial dilution and transfer of the HMT inhibitor sinefungin was also performed using the liquid handler's 8-channel head. During HMT EC_{80} determination, cofactor, substrate, stop buffer and detection reagent additions were automated using a non-contact dispenser. Dispensing of the EC_{80} concentration of HMT enzyme and all assay components were automated during HMT inhibitor IC_{50} determination. The G9a EC_{80} was determined to be approximately 80 ng/mL and used for subsequent inhibition studies, while the sinefungin IC_{50} value was determined to be 14.5 µM, showing excellent correlation with published values.

Introduction

The posttranslational modification of histones has proven to be critical for modulation of chromatin structure through direct interactions with DNA and indirectly via molecular interactions with nuclear proteins. Modulation of chromatin structure has been shown to be necessary for such processes as gene regulation, repair and cell cycle progression. The increased risk of onset of several diseases such as cancer, obesity, diabetes, and cardiovascular disease have more recently been linked to aberrations in activity levels of histone modifying enzymes and ensuing epigenetic changes[1,3]. Histone modifications include covalent modifications of the long, unstructured N-termini (histone tail) such as methylation, acetylation, ubiquitination, phosphorylation, ADP-ribosylation, citrullination, and SUMOylation (Figure 1). Several classes of enzymes are responsible for carrying out such modifications. One such class of enzymes, histone methyltransferases, is responsible for catalysis of transfer of one or more methyl groups to lysine or arginine residues on the histone tail. It has been shown that loss of monoacetylation of lysine 16 and trimethylation of lysine 20 in the histone H4 tail is associated with human tumors and represent a nearly universal marker for malignancy[2]. Thus, it is becoming clear that histone modifying enzymes represent an increasingly important class of drug targets for several disease states as well as having prognostic value[4].

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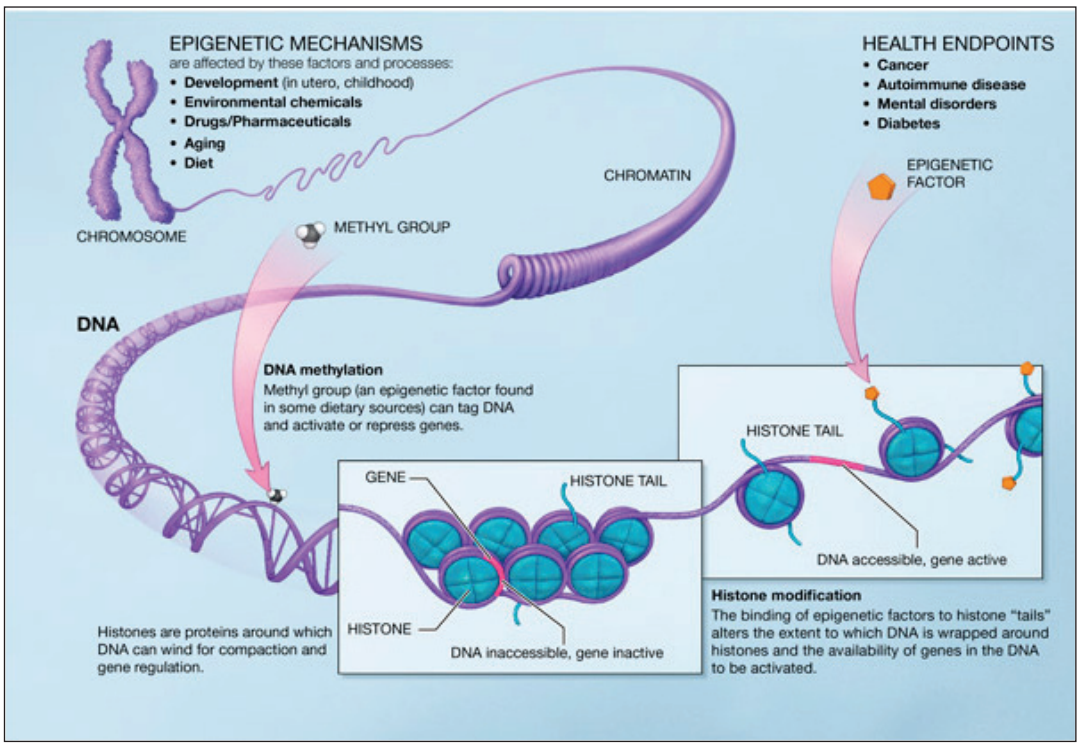

Figure 1. Histones play a central role in chromosomal structure affecting gene expression regulation. Epigenetic factors act as histone modulators, in turn controlling epigenetic mechanisms such as development and aging.

Here we demonstrate the combination of a fluorescence polarization-based assay with liquid handling and dispensing instrumentation and a multi-mode microplate reader, which may be used to monitor histone methyltransferase (HMT) G9a biological activity. A recently developed generic MT assay method uses fluorescent immunodetection of AMP, which is formed from the MT reaction product SAH in a dual enzyme coupling step (Figure 2).

The Transcreener® EPIGEN Methyltransferase Assay combines the extensively validated Transcreener AMP²/GMP² Antibody with coupling enzymes that convert SAH to AMP. The Alexa633 Tracer bound to an AMP² Antibody is displaced by AMP, the invariant product generated during the enzyme reaction. The displaced tracer freely rotates leading to a decrease in fluorescence polarization.

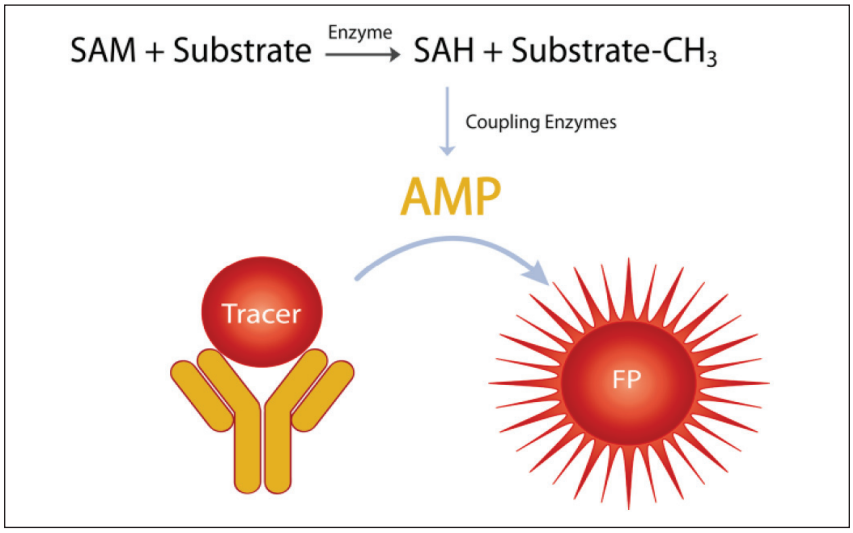

Figure 2. The Transcreener EPIGEN Methyltransferase Assay Principle. SAH produced by the target methyltransferase is converted to AMP by coupling enzymes which allows homogenous fluorescent polarization detection using the Transcreener AMP²/GMP² Assay.

Materials and Methods

The Transcreener EPIGEN Methyltransferase Assay Kit (Cat. No. 3017-1K) was a gift from BellBrook Labs, LLC. (Madison, Wisconsin, USA), and the assay was performed per the manufacturer's protocol. Workflows are summarized in Figure 3. Human recombinant G9a histone methyltransferase (HMT) (Cat. No. 51001) was purchased from BPS Biosciences (San Diego, California, USA), histone H3 (1-25), amide (Cat. No. 83643A) from AnaSpec (Fremont, California, USA), and sinefungin from Enzo Life Sciences (Plymouth Meeting, Pennsylvania, USA). Methyltransferase buffer (MT buffer) (50 mM Tris, pH=8.5, 5 mM $MgCl_2$, 1% DMSO, 4 mM DTT) was prepared daily.

Optimization of Assay Window

Two 11-point S-adenosylmethionine (SAM)/SAH standard curves, representing from 100% to 1% SAM conversion, were prepared along with zero substrate points, at 1 µM and 10 µM. 10 µL of each prepared standard curve point was transferred in column format to a 384-well assay plate using a manual multi-channel pipettor resulting in 24 replicates. 5 µL of stop buffer and detection reagent were added using a 1 µL cassette and MultiFlo™ Microplate Dispenser (BioTek Instruments, Inc., Winooski, Vermont, USA). Following a one-hour incubation at room temperature, the plate was read on the Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instrument, Inc., Winooski, Vermont, USA) using the automatic gain adjustment and xenon flash lamp with variable flash counts from 1 to 15 flashes in fluorescence polarization mode with a Ex. 620/40 nm, Em. 680/30 nm filter pair and 660 nm dichroic mirror. The reader was controlled by Gen5™ Data Analysis Software with additional data analysis performed using Microsoft® Excel® spreadsheet software (Redmond, California, USA) or GraphPad Prism® (GraphPad Software, LaJolla, California, USA). The Z'-factor was calculated at each percent conversion standard to determine the minimum substrate concentration exhibiting a sufficient assay window for high-throughput screening (HTS) applications.

Histone Methyltransferase Enzyme Titration

The Precision Microplate Pipetting System (BioTek Instruments, Inc., Winooski, Vermont, USA) was used to perform an 11-point 1:2 serial dilution, with a zero point, in a 96-well assay plate starting with an initial G9a concentration of 5 µg/mL. Eight 7 µL replicates of each dilution were transferred to a 384-well assay plate. Four replicates received 3 µL of either 2 µM SAM or 2 µM SAM plus 10 µM H3 (SAM/H3) peptide substrate dispensed using a MultiFlo dispenser. The ΔmP value of each G9a titration point was background corrected by subtracting the ΔmP of G9a plus SAM alone from the ΔmP of the reaction containing G9a plus SAM/H3 peptide. The data was analyzed in Gen5 and GraphPad Prism for EC_{80} determination for use in subsequent experiments.

Enzyme Inhibitor Titration

An 11-point 1:2 serial dilution was performed, with a zero point, in a 96-well assay plate starting with an initial concentration of sinefungin of 250 µM using the Precision Microplate Pipetting System. Eight 5 µL replicates of each dilution were transferred to a 384-well assay plate. The EC_{80} concentration of G9a in a volume of 2 µL was added to each well containing inhibitor. Four replicates received 3 µL of either SAM or SAM/H3 peptide substrate. All additions were accomplished using the MultiFlo Microplate Dispenser. Background correction was performed as previously described. The data was analyzed in Gen5 and GraphPad Prism for IC_{50} determination.

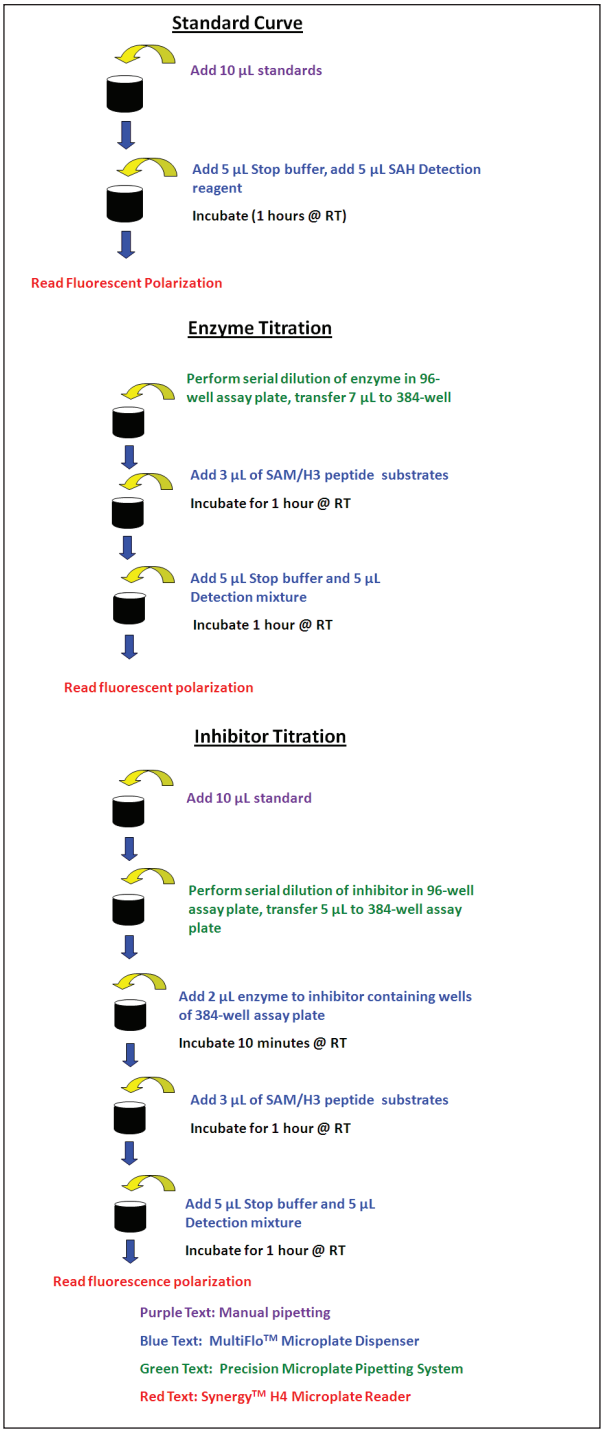

Figure 3. Transcreener EPIGEN Methyltransferase Assay workflow.

Results and Discussion

Assay Window Optimization

Assay window optimization is reader dependent and thus verified by use of standard curves which mimic conversion of SAM to SAH. Two substrate concentrations, 1 µM and 10 µM, were used to construct standard curves for determination of the minimum allowable substrate concentration resulting in an adequate assay window, as well as, Z'-factor determination to verify the robustness of the assay conditions. The results of the assay window optimization can be depicted as initial velocity curves (Figure 4). High sensitivity and low backgrounds were achieved as indicated by the consistent data measurements achieved across the range of standards tested and flashes used. Excellent assay performance is still evident at low substrate concentrations when measured in combination with a low number of flash counts when employing automated methods.

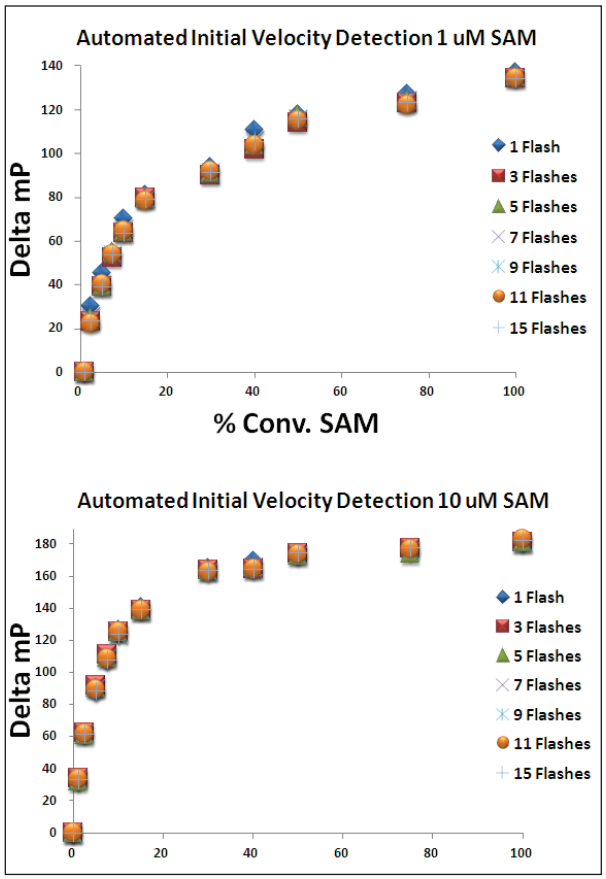

Figure 4. Initial velocity detection of 1 µM and 10 µM SAM. Comparison of the affects of substrate concentration and flash count on assay window.

Assay validation requires a minimum polarization shift of 60-100 mP units and a Z' value ≥0.5 to be suitable for HTS applications. Both criteria can be easily achieved by using a SAM concentration of >1 µM in combination with automated methods as seen in Figure 4 and Table 1. Further experimentation relied on a SAM concentration of 2 µM. It was determined that 5 flashes resulted in data with sufficient Z' values while minimizing assay plate read time.

% SAM Conversion	1 uM	10 uM	
1	-0.22	0.27	Z'Factor
2.5	0.33	0.55	
5	0.42	0.71	
7.5	0.53	0.75	
10	0.64	0.81	
15	0.67	0.82	
30	0.72	0.84	
40	0.74	0.80	
50	0.77	0.87	
75	0.79	0.85	
100	0.82	0.84	

Table 1. Representative Z' values at percent SAM conversion are showing the comparison between two substrate concentrations of lower limit of detection (yellow) and initial values that are ≥0.5 (green) at 5 flashes using automated methods. It was determined that 2 µM SAM at 5 flashes would provide sufficient limit of detection and Z'-factor for inhibition studies.

Histone Methyltransferase Enzyme Titration

G9a enzyme was serially diluted and assayed to determine the effective concentration (EC) for use with subsequent experiments. The optimal concentration should yield a sufficient assay window and initial velocity conditions (substrate consumption ≤20%). The background subtracted G9a response in ΔmP was plotted against the log G9a concentration resulting in a characteristic sigmoidal dose-response curve as seen in Figure 5. A shift of approximately 135 mP defined the assay window, which is well above the minimum required polarization shift of 60-100 mP. The EC_{80} concentration was determined to be ~80 ng/µL which agrees well with published values (Reactions Biology Corp., Malvern, Pennsylvania, USA).

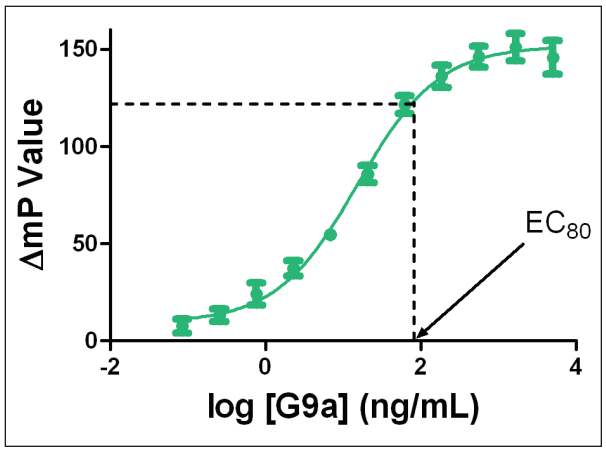

Figure 5. EC_{80} concentration for use in inhibition experiments was determined from the enzyme titration curve.

Enzyme Inhibitor Titration

A SAM/SAH standard curve was run in parallel with the inhibitor titration, allowing for quantitative data analysis through conversion of polarization values to product formation values. A known inhibitor of the G9a histone methyltransferase, sinefungin, was serially diluted and assayed to determine the IC_{50} (Figure 6). The background corrected ΔmP response was converted to product formed, picomoles (pmol) of SAH, using the standard curve. SAH was plotted against the log sinefungin concentration, yielding a typical dose response curve. The IC_{50} was determined to be 14.5 µM which shows excellent correlation with published values (Reactions Biology Corp., Malvern, Pennsylvania, USA).

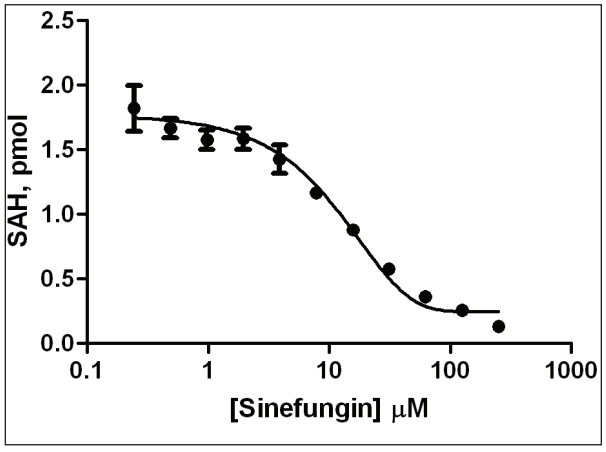

Figure 6. A dose response curve was generated for G9a methyltransferase sensitivity with the known inhibitor sinefungin yielding an IC_{50} value of 14.5 µM.

Conclusion

The Transcreener EPIGEN Assay can be performed using simple, inexpensive automated methods resulting in higher throughput and ease-of-use. The pharmacology of a known compound generated using the MultiFlo™ Microplate Dispenser for enzyme and reagent dispensing, and Precision™ Microplate Pipetting System for serial dilution and compound transfer, agree with previously published values (Reactions Biology Corp., Malvern, PA, USA). The use of automated methods show a robust Z'-factor determination indicating excellent assay performance as well as the additional benefit of increased throughput.

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